Mechanisms of biohalogenation and dehalogenation

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Mechanisms of biohalogenation and dehalogenation

Edited by D.B. Janssen, K. Soda and R. Wever

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Preface and Acknowledgements

This book is based on portions of a colloquium of the Royal Netherlands Academy of Arts and Sciences: "Mechanisms of Biohalogenation and Dehalogenation" which was held in Amsterdam during the period September 16–18, 1996. This colloquium followed an international conference previously held in the Netherlands in 1993 on naturally produced organohalogens. This conference had a strong emphasis on analysis, characterisation, distribution and budgets and it was strongly felt that there was a lack of knowledge of the mechanisms underlying the biosynthesis and breakdown of organohalogens. The idea was conceived that bringing together scientists working on halogenating enzymes and dehalogenating enzymes would be fruitful. Indeed, this was the case and the meeting which was restricted to 50 invited scientists was a great success. This led us to bring together the information presented at the meeting. In this volume the occurrence of natural organohalogens is discussed and it particular highlights and reviews the biochemical research on enzymes involved in their biosynthesis (haloperoxidases) and the enzymes involved in the breakdown (dehalogenases). Further, the potential commercial application of these enzymes and micro-organisms are discussed. The field has experienced a rapid growth in recent years in particular since three dimensional structures of the enzymes that catalyse such reactions have become available. It is expected that the introduction of modern molecular biology techniques to modify the enzymes in their properties and catalytic efficiency will lead to the development of efficient catalysts that may be used in specific industrial halogenation and dehalogenation reactions. We are sure that the contents of this book will stimulate further research in this direction.

We are grateful to the Royal Netherlands Academy of Arts and Sciences supporting us in organising the meeting. Further, we gratefully acknowledge the support of Ciba-Geigy Foundation (Japan) for the Promotion of Science, Euro Chlor representing the chlor-alkali industry, DSM Research, the Netherlands Society for Biochemistry and Molecular Biology, the Netherlands Biotechnological Society and Unilever Research Laboratory, Vlaardingen.

The editors

G.W. Gribble

A Survey of Natural Organohalogen Compounds

Abstract

New naturally occurring organohalogen compounds continue to be discovered. As of mid-1996 the number of such distinct compounds is 2,730, most of which contain chlorine or bromine. These organohalogens are produced by marine and terrestrial plants, bacteria, fungi, lichens, insects, marine animals, some higher animals, and a few mammals. The total number of natural organohalogens may surpass 3,000 by the turn of the century.

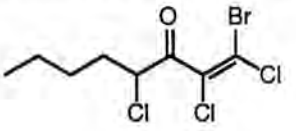
Introduction

Forty years ago, the few known naturally occurring organohalogen compounds were considered aberrations. In 1968, Fowden wrote: 'present information suggests that organic compounds containing covalently bound halogens are found only infrequently in living organisms' (Fowden, 1968). Over the past decade, it has become clear that many organisms use halogen as a normal component of their biochemical processes and synthesize organo-halogens for survival. This extraordinary explosion of information was the subject of the first 'International Conference on Naturally Produced Organohalogens' in The Netherlands in 1993. A comprehensive review of all known natural organohalogen compounds has recently appeared (Gribble, 1996a). Earlier reviews on various aspects of this field are cited in this monograph. The present chapter attempts to highlight the fantastic diversity of organohalogen chemicals in living organisms and the remarkable synthetic virtuosity of nature. All references to organohalogens that are not explicitly cited herein are found in Gribble (1996a, 1996b).

Simple Organohalogens

A vast array of simple halogenated alkanes and other low molecular weight organohalogen compounds is known to be produced by living organisms. For example, chloromethane, which is also produced from natural combustion sources, is found in many species of wood-rotting fungi, marine algae, phyto-

plankton, giant kelp, the ice plant, some evergreen trees, mushrooms, potato tubers, and a bryozoan. Both tri- and perchloroethylene are produced by 26 species of algae. Nearly 100 organohalogen compounds have been isolated from the edible Hawaiian red seaweed *Asparagopsis taxiformis*, which is prized for its flavor and aroma by native Hawaiians. Examples include the octenone **1** from the red alga *Bonnemaisonia asparagoides*, dichloroacetamide (**2**) from *Marginisporum aberrans*, and the vinyl chloride **3** from a blue-green alga. Neocarzillin A, (**4**) which is highly cytotoxic to K562 leukemia cells, is produced by *Streptomyces carzinostaticus* (Nozoe *et al.*, 1992). Caldariomycin (**5**), from the fungus *Caldariomyces fumago*, was one of the first naturally occurring organohalogen compounds to be discovered. The novel calmodulin inhibitors KS-504a (**6**) and related cyclopentanes have been isolated from the fungus *Mollisia ventosa* and contain up to 69% chlorine by weight.



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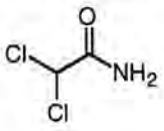

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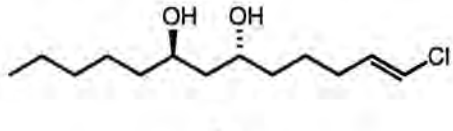

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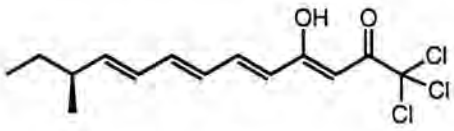

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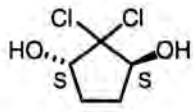

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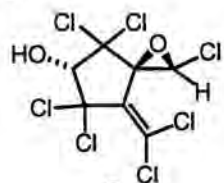

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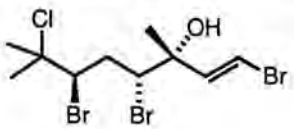
Terpenes

The first report of a halogen-containing monoterpene appeared in 1973 with the isolation of **7** and **8** from the sea hare *Aplysia californica*. These compounds are probably derived from the animals' algae diet. Numerous other halogenated monoterpenes have been identified in marine organisms, and several of these compounds, for example telfairine, have potent insecticidal activity.



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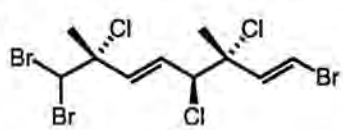

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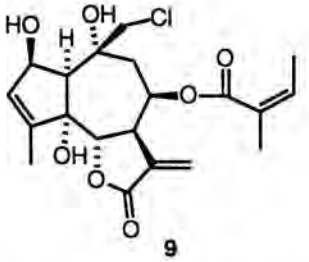
Myriad chlorine-containing terrestrial sesquiterpenes have been discovered, particularly possessing the guaianolide skeleton. Eupachlorin (**9**) from *Eupatorium rotundifolium* was one of the first such compounds to be isolated. Asian bracken ferns contain several chlorinated pterosins, such as pterosin F (**10**).



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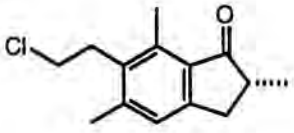

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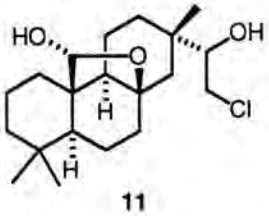
Although most halogen-containing diterpenes are marine-derived, a few terrestrial examples are known. A recent example is **11** from the Brazilian plant *Vellozia bicolor* (Pinto *et al.*, 1991). Interestingly, the corresponding epoxide is not converted to **11** under the isolation conditions. The sea hare *Aplysia dactylomela* produces dactylomelol (**12**), and the sponge *Acanthella* sp. has been a rich source of diterpene isonitriles such as kalihinol A (**13**).



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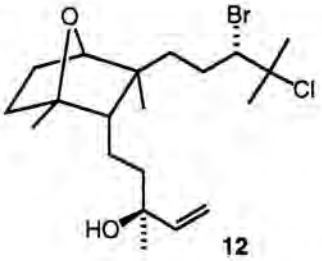

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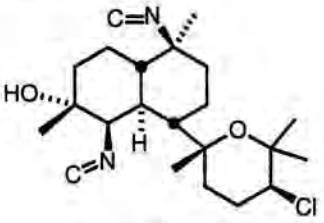

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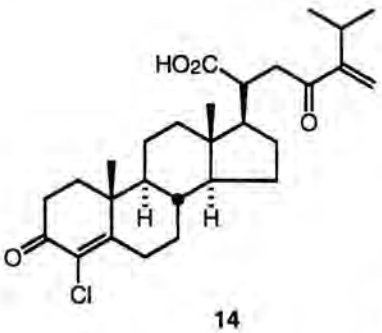
Steroids

Several examples of terrestrial plant chlorine-containing steroids are known, but the most interesting are the Maui sponge metabolites such as kiheisterone C (**14**) (Carney *et al.*, 1993), and the German cockroach pheromones such as blattellastanoside A (**15**) (Sakuma and Fukami, 1991).



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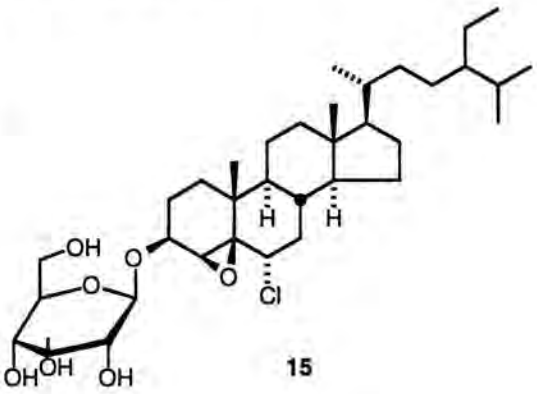
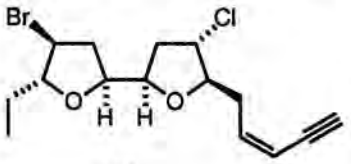


15

An extraordinary observation is the *in vitro* formation of three cholesterol chlorohydrins from cholesterol, chloride, hydrogen peroxide, and myeloperoxidase. Since myeloperoxidase is a component of human atherosclerotic lesions, cholesterol chlorohydrins might play a role in this disease (Heinecke *et al.*, 1994).

Marine Acetogenins

A very large number of halogenated marine nonterpenoid C15 acetogenins are known, particularly from *Laurencia* red algae. For example, notoryne (**16**) is produced by the Japanese red alga *Laurencia nipponica* and intricenyne (**17**) is found in *L. intricata*. The Guam 'bubble shell' (*Haminoea cymbalum*) contains kumepaloxane (**18**), a feeding deterrent against carnivorous fishes.



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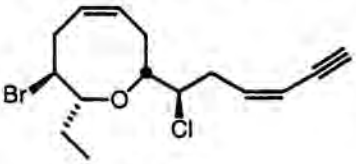

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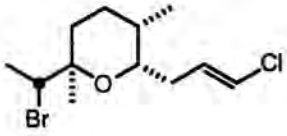

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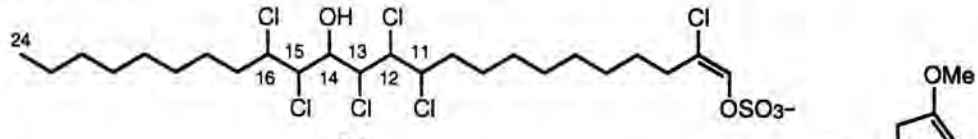
Fatty Acids

Numerous chlorinated fatty acids are known from fungi and phytoflagellates, some of which contain six chlorine atoms. For example, malhamensilipin A (**19**) is found in the chrysophyte *Poterioochromonas malhamensis* (Chen *et al.*, 1994). The blue-green alga *Lyngbya majuscula* has been a rich source of novel fatty acid derived amides such as malyngamide A (**20**). The Okinawan sponge *Xestospongia* sp. contains 17 brominated fatty acids (Li *et al.*, 1995).



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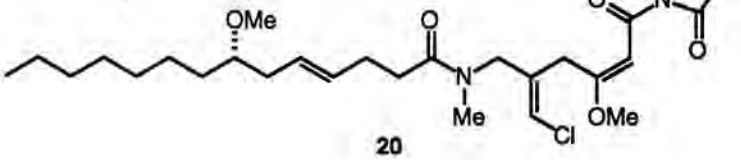

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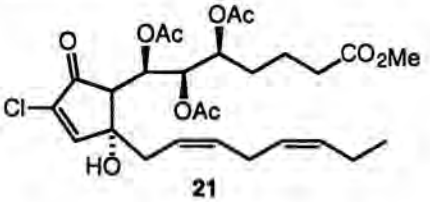
Prostaglandins

Nearly 30 chlorine-containing prostaglandins have now been isolated from several marine animals. These compounds possess pronounced anticancer

activity. For example, the octocoral *Telesto riisei* produces punaglandin 1 (**21**) and the brown alga *Egregia menziesii* has yielded egregiachloride A (**22**) (Todd *et al.*, 1993).



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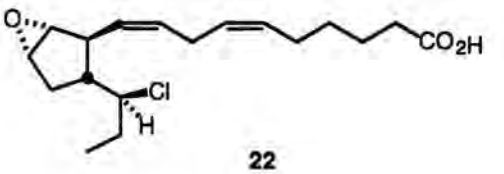

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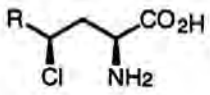
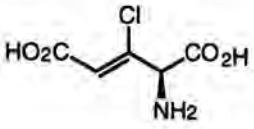
Amino Acids and Peptides

Several simple halogenated amino acids and peptides have been found in microorganisms such as *Streptomyces* and *Pseudomonas*. Some are shown here (**23-27**).



23 R = Cl
24 R = CH_3



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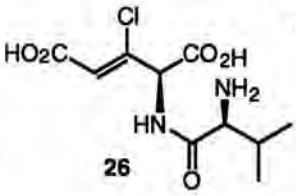

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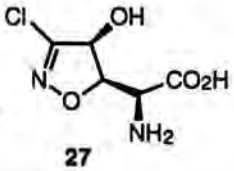
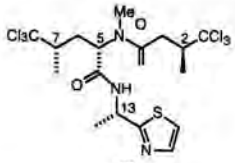


27

The marine sponge *Dysidea herbacea* has proven to be a rich source of novel amino acid and peptide-derived metabolites such as dysidin and dysidenin (**28**). These trichloromethyl compounds may be a source of chloroform in the oceans. Cryptophycin A (**29**) is one of many related *Nostoc* sp. blue-green algae metabolites with excellent anticancer activity against solid tumors (Golakoti *et al.*, 1995). The *threo*-4-chlorothreonine amino acid is found in several *Pseudomonas syringae* cyclic peptides (Fukuchi *et al.*, 1992). The fungal pathogen *Periconia circinata* produces several interesting chlorine-containing peptides such as peritoxin A (**30**) (Macko *et al.*, 1992).



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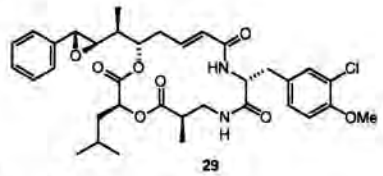

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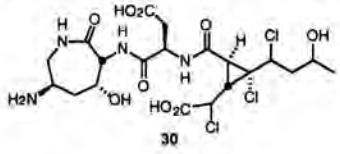

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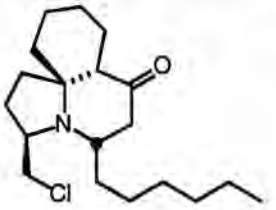
Alkaloids

Despite the enormous number of known terrestrial plant alkaloids, only a few halogen-containing examples have been discovered. Some early examples include jaconine (*Senecio jacobaea*), doronine (*Doronicum macrophyllum*), lolidine (*Lolium cuneatum*), and acutumine (*Sinomenium acutum*). The Tasmanian ascidian *Clavelina cylindrica* produces the novel alkaloids cylindricine A (**31**) and B (Blackman *et al.*, 1993). The Asian folk medicine plant *Houttuyniae cordata* has yielded 7-chloro-6-demethylcepharadione B (**32**) (Jong and Jean, 1993), and epibatidine (**33**), which is a potent analgesic, is secreted by the Ecuadorian frog *Epipedobates tricolor* (Spande *et al.*, 1992).



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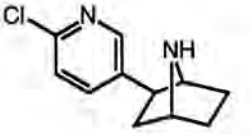

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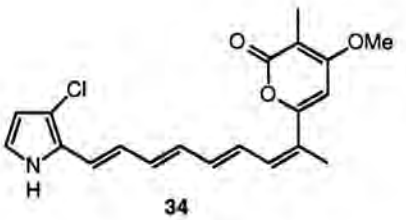
Heterocycles

The high reactivity of heterocycles such as pyrroles and indoles towards electrophiles portends the large number of naturally occurring halogenated electron-rich heterocycles. Several chlorinated pyrroles are produced by *Pseudomonas* spp., such as pyoluteorin and pyrrolnitrin, and the optically active neopyrrolomycin is found in cultures of a *Streptomyces* sp. The fungus *Auxarthron umbrinum* produces rumbrin (**34**), which may be useful in the treatment of ischemia (Yamagishi *et al.*, 1993). The sponge *Phorbas aff. clathrata* contains four chlorinated phorbazoles (e.g., **35**) (Rudi *et al.*, 1994), and a set of novel pyralomicins, such as **36**, is produced by *Actinomadura spiralis* (Kawamura *et al.*, 1995).



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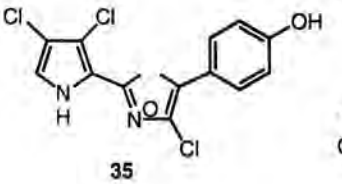

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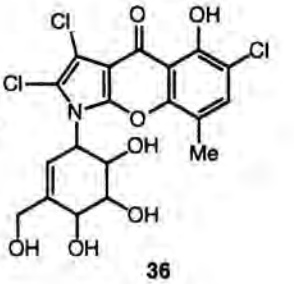

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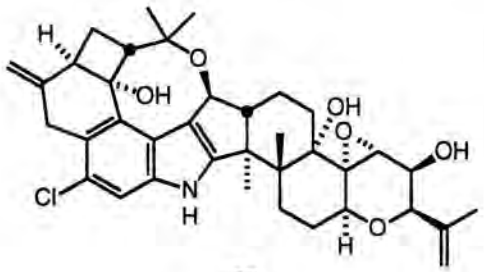
Halogenated indoles are widespread in living systems. The simple 3-chloroindole is found in the acorn worm *Ptychodera flava laysanica*, and the New Zealand red alga *Rhodophyllis membranacea* produces eight novel chlorinated indoles including 2,3-dichloro-7-bromo-indole. Several terrestrial plants (green peas, fava bean, grasspea, sweet pea, lentil, vetch) produce 4-chloroindole-3-acetic acid and the methyl ester as growth hormones. The fava bean also contains 4-chloro-6-methoxyindole which is thought to be the precursor of a potent mutagen that forms during intragastric nitrosation (Brown *et al.*, 1992). The blue-green alga *Hapalosiphon fontinalis* is the source of a dozen chlorinated isonitriles, and related compounds are produced by *Fischerella* spp. blue-green algae (Park *et al.*, 1992). The fungus *Penicillium crustosum* produces three 6-chloroindole penitrem metabolites of almost incredible molecular complexity (e.g., penitrem A (**37**)). The bryozoan *Chartella papyracea* has yielded the stunningly complex chartelline A (**38**) and several related halogenated indoles. In contrast, the bryozoan *Amathia convoluta* contains four indoles such as convolutamydine B (**39**) (Zhang *et al.*, 1995). More recently, the bryozoan *Securiflustra securifrons* has been found to contain four chlorinated securamines, e.g., **40** (Rahbaek *et al.*, 1996).



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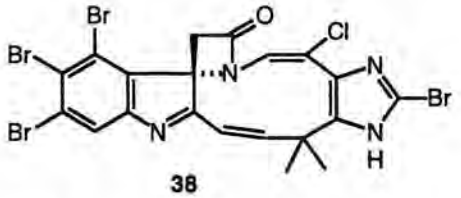

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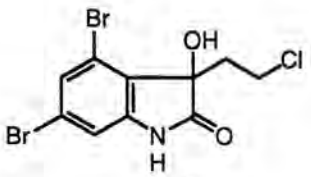

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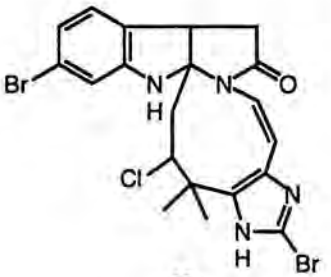

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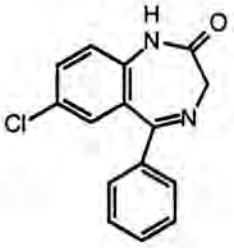
Surprisingly, seven chlorine-containing benzodiazenes, such as **41**, are found in wheat and potato tubers (Klotz, 1991), and benzodiazepines, including several chlorine-containing examples, are found in human and animal brains (Medina *et al.*, 1993). There is strong evidence of the *in vivo* formation of these chlorinated benzodiazepines in the mammalian brain. Several nucleic acid bases contain chlorine, such as **42** from *Streptomyces rishiriensis*, and kumusine (**43**) from a *Theonella* sp. sponge (Ichiba *et al.*, 1995) and the sponge *Trachycladus laevispirulifer* (Searle and Molinski, 1995).



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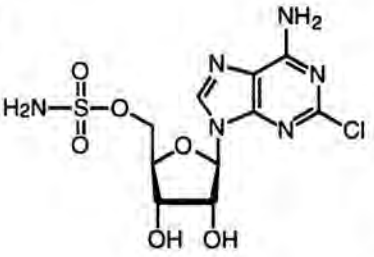

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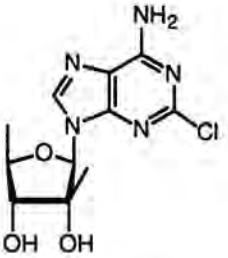

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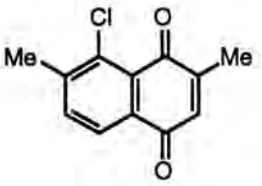
Macrolides

Several halogen-containing macrolides are known. Chlorothricin and its derivatives are produced by *Streptomyces* spp. and the well-known maytansinoids are found in numerous microorganisms. The simpler monorden (radicidol) is produced by *Monosporium bonorden* and *Nectria radicicola*, and the related 6-chlorodehydrocurvularin was extracted from the fungus *Cochliobolus spicifer* (Ghisalberti and Rowland, 1993). A collection of remarkably active antitumor sponge metabolites, the spongistatins, have been discovered in *Spirastrella spinispirulifera*, some of which contain the chlorovinyl moiety (Pettit *et al.*, 1993).

Quinones

Many aromatic quinones of varying complexity contain chlorine and a few contain bromine. The simple 8-chlorochimaphilin (**44**), which has antibiotic activity, was recently isolated from *Moneses uniflora* (Saxena *et al.*, 1996). A series of eight chlorinated napyradiomycins (e.g., **45**) have been isolated from *Chainia rubra* (Hori *et al.*, 1993) and four extraordinary chloropalmarumycins (e.g., **46**) were found in the West Borneo forest soil microbe *Coniothyrium* sp. (Krohn *et al.*, 1994).



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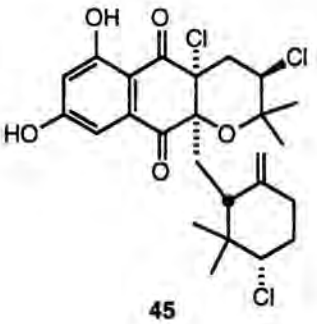

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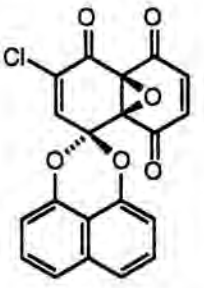

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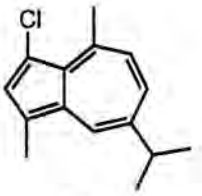
Aromatic Compounds

Although most of the known halogenated aromatic compounds are phenolic, a few are simple aromatic derivatives. For example, 1,2,3,4-tetrachlorobenzene is a major component of needlerush oil (*Juncus roemerianus*) and a deep sea gorgonian has yielded azulene **47**. The novel nostocyclophanes (e.g., **48**) are produced by the blue-green alga *Nostoc linckia* (Chen *et al.*, 1991), and 3-chloroanthranilic acid is found in cultures of *Pseudomonas aureofaciens*.



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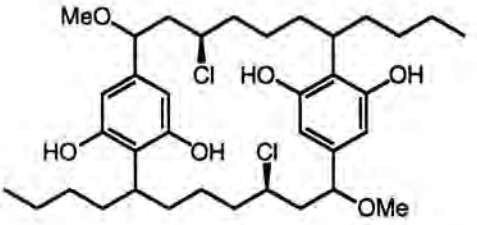

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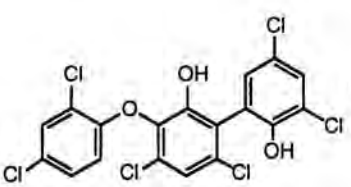
Phenols

The great reactivity of phenols in electrophilic substitution reactions has allowed nature to produce an enormous array of natural chlorinated phenols, both simple and complex. A *Penicillium* sp. produces 2,4-dichlorophenol and at least a dozen species of ticks biosynthesize 2,6-dichlorophenol as a sex pheromone. Chloride labeling studies confirm the biosynthesis of this compound within the insect. Amudol is found in *Penicillium martinsii*, and several other chlorine-containing benzyl alcohols and benzaldehydes are produced by white-rot and other fungi (Spinnler *et al.*, 1994). There is also mounting evidence that 2,4,6-trichlorophenol is a natural product of soil microbes (Hodin *et al.*, 1991). The Florida acorn worm *Ptychodera bahamensis* contains four chloro- and bromophenols (Corgiat *et al.*, 1993). Some chlorinated tyrosines are found in the proteins of locusts and molluscs, where they are believed to improve adhesion between protein fibers and sheets. Several chlorine-containing diphenyl ethers have been reported, mainly from fungi and algae. The freshwater fungus *Kirschsteiniothelia* sp. produces **49** (Poch *et al.*, 1992), and the terrestrial blue-green alga *Fischerella ambigua* has afforded ambigol A (**50**) (Falch *et al.*, 1993). This latter compound inhibits HIV reverse transcriptase. A number of chlorinated fucols have been extracted from the brown alga *Analipus japonicus*. The toxic mushroom *Russula subnigricans* contains seven chlorinated russuphelins, such as russuphelol (**51**) (Ohta *et al.*, 1995).



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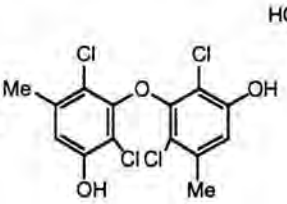

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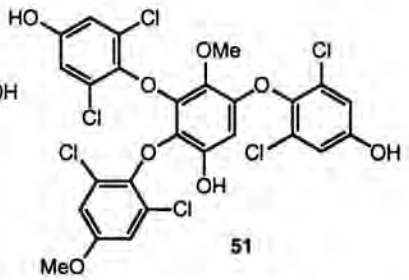

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Other natural chlorine-containing phenolic derivatives include transformed tyrosines from sponges, depsides and depsidones from lichens, and xanthones and anthraquinones from lichens and fungi. A remarkable observation is the detection of chlorotyrosine in low-density lipoproteins isolated from human atherosclerotic lesions (Heinecke, 1996). Perhaps the most medicinally important organochlorine compound is vancomycin, the glycopeptide antibiotic and the drug of choice to treat methicillin-resistant *Staphylococcus aureus* infections, particularly those that occur in hospitals. Most of the 200 known glycopeptides contain chlorine (Nagarajan, 1993).

Miscellaneous

A fitting conclusion to this brief survey is to mention the isolation of prymnesin-2 from the red tide alga *Prymnesium parvum* (Igarashi, *et al.*, 1996). This enormous organohalogen, $C_{96}H_{136}Cl_3NO_{35}$, is a potent ichthyotoxin and the first red tide toxin found to contain chlorine.

Summary

The number of reported organohalogens from living organisms continues to increase. These discoveries result from our advances in collection, isolation, bioassay, and spectroscopic identification techniques. The total number of 2,730 natural organohalogen compounds can be divided into 1,600 organochlorines, 1,570 organobromines, 90 organoiodines, and 20 organo-fluorines. Since many of these compounds, especially those from marine sources, contain both chlorine and bromine, these are included in both categories. As only a small percentage of living organisms have been examined for their chemical content, it is certain that a large number of new natural organohalogen compounds is awaiting discovery. Moreover, since mammals, including humans, utilize in vivo halogenation in white blood cells as part of the immune process, it is only a matter of time before additional organohalogen compounds are found to occur naturally in humans.

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D.B. Harper

Halogenated Methanes - Biological Sources and Physiological Role

Abstract

Current knowledge concerning the environmental abundance and biological origin of naturally-occurring halogenated methanes is reviewed and an assessment made of the relative contributions of fungi, algae and higher plants to the global flux of these compounds. The mechanisms by which mono- and polyhalogenated methanes are biosynthesized are outlined and the metabolic role of chloromethane in fungi and higher plants is discussed.

Introduction

The recognition of the role of man-made halogenated gases in particular the chloro-fluorocarbons in catalysing ozone destruction in the upper atmosphere and in enhancing the greenhouse effect in the lower atmosphere has focused considerable attention on the identification of natural sources of volatile halocarbons in the atmosphere and on the assessment of their global fluxes.

The gaseous monohalomethanes, in particular chloromethane (CH_3Cl), are the most important of these compounds in terms of atmospheric abundance but there are substantial natural fluxes of other less volatile polyhalogenated compounds such as $CHBr_3$. Table 1 summarizes recent data regarding environmental concentrations of the most significant of these compounds and the generally accepted values for the atmospheric residence time of each. Estimates of global inputs to the atmosphere from various natural and where applicable anthropogenic sources are also included. The origin and flux of each halomethane in Table 1 is considered below in more detail and the possible biochemical mechanisms involved in their synthesis are described. In the final section the metabolic role of CH_3Cl in fungi and higher plants is discussed.

Chloromethane

CH_3Cl is still the most abundant volatile halocarbon in the atmosphere, its concentration in 1995 exceeding that of the most significant of the man-made chlorofluorocarbons, CF_2Cl_2, which is present in the atmosphere at 530 pptv

Table 1. Environmental concentrations and estimated global atmospheric inputs of the most abundant naturally-occurring halomethanes.

Halomethane	Air concn. (pptv)	Seawater concn. (ng/litre)	Atmos. residence time (years)	Global input to atmosphere (10^5 ton/year)
CH_3Cl	550–630	4–12	1.5	35–50 Natural: 3–20 Oceanic 7–14 Biomass burning 0–20 Other terrestrial 0.3 Anthropogenic
CH_3Br	10	0.4–1.2	0.8	0.8 Natural: 0.3–0.9 Oceanic 0.3 Biomass burning 0.6 Anthropogenic: 0.30–0.65 Fumigation 0.05–0.22 Vehic. emissions
CH_3I	2–20	0.6–20	0.02	3–13 Natural (oceanic)
$CHBr_3$	1–15	1–10	0.04	1–20 Natural (algae)

See text for references

(Montzka *et al.*, 1996). At the present time 15–20% of the chlorine contributing to ozone destruction in the stratosphere is derived from CH_3Cl (Prather & Watson, 1990; Montzka *et al.*, 1996). Atmospheric CH_3Cl is overwhelmingly of natural origin with between 3500–5000 ktonnes/y arising from biological and chemical processes in the marine and terrestrial environments; manmade emissions of 30 ktonnes/y are of little consequence in this context (Edwards *et al.*, 1982; Koppmann *et al.*, 1993). During low temperature combustion of biomass, particularly foliage, a small proportion of the Cl^- present is volatilized as CH_3Cl. Thus considerable quantities can be emitted during forest fires and as a product of slash-and-burn agriculture in the tropics although current estimates indicate that this source is unlikely to contribute more than 25% of the natural flux (Andreae, 1991). Whilst volcanic activity has been implicated by some studies as a source of atmospheric CH_3Cl, thermodynamic calculations by Symonds *et al.* (1988) have shown that enhanced CH_3Cl concentrations found in volcanic plumes can be attributed to the combustion of vegetation by lava flows rather than direct volcanic emission.

Since the first reports of the presence of CH_3Cl in the atmosphere it has been widely presumed that the vast bulk of atmospheric CH_3Cl is derived from an oceanic source with estimates of an annual flux of the order of 5 million tonnes (Singh *et al.*, 1983; Koppmann *et al.*, 1993). Production abiotically by mechanisms such as the reaction of Cl^- with biologically formed CH_3I and CH_3Br (Zafiriou, 1975) or dimethylpropiothetin (White, 1982a) has been proposed but there is little evidence that either of these reactions has significant impact in the marine environment. Indeed the failure to find any relationship between oceanic concentrations of CH_3I and CH_3Cl suggests independent

origins for the two compounds (Singh *et al.*, 1983). However, CH_3Cl can be formed in the oceans by direct biosynthesis by marine macroalgae and phytoplankton. On the basis of field measurements Manley and Dastoor (1987) estimated that the giant kelp, *Macrocystis pyrifera*, a frequently dominant primary producer in waters off the Californian coast, released CH_3Cl, CH_3Br and CH_3I at rates of 160, 9 and 1 ng/g f.wt/day respectively. Subsequently CH_3Cl release of 1.5 ng/g f.wt/day by the red alga, *Endocladia muricata*, was reported by Wuosmaa & Hager (1990) who successively isolated and purified an *S*-adenosylmethionine/halide ion methyltransferase from the organism. The enzyme could also methylate Br^- and I^- but the kinetic parameters of the enzyme precluded the formation of any halomethane other than CH_3Cl in seawater. Nevertheless, even if it is assumed that the CH_3Cl release rate observed by Manley & Dastoor (1987) is typical of all marine algae, the global standing crop of marine algae of 58 million tonnes cannot be responsible for the production of more than 3000 tonnes/y of CH_3Cl, an insignificant contribution to the global CH_3Cl flux.

Several workers have suggested that marine phytoplankton may be a major oceanic source of CH_3Cl. Such an origin would help to explain the elevated atmospheric CH_3Cl concentrations that occur over equatorial regions and during springtime at higher latitudes (Khalil & Rasmussen, 1981). The first direct evidence of release of CH_3Cl by phytoplankton cultures has recently been obtained by Moore and co-workers (Moore *et al.*, 1995; Tait & Moore, 1995) who have demonstrated emission by cultures of both warm-water and cold-water diatoms. The highest rates of CH_3Cl production were observed during and following phytoplankton senescence. As the cultures were non-axenic the possibility that associated bacteria were involved in emission cannot be dismissed. The continuation of CH_3Cl release after death and even removal of cells from the cultures suggests that CH_3Cl production may proceed via a precursor released by the phytoplankton which undergoes bacterial or chemical degradation to CH_3Cl. Overall rates of biosynthesis by cultures were very low with maximum aqueous phase concentrations of about 1 nM observed after 30 days culture. When scaled to phytoplankton abundance in the oceans this rate of release cannot account for more than 0.5% of the global flux of CH_3Cl to the atmosphere apparently eliminating phytoplankton as a significant source of atmospheric CH_3Cl. Interestingly Tait *et al.* (1994) and more recently Moore *et al.* (1997) on the basis of observations made in the NW Atlantic and Pacific oceans and a critical reappraisal of previous data collected by other workers have concluded that the flux of CH_3Cl from the oceans is very much less than hitherto estimated. Indeed large areas of the ocean particularly cooler waters appear undersaturated with CH_3Cl and may represent a sink for the compound. A net annual flux from the oceans of only 500 ktonnes was calculated suggesting that, contrary to previous supposition, the terrestrial rather than the marine environment may in fact be the major source of atmospheric CH_3Cl.

Several terrestrial biological sources of CH_3Cl have been identified over the last 15 years. CH_3Cl is released during the rotting of wood by many polypore

fungi (Harper, 1985; Harper & Kennedy 1986; Harper *et al.*, 1988). The biosynthetic trait is highly developed amongst the Hymenochaetaceae, a survey revealing that of 63 species from 6 genera in this family, 54% released CH_3Cl when grown in the presence of Cl^-. Biosynthesis is particularly well established in *Phellinus* and *Inonotus*, widely distributed genera characterized by bracket-like perennial fruiting bodies on wood of trees of tropical and temperate origin. The extent of conversion of Cl^- to CH_3Cl during fungal growth is dependent on the species and the growth substrate with production being particularly favoured by cellulose-based media. Amongst CH_3Cl releasing species examined, approximately 60% were capable of converting more than 10% of Cl^- present to CH_3Cl and with many species yields were much higher. As the enzyme system responsible for CH_3Cl biosynthesis apparently possesses a high affinity for Cl^-, the percentage conversion of Cl^- to CH_3Cl is comparatively insensitive to Cl^- concentration in the medium. Thus with cellulose as carbon source for growth of *Phellinus pomaceus* yields of CH_3Cl ranged from 75% at 0.5 mM Cl^- to 90% at 50 mM Cl^-.

It is difficult to extrapolate from laboratory data of this nature to a rate of global emission of CH_3Cl by wood-rotting fungi especially as the distribution of vegetative hyphae of such fungi does not necessarily correspond to that of their fruiting bodies with consequent uncertainty regarding the nature of the growth substrate. The surprisingly limited information available in the literature on the Cl^- content of wood indicates concentrations can range from 0.3 to 1,150 ppm. Unfortunately the data on wood of tropical species is exceedingly sparse and, as it appears that the Cl^- content of such wood is at least a magnitude higher than that of temperate species, further measurements are essential if an accurate assessment of the potential CH_3Cl flux from fungi is to be made. An additional complicating factor alluded to above is that wood-rotting fungi are in many cases capable of vegetative growth on non-woody plant detritus both in and on the soil; such material can contain 200 to 10,000 ppm Cl^-. Despite these difficulties Watling & Harper (unpublished) using the data available have attempted to estimate the order of magnitude of the global emission of CH_3Cl from fungi. On the basis of published surveys of the frequency of occurrence of *Phellinus* and *Inonotus* species in tropical and temperate forests and making the conservative assumption that vegetative growth of these species is confined to woody tissue the annual global flux of CH_3Cl from fungi is assessed at 500 ktonnes of which over 95% is derived from tropical forests.

The biosynthetic route by which CH_3Cl is formed in *Phellinus* spp. is still unclear. Investigations by White (1982b) and Harper & Hamilton (1988) using labelled precursors have demonstrated that CH_3Cl arises from the *S*-methyl group of the amino acid methionine in *P. pomaceus*. Whilst several workers (White, 1982b; Wuosmaa & Hager, 1990) have postulated that *S*-adenosyl-methionine (SAM) may be the actual methyl donor the presence of biosynthetically significant quantities of SAM/halide ion methyltransferase in cell free extracts has not yet been substantiated. The metabolic role of CH_3Cl in fungal metabolism renders such an intermediate unlikely.

Higher plants represent another terrestrial source of CH_3Cl which may make a significant contribution to the global flux. CH_3Cl production by higher plants was first reported by Varns (1982) who showed that freshly harvested tubers of the potato (*Solanum tuberosum*) released CH_3Cl. The rate of emission varied with the date of harvest and reached a maximum of 2.5–17 ng/g f.wt/day within 2 or 3 days of harvest falling to 2–10% of the maximum a week after harvest. On cutting or wounding the tissue, release rates rose five fold higher than the maximum rate observed immediately post harvest. A survey of tubers of 60 varieties of potato within 48h of harvest by Jeffers & Harper (1996) demonstrated that CH_3Cl emission rates vary widely with cultivar ranging from <4 to 590 ng/g f.wt/day (mean 22 with 42 var. <4 and 11 var. >25). However these workers found no evidence for increased emission on cutting or wounding tissue. Surprisingly Varns explained CH_3Cl release by tubers by postulating that developing potato tissue possesses a mechanism for accumulating natural CH_3Cl from the atmosphere and that the compound so sequestered is liberated on damage to the mature tuber. However work in this laboratory (Jeffers & Harper, 1996) using ^2H-labelled precursors has established unequivocally that CH_3Cl can be synthesized de novo in potato tuber tissue from the *S*-methyl group of either L or D-methionine so it seems probable that CH_3Cl is a metabolic intermediate in some biochemical process in tuber tissue. Further investigations aimed at defining the role of CH_3Cl in the potato tuber are described in the section on 'Metabolic role of CH_3Cl'.

Emission of CH_3Cl by several species of the Pinaceae (pencil cedar, evergreen cypress and Northern White cedar) was noted by Isidorov (1990) but no attempt was made to quantify release rates. Wuosmaa & Hager (1990) reported production of CH_3Cl by whole cells of *Mesembryanthemum crystallinum* at a rate of 0.95 ng/g f.wt/day on incubation with 100 mM KCl. *M.crystallinum* is a succulent species growing on saline soils in Californian coastal areas and the authors of the report therefore suggested that a survey of CH_3Cl release by other halophytes might be rewarding. Although Wuosmaa & Hager did not themselves implement this proposal, Saini *et al.* (1995) later conducted a survey of halomethane emission in 118 species of herbaceous plant (97 species from Montreal Botanic Garden and 21 halophytic species from saline habitats in the St Lawrence Gulf region). As the rate of methylation of I^- was 2300 fold higher than that of Cl the assay for halomethane emission involved measurement of CH_3I production on incubation of leaf discs with KI^-. For 31 species CH_3I production was below the limit of detection (0.5 nmole/g f.wt/day) but all other species yielded detectable amounts of the halomethane. The emission rates of these species which represented 44 families from 33 orders ranged over four orders of magnitude from 0.5 to 4510 nmole/g f wt/day. Measured emission rates were not corrected for recovery and the authors calculated that actual emission rates were at least 2 or 3 fold greater. Paradoxically in the light of the speculations of Wuosmaa & Hager (1990), all halophytic species had relatively low emission rates (mean 25 nmole/g f.wt/day). Non-halophytes showed a higher level of release with maximum activity being displayed in the order Capparales

represented by 15 species of Brassicaceae and 1 of Resedaceae (mean 145 nmole/g f.wt/day). The methyltransferase enzyme mediating halide methylation has been isolated from *Brassica oleracea* and utilizes SAM as methyl donor (Attieh *et al.*, 1995). Significantly, it appears capable of methylating sulphide in addition to halide ion. In whole leaf discs the relative rates of I^-, Br^-, Cl^- and HS^- methylation by the enzyme are 1000:28:0.43:598 respectively and the Michaelis constants of the purified enzyme with these substrates are 1.3, 29. 85 and 4 mM respectively indicating that I^- and HS^- are by far the best substrates *in vitro*. Whilst I^- is unlikely to be a major substrate for the enzyme *in vivo* given that its concentration in plant tissue lies in the range 0.4–16 μM (Bollard, 1983), the possibility that HS^- is the normal substrate for the enzyme in plant tissue cannot easily be dismissed. Unfortunately information on the concentrations of sulphide ion in plant tissue is not readily available in the literature but levels are probably very low as the anion is highly toxic. The Brassicaceae, the family showing the highest methyltransferase activity, are very rich in several sulphur-containing secondary metabolites including non-protein *S*-amino acids and *S*-containing glucosinolates. The enzyme may have a role in detoxifying HS^- released by hydrolysis of these metabolites after cellular damage. Nevertheless, the high concentration of Cl^- in plant sap (5 to 70 mM for non-halophytic plants (Cram, 1976)) renders it unlikely that methylation by the methyltransferase *in vivo* is entirely restricted to HS^- but the extent to which Cl^- is attacked is not quantifiable on present information. Accurate estimation of the global CH_3Cl flux from higher plant sources must therefore await measurement of rates of CH_3Cl emission by intact plants in situ under normal growing conditions. Such a study should preferably involve a broad range of species of herbaceous plant and tree representative of the principal terrestrial ecosystems.

Bromomethane

Atmospheric CH_3Br is the main source of stratospheric bromine. On a molar basis bromine is about 50 fold more effective than chlorine in destroying stratospheric ozone. Consequently 20–25% of the ozone hole that develops over the Antarctic in spring can be attributed to reactions involving bromine (Butler, 1995). In contrast to CH_3Cl, CH_3Br has significant anthropogenic sources principally emissions during soil fumigation and during combustion of leaded petrol. The contribution of these sources and industrial activities to the global flux of CH_3Br has been variously estimated at 30–90 ktonnes/y i.e. between 20 and 65% of the total budget (Butler 1995; Lobert *et al.*, 1995). One of the major natural sources of CH_3Br is biomass burning. CH_3Br, like CH_3Cl, is formed during smouldering combustion of vegetation and it has been calculated that annual global emissions from savannah and forest fires fall in the range 10–50 ktonnes with a best estimate of 30 ktonnes (Mano & Andreae, 1994).

Estimates of natural emissions particularly biological are complicated by the discovery recently of large natural sinks for CH_3Br in both the oceans (Lobert *et al.*, 1995) and the soil (Shorter *et al.*, 1995) which have resulted in the revision downwards of the atmospheric lifetime for CH_3Br from 1.5 y to about 0.8 y. Historically a large proportion of global CH_3Br emissions have been attributed to the oceans but the simultaneous production and degradation of CH_3Br in the marine environment makes accurate assessment of the net oceanic efflux difficult. Most of the open ocean is undersaturated with CH_3Br but areas of upwelling are near equilibrium with the atmosphere and coastal waters are supersaturated with the compound (Lobert *et al.*, 1995). Nevertheless even in the undersaturated regions some CH_3Br production must occur in order to sustain observed concentrations given the known rate of chemical degradation. Overall observed saturations suggest a net oceanic emission of 60 ktonnes/y, the most probable source of which is biological. Although macroalgae are capable of releasing CH_3Br in addition to CH_3Cl (Manley & Dastoor, 1987) the quantities formed are insufficient to account for observed concentrations. Also the global distribution of macroalgae does little to explain CH_3Br emission form the open ocean. Whilst marine phytoplankton have been shown to release a variety of brominated methanes (see section on 'Bromoform') there is no evidence of substantial CH_3Br emission. The abiotic reaction of Br^- with CH_3I analogous to the reaction of Cl^- with the compound is another possible mechanism for oceanic CH_3Br generation but seems improbable as the concentration of Br^- in seawater is such that the reaction proceeds only at 10% the rate with Cl^-.

Iodomethane

The sole source of atmospheric CH_3I appears to be the ocean. Total CH_3I emissions to the atmosphere are estimated to be in the range 300 to 1300 ktonnes/y (Rasmussen *et al.*, 1982; Singh *et al.*, 1983; Reifenhauser & Heumann, 1992) and are believed to be mainly biological in origin. Enhanced CH_3I concentrations have been recorded in the vicinity of beds of macroalgae (Lovelock, 1975; Manley & Dastoor, 1987; Nightingale *et al.*, 1995) and release rates by tissue under laboratory conditions of $0.2-17$ ng/g f.wt/day (mean ~ 1 ng/g f.wt/day) have been measured in a variety of brown, red and green macroalgae (Manley *et al.*, 1992; Schall *et al.*, 1994; Nightingale *et al.*, 1995). Macroalgal decay by bacteria may also generate CH_3I as Manley & Dastoor (1988) have demonstrated low rates of CH_3I production when microbial populations from decaying kelp are cultured on powdered kelp in the presence of seawater. However, rates of CH_3I production by macroalgae either by direct biosynthesis or during decay are at least 3 orders of magnitude less than the estimated oceanic flux so, unless there is a large seasonal effect on release or as yet unidentified species are much more prolific producers, the principal biogenic source of CH_3I must be sought elsewhere in the marine environment.

Atmospheric CH_3I levels are markedly higher (10–20 pptv) in oceanic regions of high biological productivity such as the areas off the Peruvian, S. African and Icelandic coasts (Rasmussen *et al.*, 1982). Although these areas comprise only 10% of the ocean surface they may release as much as 80% of the total CH_3I flux. In such areas of open ocean CH_3I is presumably generated by as yet unidentified species of phytoplankton. Campos *et al.* (1996) noted some seasonality in the CH_3I flux in the North Sea with minimum emissions in winter. A correlation was also observed by Nightingale (1991) between CH_3I in seawater and chlorophyll a concentrations (taken as a measure of phytoplankton biomass). Notwithstanding these observations significant amounts of CH_3I continued to be produced in winter even though phytoplankton productivity had fallen to very low levels indicating that whilst CH_3I emission in this region is related to the seasonal cycle of primary productivity, the linkage is neither direct nor simple. One possible abiotic source of CH_3I in the ocean which might explain the correlation of CH_3I concentration with biological productivity is the reaction of I^- with dimethylsulphonium compounds such as dimethylpropiothetin which are biosynthesized and excreted by many marine algae and phytoplankton (White, 1982a; Brinkmann *et al.*, 1985). However, Moore *et al.* (1993) have cast doubt on the feasibility of this route when both reactants are at realistic concentrations.

Bromoform

Atmospheric $CHBr_3$ is entirely derived from the oceans but the magnitude of the flux is still not certain with estimates ranging up to 2000 ktonnes/y (Penkett *et al.*, 1985; Liss, 1986; Schall & Heumann, 1993). Moore *et al.* (1993) have computed a global source of 80 ktonnes/y from coastal waters but have argued that the present uncertainties in measurements of air and water concentrations and in Henry's Law constants are so great that it is not possible to establish whether pelagic surface waters are a source or a sink for the compound. Regardless of the scale of the flux, biosynthesis by marine algae is undoubtedly a major source of $CHBr_3$ with macroalgal release rates of up to 45 μg/g dry wt/day observed (Gschwend *et al.*, 1985; Manley *et al.*, 1992; Schall *et al.*, 1994; Nightingale *et al.*, 1996). Thus, in areas with extensive algal beds, eg off the coasts of Atlantic islands such as the Azores, Bermuda and Teneriffe or in Arctic fjords such as Svalbard, high concentrations of $CHBr_3$ have been measured with levels of up to 450 pptv in air and 80 ng/l in seawater (Class *et al.*, 1986; Dyrssen & Fogelqvist, 1981; Fogelqvist, 1985). On the basis of observed rates of volatile halocarbon release by a variety of temperate macroalgae, global production of volatile polybromomethanes predominantly $CHBr_3$, has been calculated at between 10 and 200 ktonnes/y (Gschwend *et al.*, 1985; Manley *et al.*, 1992; Itoh & Shinya, 1994). The mechanism of biosynthesis has been investigated in the red alga *Bonnemaisonia hamifera* (Theiler *et al.*, 1978) and the green alga *Penicillus*

capitatus (Beissner *et al.*, 1981). and has been shown to involve successive bromination of the α carbon atom of a β-keto acid catalysed by a bromoperoxidase to yield a di- or tri-brominated ketone which undergoes hydrolysis to form CH_2Br_2 or $CHBr_3$.

Although macroalgae are obviously an important source of $CHBr_3$ in coastal waters they cannot be responsible for $CHBr_3$ production in the open ocean. Measurements of seasonal variations in the $CHBr_3$ concentrations in and above surface waters of the open sea in the Arctic clearly implicate phytoplankton as a source of $CHBr_3$ (Fogelqvist, 1985; Cicerone *et al.*, 1988; Sturges & Barrie, 1988; Krysell, 1991). Sturges *et al.* (1992) have shown that algal communities living on the bottom of polar ice (mainly pennate diatoms) can generate substantial quantities of $CHBr_3$ and an annual global flux of 10–150 ktonnes from this source has been proposed — more than adequate to account for atmospheric $CHBr_3$ levels in polar regions. Recently Tokarczyk & Moore (1994) examined unialgal cultures of a number of warm- and cold-water phytoplankton and showed $CHBr_3$ release by several species with rates of up to 230 ng/g f.wt/day recorded for a *Nitzschia* sp. These rates are 10–1000 times lower than those found in macrophytes but the potential global flux from such phytoplankton is very much greater as they occupy the entire ocean surface in contrast to macrophytes which are located in only 0.5% of the total area.

Direct biological synthesis is not necessarily the only route by which $CHBr_3$ is formed in the marine environment. Release of HOBr directly into seawater by a vanadium bromoperoxidase situated on the surface of the thallus of the brown alga, *Ascophyllum nodosum*, can occur. (Wever *et al.*, 1991). $CHBr_3$ may therefore arise abiotically in the oceans by reaction of HOBr with dissolved organic matter giving rise to unstable brominated compounds which decompose to $CHBr_3$ by the haloform reaction. Seasonal variations in bromoperoxidase activity in various brown and red algae correlate well with fluctuations in $CHBr_3$ concentrations in Arctic waters (Wever, 1988; Itoh & Shinya, 1994).

Other halomethanes

Large biological fluxes may exist for several other halomethanes occurring in the atmosphere. $CHCl_3$ is present in the atmosphere at 20 pptv and the total global emission is estimated at 600–800 ktonnes/y of which 300 ktonnes/y can be ascribed to anthropogenic sources (Khalil *et al.*, 1983; Kindler *et al.*, 1995). Little is known regarding the origin of the natural component although Khalil *et al.* (1990) has calculated that termite mounds might produce 10–100 ktonnes $CHCl_3$ annually and Nightingale *et al.* (1995) has demonstrated that certain seaweeds can release $CHCl_3$.

The polyhalogenated methanes CH_2BrCl, $CHBr_2Cl$ and CH_2Br_2 present in the atmosphere are undoubtedly largely biological in origin produced by algae by a mechanism similar to that yielding $CHBr_3$. Atmospheric concentrations are

usually in the 1–3 pptv range but CH_2Br_2 can occur locally in concentrations up to 60 pptv in the Arctic spring (Berg *et al.*, 1984, Class *et al.*, 1986; Reifenhauser & Heumann, 1992).

Metabolic role of CH_3Cl

Fungi

Initial investigations of fungal production of CH_3Cl suggested that the compound was a stable end product of metabolism whose biosynthesis was confined to the period after exponential growth, characteristics typical of a secondary metabolite (Harper, 1985; Harper & Kennedy, 1986). Unexpectedly, subsequent work has led to the surprising conclusion that CH_3Cl plays a far more fundamental role in fungal metabolism. Many of the *Phellinus* species which release CH_3Cl can also biosynthesize methyl esters of benzoic and furoic acids (Harper & Kennedy, 1986; Harper *et al.*, 1988). This observation provoked speculation that the formation of these esters was linked to CH_3Cl biosynthesis as both processes were inhibited by the pseudohalide ion, SCN^- (Harper & Kennedy, 1986) and the patterns of incorporation of the *S*-methyl group of labelled methonine into methyl benzoate and CH_3Cl during growth of *Phellinus pomacecus* were remarkably similar (Harper and Hamilton, 1988). Additional evidence of a biochemical relationship was provided by the finding that the occurrence of methyl benzoate as a natural product in the genus *Phellinus* was restricted to CH_3Cl-producing species (Harper et al, 1988).

By incubating washed mycelia of *P. pomaceus* with appropriately ^2H-labelled precursors Harper *et al.* (1989) were able to demonstrate that CH_3Cl acted as a methyl donor in the methylation of benzoic and furoic acids during the growth phase. The transmethylation system exhibited a broad substrate specificity methylating the carboxyl group of a wide range of aromatic and aliphatic acids. CH_3Br and CH_3I, in addition to CH_3Cl, could act as methyl donors. A biochemically distinct CH_3Cl-utilizing system capable of methylating phenols and thiophenol was also identified in the fungus. The CH_3Cl-utilizing methylation reactions catalysed by intact mycelia of *P. pomaceus* are summarized in Fig 1. The kinetics of methylation by CH_3Cl were examined in some detail and evidence obtained for the existence of a membrane-bound CH_3Cl biosynthesis and utilizing complex (McNally *et al.*, 1990; McNally and Harper, 1991). A study of the activity of the carboxyl-methylating system during growth in relation to the extent of incorporation of the C^2H_3 group from exogenous C^2H_3Cl into methyl benzoate was conducted by Harper *et al.* (1989). The results of this investigation clearly indicated that during the early growth phase CH_3Cl biosynthesis was closely coupled to its utilization in methyl ester biosynthesis in the complex, but that in the late trophophase and early idiophase the system became less tightly channelled allowing leakage of CH_3Cl from the complex and subsequent emission by the fungal culture. Normally a compound released for

Carboxyl methylation

where R can be a range of alkyl and aryl groups

Phenol methylation

where X can be a variety of substituents

Fig. 1. CH_3Cl-utilizing methylation reactions occurring in *P. pomaceus* (Harper *et al.*, 1989)

the first time at this stage of the growth cycle would be classified as a secondary metabolite but in this instance emission during the idiophase would seem simply a reflection of the breakdown of the strict co-ordination of biosynthesis and utilization of the compound which governs its role as a primary metabolite.

An important corollary of this rationale for CH_3Cl release is that, if tight coupling of biosynthesis and utilization of the CH_3Cl is maintained throughout the growth cycle of a species, no emission of CH_3Cl will occur so raising the possibility that the use of CH_3Cl as a methyl donor may not be restricted to the fungal species which release CH_3Cl in the Hymenochaetaceae. Accordingly Harper *et al.* (1990) investigated the biosynthesis of veratryl alcohol (3, 4-dimethoxybenzyl alcohol) in the non-Hymenochaetaceous white-rot fungus *Phanerochaete chrysosporium.* This species which is frequently employed as a model organism in studies of lignin degradation does not release CH_3Cl at any stage of growth. Veratryl alcohol is a secondary metabolite produced by many white-rot fungi which is believed to play a central role in lignin degradation. The 3- and 4-0-methyl groups of this key compound are known to be derived ultimately from methionine. Harper *et al.* (1990) measured the incorporation of the C^2H_3 group into the *O*-methyl groups of veratryl alcohol isolated from *P. chrysosporium* cultures supplemented with C^2H_3Cl or L-(*methyl*-$^2H_3$) methionine and showed that C^2H_3Cl was as effective a precursor as L-methionine. Further evidence that CH_3Cl is involved in veratryl alcohol biosynthesis was afforded by a study of the effect of supplementation of fungal culture medium with CH_3Cl (Harper *et al.*, 1991). In the presence of 0.6 mM CH_3Cl not only was veratryl alcohol biosynthesis induced earlier in the growth cycle but peak concentrations of the idiolyte were significantly increased. In more recent work on *P. chrysosporium* Harper *et al.* (1996) using ^2H-labelled precursors compared the efficacies of CH_3Cl and L-methionine as methyl donors in the biosynthesis of veratryl alcohol with that of SAM, the conventional biological methylating agent. High C^2H_3 incorporation into the *O*-methyl groups was recorded when either labelled L-methionine or C^2H_3Cl was present

but no significant labelling was detected when labelled SAM was added. Incorporation of C^2H_3 from C^2H_3Cl was strongly antagonized by unlabelled L-methionine; conversely C^2H_3-incorporation from labelled L-methionine was reduced by C^2H_3Cl. These results suggest that L-methionine is converted either directly or via an intermediate (which is not SAM) to CH_3Cl which is utilized as a methyl donor in veratryl alcohol biosynthesis. Despite these observations methylation of phenolic substrates in *P. chryosporium* does not appear to be exclusively dependent on CH_3Cl as the organism contains at least two SAM-dependent phenol *O*-methyltransferases (Coulter *et al.*, 1993b; Jeffers *et al.*, 1997). Comparison of the relative utilization of CH_3Cl and SAM in the methylation of acetovanillone and other phenolic substrates by *P. chrysosporium* at different stages of growth confirms the existence of two biochemically distinct routes for *O*-methylation (Coulter *et al.*, 1993a; Harper *et al.*, 1996):-

(a) Via SAM-utilizing enzymes which can be inhibited by *S*-adenosylhomocysteine (SAH) and are induced early in the growth cycle. Two such enzymes with a pH optima of 7 to 8 have been purified from cell-free extracts. Their exact role is unclear but definitely does not include veratryl alcohol biosynthesis.

(b) Via a CH_3Cl-dependent system whose activity is not suppressed by SAH. The enzyme which is absent in early growth attains peak activity in mid-growth phase and is employed in veratryl alcohol biosynthesis. It has a pH optimum of 6 but is not detectable in cell-free extracts signifying that it is highly labile or membrane bound.

The biochemical justification for the exclusive utilization of CH_3Cl as a methyl donor in veratryl alcohol biosynthesis is not immediately apparent and further investigations are required to establish whether CH_3Cl plays a role in other methylation reactions in wood-rotting fungi and indeed whether its utilization as a metabolic intermediate is widespread in the fungal kingdom. It is conceivable that CH_3Cl is exploited as a methyl donor in methylation reactions which do not require a donor as highly activated as SAM. Such a strategy may have considerable advantage to an organism producing large amounts of a methylated product with a rapid turnover such as veratryl alcohol since the energy costs associated with the formation of CH_3Cl from methionine may be substantially less than those for SAM.

Higher plants

The demonstration by Jeffers and Harper (1996) using ^2H-labelled precursors that CH_3Cl released by the potato tuber is derived from the *S*-methyl group of methionine rather than from CH_3Cl passively accumulated from the atmosphere as suggested by Varns (1982) indicates that CH_3Cl is either a metabolic intermediate in the tuber or a by-product of a metabolic process. The restriction of CH_3Cl emission by tubers at normal temperatures to a comparatively short period immediately postharvest suggests that the compound may participate in some process associated with maturation of the tuber. One of the major changes

occurring during this phase is the suberization of the periderm. It is interesting to note that a large proportion (40–45%) of suberin in the periderm consists of phenolic components. Studies of phenolic metabolism during suberization have shown that deposition of the phenolic matrix begins approximately 3 days after wounding and continues at an increasing rate for several days eventually ceasing after 10–12 days (Cottle & Kolattukudy, 1982). The most abundant components of the polymeric phenolic matrix are methylated monomer units such as vanillin.

In investigations by Jeffers and Harper (1996) in which tubers were incubated in an atmosphere of $^{14}CH_3Cl$, significant amounts of ^{14}C were fixed in the tubers in an involatile form predominantly ($\sim$80%) in the outer 4–5 mm. Autoradiography revealed that much of the ^{14}C in this outer tissue was located in the superficial 0.5 mm of the tuber consistent with its presence in the suberized periderm. Possible utilization of CH_3Cl in biosynthesis of suberin was investigated by Jeffers and Harper (1996) by incubation of potato tuber tissue in an atmosphere of C^2H_3Cl followed by examination of C^2H_3-incorporation into the suberin phenolics such as vanillin and isovanillin by GC/MS after oxidative degradation of suberin. Disappointingly no incorporation of C^2H_3 was detected so, despite the plausibility of a role for CH_3Cl in plants analogous to that in fungi, the compound does not appear to be involved in methylation of suberin phenolics.

In the only other higher plant halomethane – emitting tissue investigated in any depth, the cabbage leaf disc system studied by Saini *et al.* (1995), it appears that CH_3Cl is released as a by-product of an enzyme whose principal substrate is HS^- (Attieh *et al.*, 1995). It is not yet possible to draw any conclusion as to whether CH_3Cl released by leaf tissue of other plant species examined by Saini *et al.* (1995) has a similar origin or plays a more fundamental role in the plant.

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Organohalogen Metabolites of Basidiomycetes

Abstract

The biological capacity for organohalogen synthesis is widespread among basidiomycetes. Up to 79 organohalogen metabolites have been identified from 46 genera of basidiomycetes. Three of the most common occurring basidiomycete genera in The Netherlands were found to be responsible for the highest levels of organohalogen production. Based on the biodiversity of species producing chlorinated anisyl metabolites (CAM), they represent the most ubiquitous group of organohalogens from basidiomycetes. Phenylalanine and 3-chloro-4-hydroxybenzoate were implicated as possible precursor of CAM biosynthesis. Organohalogens were shown to be significantly produced when basidiomycetes were cultured on natural lignocellulosic substrates. Likewise, CAM could be detected in the environment and were highly concentrated in rotting wood and forest litter colonized by common fungi. Considering the ecological importance of basidiomycetes as decomposers of forest litter, we conclude that they are a major source of natural organohalogens in terrestrial environments.

Introduction

Basidiomycetes are higher fungi that produce macroscopic fruiting bodies (mushrooms). These organisms play a key role in the ecology degrading lignocellulosic debris such as wood, straw and litter. Also basidiomycetes are known to constitute a major fraction of the living biomass degrading forest litter. Approximately a ton dry weight of basidiomycete mycelium is estimated to be produced annually per hectare in a typical temperate hardwood forest (Swift, 1982). Over the years, several basidiomycetes were reported to produce de novo organohalogen metabolites indicating that this group of organisms might be an important source of naturally occurring organohalogens in terrestrial environments (De Jong *et al.*, 1994a). Additionally, organohalogen production has been shown to be associated with the decay of forest litter (Oberg *et al.*, 1996a). The objective of this manuscript is to review the literature and report on our ongoing

studies demonstrating the widespread capacity among basidiomycetes to produce organohalogens in the environment.

Organohalogen screening

Two studies have been conducted screening basidiomycetes for bulk organohalogen parameters. Either the production of adsorbable organic halogens (AOX) or total organic halogens (TOX) were measured (Verhagen *et al.*, 1996; Oberg *et al.*, 1997). The combined results of both studies screened 200 strains and evidence was found for organohalogen production in 51% of the strains, indicating a widespread capacity for organohalogen biosynthesis among basidiomycetes. Approximately 10% of all strains produced organohalogens at remarkably high concentrations, ranging from 5 to 67 mg AOX/l in the extracellular culture fluid. The top AOX producers were predominated by species from three genera: *Bjerkandera*, *Mycena* and *Hypholoma* (Verhagen *et al.*, 1996). These genera are reported to be the 6th, 2nd and 1st most common occurring basidiomycetes in The Netherlands; respectively (Nauta and Vellinga, 1995).

Organohalogen metabolites

In 1995, we reviewed the literature indicating that 53 halogenated metabolites have been reported as de novo products of basidiomycetes (Field *et al.*, 1995). Since then, there have been new reports and newly found reports published prior to 1995 which brings the total to 79 halometabolites identified from 46 genera of basidiomycetes. Compared with the data from the AOX and TOX screenings, an additional 21 genera are known to produce organohalogens, although no metabolites have yet been identified.

The organohalogen metabolites of basidiomycetes can be classified as halomethanes, miscellaneous halogenated aliphatics and halogenated aromatics (Field *et al.*, 1995). Six halomethane metabolites are known from 11 genera, ranging from chloromethane to dichloroiodomethane. Up to 8 other chlorinated aliphatics metabolites have been described from 4 genera: pinicoloform from *Resinicium pinicola*; lepiochlorin from *Lepiota* sp.; 1-chloro-heptadecyne from *Lactarius* sp. and 5 different chlorinated pentenoic or hexenoic amino acids from the genus *Amanita* (references in Field *et al.*, 1995; Anonymous, 1993; Ohta *et al.*, 1987; Hatanaka *et al.*, 1994). As many as 65 halogenated aromatic compounds have been identified from 34 genera of basidiomycetes. Common occurring chlorinated aromatic metabolites of basidiomycetes are illustrated in Figure 1 and the genera producing these are listed in Table 1. The most ubiquitous of these are the chlorinated anisyl metabolites (CAM) which represent 9 metabolites that are known from 16 genera. CAM are produced at relatively high concentrations (ranging from 10 up to 108 mg/l) in many strains including

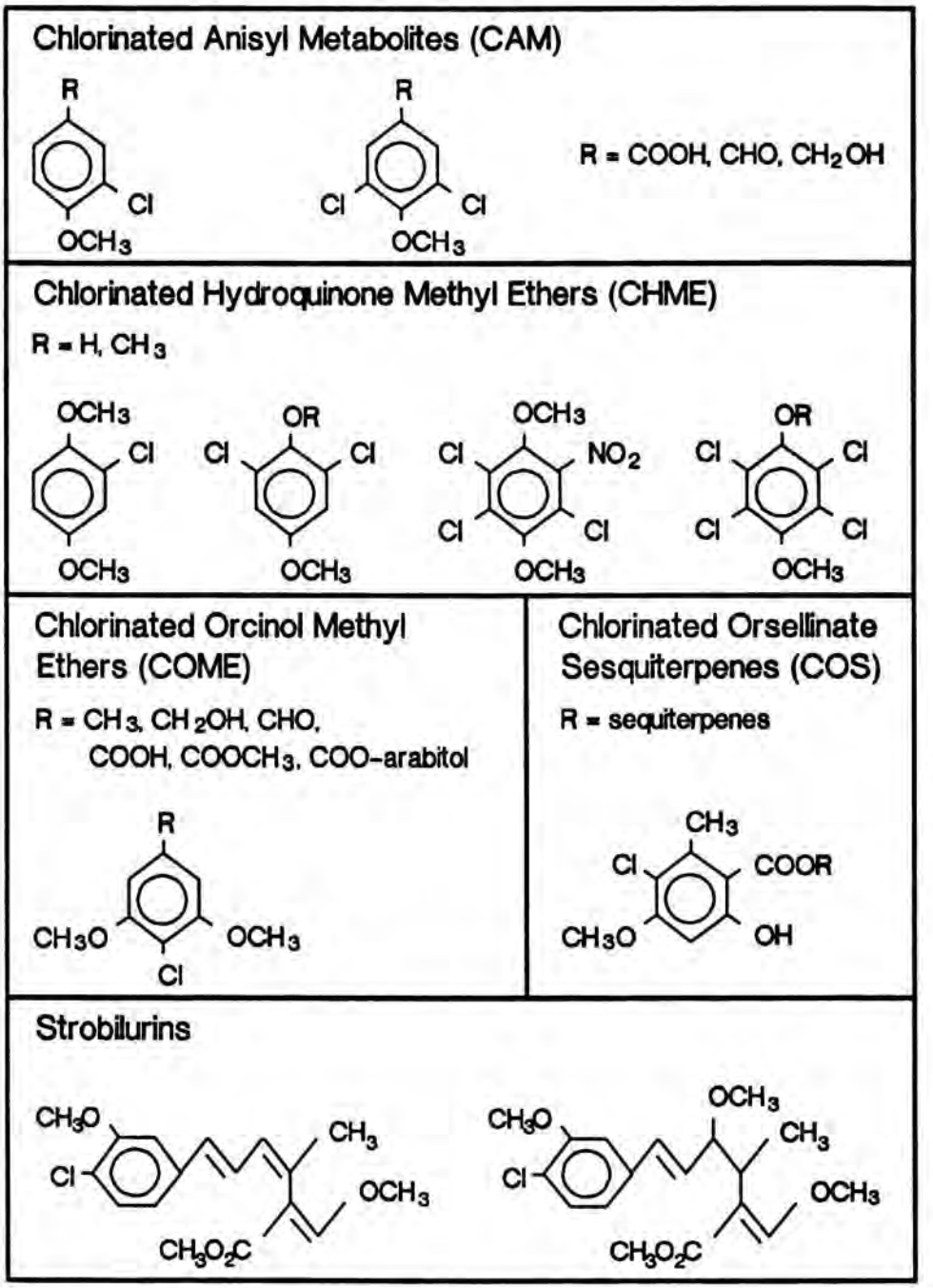

Fig. 1. Structures of common chlorinated aromatic metabolites produced de novo by basidiomycetes (literature references in Table 1).

Table 1. Basidiomycete genera producing ubiquitous chlorinated aromatic metabolites.

Metabolite Family*	Genera	Reference***
CAM	*Bjerkandera*	1, 2
	Daedaleopsis (t)**	3
	Formes (t)	3
	Hypholoma	1, 2
	Ischnoderma (t)	3
	Lepsita	1, 4
	Mycena	2
	Oudemansiella	1
	eniophora	2
	Phellinus	2
	Pholiota	1, 2
	Phylloporia	2
	Pleurotus	1
	Ramaria	3
	Stropharia	1, 5
	Trametes	
CHME	*Agaricus*	1, 6
	Bjerkandera	1, 6
	Coprinus	1
	Hypholoma (t)	7
	Lepista	4
	Mycena	1
	Peniophora	6
	Phellinus	1, 6
	Psathyrella	1
	Russula	1
	Schizophyllum	8
COME	*Bjerkandera*	6
	Hericium	1, 9
COS	*Armillaria*	1, 10, 11
	Clitocybe	12
Strobilurins	*Mycena*	13
	Strobilurus	13
	Xerula	14

* abbreviations defined in Figure 1;
** trace levels of metabolite
*** 1) references cited in Field *et al.*, 1995; 2) Swarts *et al.*, 1996a; 3) De Jong *et al.*, 1994a; 4) Hjelm *et al.*, 1996; 5) Thines *et al.*, 1995; 6) Teunissen *et al.*, submitted; 7) Verhagen *et al.*, unpublished data; 8) Schwartz *et al.*, 1992; 9) Qian *et al.*, 1990; 10) Arnone *et al.*, 1988a; 11) Cremin *et al.*, 1995; 12) Arnone *et al.*, 1988b; 13) references cited in Anke, 1995; 14) Anke *et al.*, 1983

ecologically significant species. The second most important group are the 6 different chlorinated hydroquinone methyl ethers (CHME) produced by 11 genera. Furthermore, two genera of basidiomycetes produce 6 different types of chlorinated orcinol methyl ethers (COME) and another two genera produce 10 different metabolites of the structurally related chlorinated orsellinate sesquiterpenes (COS). Finally, three genera produce two types of chlorinated strobilurin metabolites.

New chlorinated aromatic metabolites identified since our last review are shown in Figure 2 (see references in figure caption). Five new metabolites were described from strains in the genus *Bjerkandera*, these included 3-chloro- or 3,5-dichloro-4-hydroxybenzoates as well as 2,4-dichlorobenzoate. 5-chloroveratraldehyde was identified from *Lepista nuda*. Two types of chlorinated 3-(4-methoxyphenyl)propanoid structures, methyl chloro-4-methoxycinnamate and trametol were identified from *Poria cinerescens* and *Trametes* sp; respectively. In addition to these, a polychlorinated tetrameric metabolite, denominated russuphelol, was described from the black pigments in *Russula subnigricans* fruiting bodies and is presumably formed from the oxidative coupling of 2,6-dichloro-4-methoxyphenol (Ohta *et al.*, 1995).

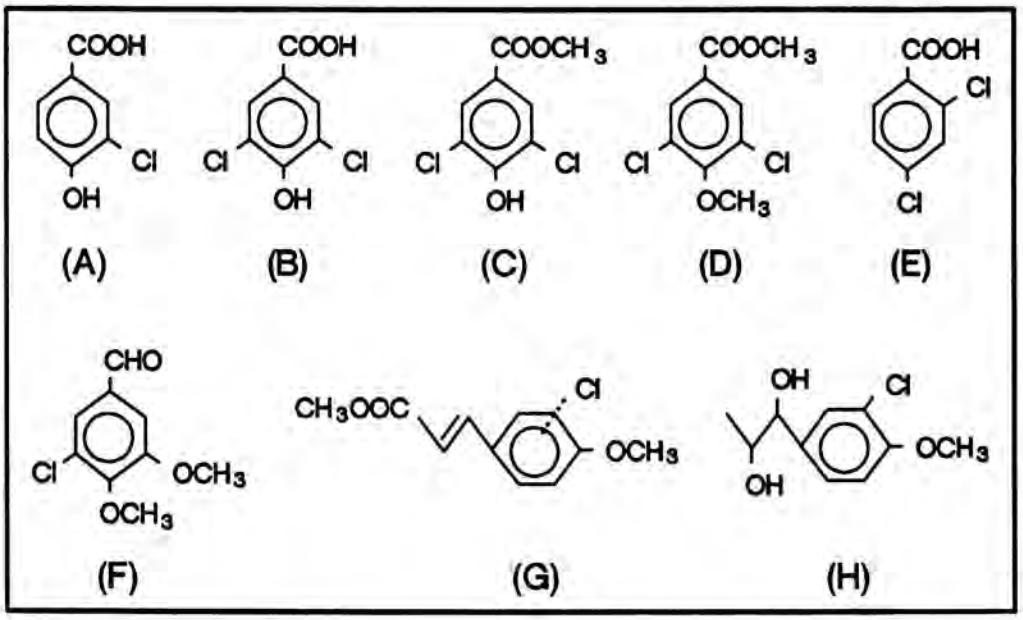

Fig. 2. New aromatic metabolites of basidiomycetes published in 1995 and 1996. (A) 3-chloro-4-hydroxybenzoate; (B) 3-5-dichloro-4-hydroxybenzoate; (C) methyl 3,5-dichloro-4-hydroxybenzoate and (D) methyl 3,5-dichloro-4-anisate reported from *Bjerkandera* spp. by Swarts *et al.* (1996b). (E) 2,4-dichlorobenzoate reported from *Bjerkandera adusta* by Oberg *et al.* (1996b). (F) 5-chloroveratraldehyde reported from *Lepsita nuda* by Hjelm *et al.* (1996). (G) methyl chloro-4-methoxycinnamate reported from *Poria cinerescens* by Oberg *et al.* (1996b). (H) trametol reported from *Trametes* sp. by Brambilla *et al.* (1995).

Biosynthesis

Not much is known about the biosynthesis of organohalogens by basidiomycetes. In the case of halomethanes, the formation of chloro-, bromo- and iodomethane probably proceeds via methylation of an inorganic halide by a S-adenosylmethionine dependent methyl transferase as has been shown to be the case for algae and plants (Wuosmaa and Hager, 1990; Attieh *et al.*, 1995). In *Amanita* spp. the chlorinated pentenoic and hexenoic amino acids probably arise via biological chlorohydrin reactions of penta- or hexadienoic amino acids which are also present (Ohta *et al.*, 1987; Hatanaka *et al.*, 1994). CAM and chlorinated 4-hydroxybenzoate metabolites would likely result from the action of chloroperoxidase since the chlorination pattern is almost exclusively ortho to the hydroxy (or methoxy) groups (Franssen and van der Plas, 1992). However, to date chloroperoxidases have not yet been isolated from basidiomycetes. The halogenating system appears to have a high affinity for chloride since increasing chloride concentrations beyond background levels in chloride limited culture medium or wood substrates did not improve organohalogen production (De Jong *et al.*, 1994a; Verhagen *et al.*, 1996). Also a preference for bromide is indicated by the formation of bromoanisyl metabolites instead of CAM in media supplemented with KBr (Spinnler *et al.*, 1994).

Evidence is accumulating that CAM are synthesized via the phenylpropanoid metabolism of phenylalanine. The addition of phenylalanine to cultures of *Bjerkandera* sp. resulted in 10-fold increases in CAM production (Mester *et al.*, 1997). Benzoate is known to be an intermediate in the biosynthesis of veratryl alcohol (3,4-dimethoxybenzyl alcohol) from phenylalanine (Jensen Jr. *et al.*, 1994). Consequently, deuterated benzoate and deuterated 4-hydroxy- and 3-chloro-4-hydroxybenzoates were tested as precursors to CAM biosynthesis. Addition of any of these compounds to the *Bjerkandera* cultures resulted in an incorporation of the deuterated label into CAM metabolites as evidenced by matching retention times and mass spectrum in the GC-MS with the exception of the difference in mass between deuterium and hydrogen (Mester *et al.*, submitted). These results combined with the fact 3-chloro-4-hydroxybenzoate is known as a de novo metabolite from *Bjerkandera* strains (Figure 2) implicate this compound as a likely intermediate in the biosynthesis of CAM. Chlorination of 4-hydroxybenzoate was demonstrated indicating that 4-hydroxybenzyl compounds are possible substrates of the halogenation system. Conversion of 3-chloro-4-hydroxybenzoate to CAM would be expected to proceed via methylation of the 4-hydroxy group and reduction of the carboxylic acid group. Both S-adenosylmethionine dependent methyl transferases and a chloromethane dependent methylating systems are known from basidiomycetes (Coulter *et al.*, 1993; De Jong *et al.*, 1994b). Aryl dehydrogenases are also common intracellular enzymes in basidiomycetes, including *Bjerkandera* (De Jong *et al.*, 1994b). However, chlorination and methylation can also occur at an earlier stage of the phenylpropanoid metabolism as is suggested by the occurrence of trametol and methyl chloro-4-methoxycinnamate as metabolites from other basidiomycetes (Figure 2).

Organohalogens from basidiomycetes in the environment

The occurrence of organohalogen metabolites in the environment is inferred from the ability of various basidiomycete strains to produce AOX or TOX when cultured on natural lignocellulosic substrates in the laboratory (Verhagen *et al.*, 1996; Oberg *et al.*, 1997). Figure 3, illustrates the extent of this production after culturing *Hypholoma fasciculare* on different natural substrates for 6 weeks. Up to 132 mg AOX/kg dry weight of forest litter substrate was produced. *Mycena metata* produced up to 193 mg AOX/kg of forest litter substrate (Verhagen *et al.*, 1996). The maximum specific rates of organohalogen production for these species on natural substrates is extremely high. Values ranging from 630 to 3200 mg AOX produced per kg of mycelium dry weight per day were found with *Hypholoma fasciculare* when colonizing wood and forest litter utilizing ergosterol measurements to estimate the mycelium biomass (Verhagen *et al.*, unpublished data). Similar specific production rates were observed by direct measurement of the biomass in defined liquid medium. The maximum rates of organohalogen production occurred during the transition of the fungal culture from primary to secondary metabolism.

Likewise, specific compounds such as CAM are also produced by basidiomycetes when cultured on wood and straw (De Jong *et al.*, 1994a; Oberg *et al.*, 1997). However, the real proof that such metabolites exist in the environment is to look at their concentrations in environmental samples. Environmental samples were collected from rotting wood and decomposed forest litter colonized by basidiomycetes. The colonies of basidiomycetes were localized by their fruiting bodies (De Jong *et al.*, 1994a; Hjelm *et al.*, 1996). These samples contained CAM compounds at concentrations ranging from 10 to 70 mg/kg dry weight for various common species from the genera, *Bjerkandera*, *Hypholoma*, *Stropharia*, *Lepista* and *Pholiota*. CAM compounds were also identified at many sites in composite samples of the forest litter (De Jong *et al.*, 1994a).

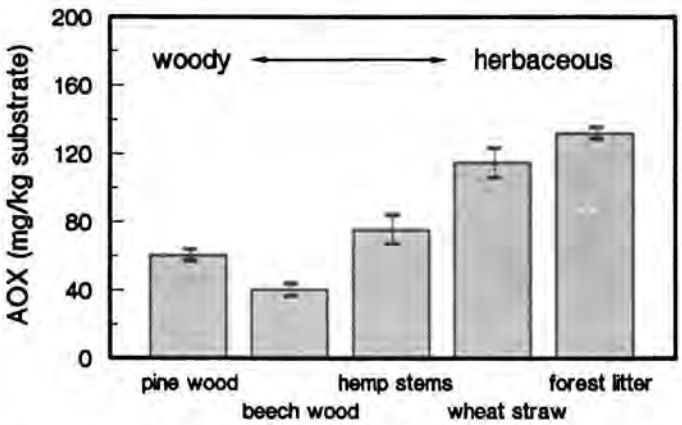

Fig. 3. The net production of adsorbable organic halogens (AOX) by *Hypholoma fasciculare* cultured for 6 weeks on various natural substrates (Verhagen *et al.*, 1996).

Conclusions

A vast number of basidiomycetes were shown to be capable of organohalogen biosynthesis. Significant production of organohalogens by basidiomycetes in the environment was demonstrated by culturing selected strains on natural substrates. Likewise, the common occurring chlorinated anisyl metabolites were found at high concentrations in environmental samples of rotting wood and forest litter. These results combined with the dominant ecological role of basidiomycetes as decomposers of forest litter suggest that they are a major source of natural organohalogens in terrestrial environments.

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K.-H. van Pée, K. Hohaus, A. Altmann, W. Burd, S. Lam, D.S. Hill, P. Hammer and J. Ligon

Novel Specific Halogenating Enzymes from Bacteria

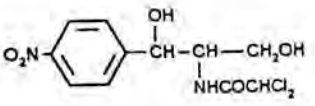
Abstract

Bacteria are known to produce a large number of halogenated metabolites. Haloperoxidases are believed to be the enzymes responsible for the incorporation of halogen into these metabolites. They need hydrogen peroxide and halide ions for the halogenation of organic compounds. Haloperoxidases oxidize halide ions with subsequent electrophilic attack of the organic substrate by the oxidized halogen species. All known haloperoxidases lack substrate and regio specificity. However, the halogenating enzymes involved in the biosynthesis of many halometabolites in bacteria must have substrate and a regio specificity. Two such enzymes have now been detected in a pyrrolnitrin-producing *Pseudomonas fluorescens* strain. They catalyze the chlorination of tryptophan and monodechloroaminopyrrolnitrin, respectively, in hydrogen peroxide-independent reactions and thus constitute a novel class of halogenases.

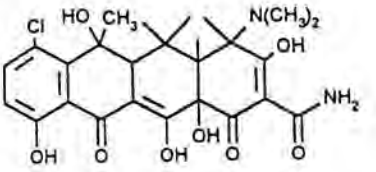
Introduction

Halogenated metabolites from bacteria include many chlorine-containing antibiotics like chloramphenicol, chlorotetracycline, vancomycin and pyrrolnitrin (Fig. 1). Although chloramphenicol, the first bacterial halometabolite detected, was already isolated in 1947 (Ehrlich *et al.*, 1947), the first halogenating enzyme from bacteria was not detected until 1984 (van Pée & Lingens, 1984). This halogenating enzyme isolated from the chloramphenicol producer *Streptomyces phaeochromogenes* was a heme-containing bromoperoxidase (van Pée & Lingens, 1985). Similar bromoperoxidases, all requiring hydrogen peroxide for the formation of carbon bromine bonds, were also isolated from *Pseudomonas* and other *Streptomyces* strains producing chlorinated metabolites (van Pée, 1990a).

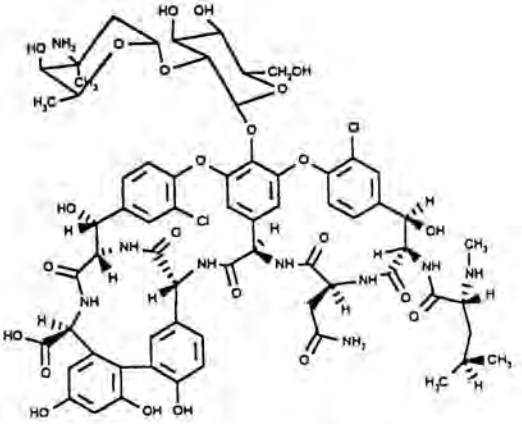
In 1986 the first bacterial chloroperoxidase was detected in the pyrrolnitrin producer *Pseudomonas pyrrocinia* (Wiesner *et al.*, 1986). This enzyme was a non-heme haloperoxidase that did not contain any prosthetic group or metal ions (Wiesner *et al.*, 1988).



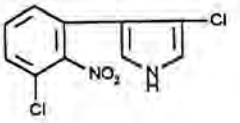
Chloramphenicol



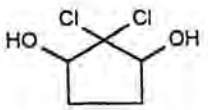
7-Chlorotetracyclin



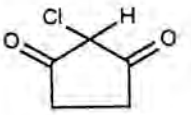
Vancomycin



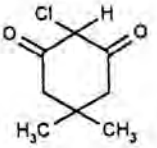
Pyrrolnitrin



Caldariomycin



2-Chloro-1,3-cyclopentanedione



Monochlorodimedone

Fig. 1. A few examples of halogenated metabolites isolated from bacteria in comparison with caldariomycin, 2-chloro-1,3-cyclopentanedione, produced by the fungus *Caldariomyces fumago*, and monochlorodimedone, the substrate used for the last 30 years to search for halogenating enzymes.

All isolated bacterial and eukaryotic haloperoxidases only showed very low or no substrate specificity at all (Franssen, 1994). However, as haloperoxidases were the only halogenating enzymes known, with the exception of some S-adenosyl methionine transferases that are involved in the formation of methyl halides (Wuosmaa & Hager, 1990), it was generally accepted that these enzymes are the ones responsible for the formation of halometabolites produced by bacteria and other organisms.

Biosynthesis of halometabolites by bacteria

Although much work was done on the biosynthesis of antibiotics like chloramphenicol, 7-chlorotetracycline and pyrrolnitrin, very little is known about the chlorination steps in these biosynthetic pathways. In chloramphenicol biosynthesis, it is not known, whether chlorination occurs before or after acetylation. Thus the structure of the substrate for the chlorinating enzyme is not known (Facey *et al.*, 1996).

In 7-chlorotetracycline biosynthesis the structure of the substrate for the chlorinating enzyme is known, however, this compound, 4-ketodedimethylanhydrotetracycline, is unstable and is thus not available for *in vitro* investigations (McCormick *et al.*, 1965). It is known, however, that the chlorinating enzyme acting on this substrate must have a substrate specificity, as chlorination is not possible at a later stage of 7-chlorotetracycline biosynthesis (McCormick, 1967). The enzyme also seems to be regiospecific, as no chlorotetracycline derivative with the chlorine atom at another than the 7-position is known.

During pyrrolnitrin biosynthesis two chlorine atoms are incorporated into the molecule. Whereas it has already been known for 15 years that monodechloroaminopyrrolnitrin is the substrate for the incorporation of the second chlorine atom, it was still under debate at what stage of the biosynthetic pathway the first chlorination step occurs (van Pée, 1996). Only recently could it be shown that chlorination of L-tryptophan to 7-chloro-L-tryptophan is the first step in pyrrolnitrin biosynthesis (Fig. 2). However, it was not known whether the two chlorination steps are catalyzed by a single enzyme or whether two different halogenases are involved in pyrrolnitrin biosynthesis.

Detection and isolation of halogenating enzymes from bacteria

The first halogenating enzyme, a chloroperoxidase, was isolated by Morris and Hager (1966) from the fungus *Caldariomyces fumago*, the producer of caldariomycin (Fig. 1). Hager et al. (1966) developed a spectrophotometric assay for the search for halogenating enzymes. They used monochlorodimedone, like 2-chloro-1,3-cyclopentanedione, an intermediate in caldariomycin biosynthesis, a 1,3-diketo-compound (Beckwith & Hager, 1963; Fig. 1), as the substrate for the search for halogenating enzymes. The monochlorodimedone assay was adopted

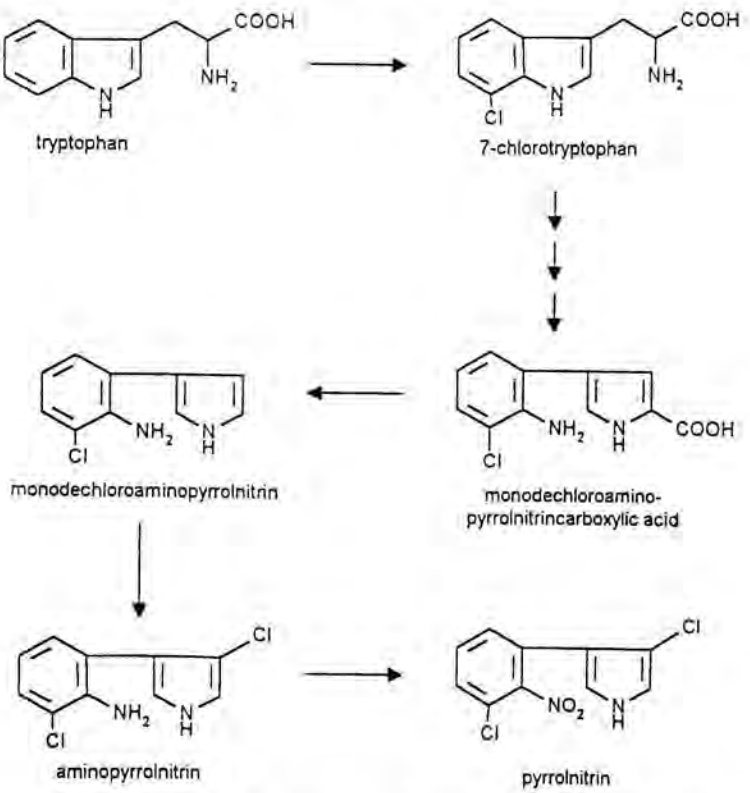


Fig. 2. Hypothetical pathway for the biosynthesis of pyrrolnitrin based on the isolation and conversion of the metabolites shown.

by all research groups working on halogenating enzymes. In addition to the use of the same organic substrate, hydrogen peroxide was generally used, too. Employing the monochlorodimedone assay, haloperoxidases were also detected in bacteria after the problem with interferring catalases was overcome (van Pée and Lingens, 1984). Isolation and characterization of several bromoperoxidases showed that these haloperoxidases had a number of similarities with chloroperoxidase from *Caldariomyces fumago* (van Pée, 1990a). They not only exhibited halogenating activity, but also showed catalase and peroxidase activities. In contrast to chloroperoxidase from *Caldariomyces fumago* none of these heme-containing bacterial haloperoxidases was able to catalyze chlorination reactions; they only had brominating activity. The lack of chlorinating activity was thought to be due to the use of the unnatural substrate. It was believed that if the natural substrates were used, these enzymes would be able to catalyze the chlorination of these substrates (van Pée, 1990b).

A few years later haloperoxidases were isolated from bacteria that were substantially different from the heme-containing haloperoxidases as they did not

contain heme or any other prosthetic group and unlike eukaryotic non-heme, vanadium-dependent haloperoxidases they did not contain any metal ions either (van Pée *et al.*, 1987; Wiesner *et al.*, 1988). Some of these non-heme haloperoxidases were identified as chloroperoxidases and they seemed to have some substrate specificity with regard to the chlorination reaction, as they did not chlorinate all the substrates they could brominate (Bongs & van Pée, 1994).

Cloning and overexpression of the genes for bacterial non-heme haloperoxidases allowed detailed investigations of these enzymes (Bantleon *et al.*, 1994). Crystallization and elucidation of the three-dimensional structure revealed that they contain a catalytical triad consisting of serine, histidine and aspartate residues and belong to the α/β hydrolases (Hecht *et al.*, 1994; Pelletier *et al.*, 1995). These findings and the observation that they required acetate or propionate for activity lead to a new hypothesis for the mechanism by which these enzymes catalyze the hydrogen peroxide-dependent halogenation of organic substrates and the oxidation of aromatic amino to nitro groups without a prosthetic group or metal ions. The first step in the catalytic cycle of these enzymes is the reaction of the serine residue of the catalytical triad with acetate with formation of a serine acetate ester. Hydrolysis of this ester by hydrogen peroxide leads to the formation of peracetic acid which then acts as the oxidizing agent that oxidizes halide ions or amino groups in an enzyme-independent reaction (van Pée, 1996; Fig. 3). As the concentration of peracetic acid formed during this reaction cycle is very low, the concentration of oxidized chloride ions is very low too, and only such organic substrates can be chlorinated that are highly susceptible to electrophilic attack, like pyrrole derivatives or indole.

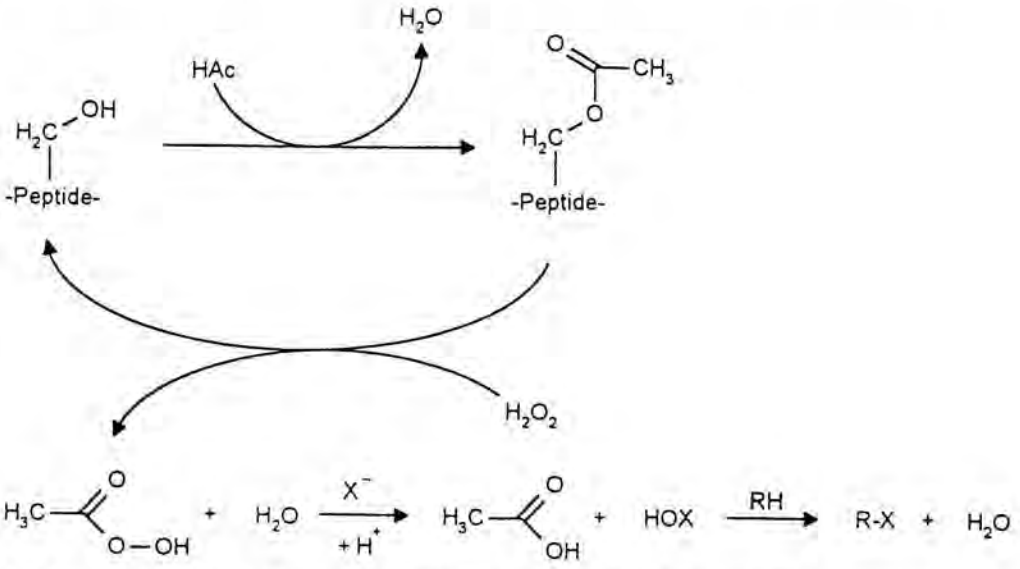


Fig. 3. Hypothetical catalytical cycle of bacterial non-heme haloperoxidases.

However, as the actual oxidation or halogenation reaction is enzyme-independent, these reactions proceed without substrate specificity. This explains why enzymes isolated from 7-chlorotetracycline-producing bacteria can also catalyze the chlorination of pyrrolnitrin and the oxidation of aminopyrrolnitrin to pyrrolnitrin.

Bacterial haloperoxidases and the biosynthesis of halometabolites

None of the heme-containing haloperoxidases isolated from bacteria were able to catalyze the chlorination of organic substrates making them unlikely candidates for the participation in the biosynthesis of chlorinated metabolites by bacteria. For the bromoperoxidase-catalase from the chloramphenicol producer *Streptomyces venezuelae* it could be shown that this enzyme was not involved in chloramphenicol biosynthesis with the help of a gene replacement experiment. Exchange of the chromosomal gene against a disrupted copy had no influence on chloramphenicol biosynthesis (Facey et al., 1996).

The non-heme chloroperoxidase isolated from the pyrrolnitrin producer *Pseudomonas pyrrocinia* was found to catalyze the chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin (Wiesner *et al.*, 1988) and the oxidation of the amino group of aminopyrrolnitrin to the nitro group of pyrrolnitrin (Kirner & van Pée, 1994; Fig. 2). This very strongly suggested the participation of this enzyme in pyrronitrin biosynthesis. However, the lack of substrate specificity due to the reaction mechanism cast considerable doubt on the involvment of this enzyme in pyrrolnitrin biosynthesis. Isolation of a highly homologous chloroperoxidase and the corresponding gene from *Pseudomonas fluorescens* strain 915, another pyrrolnitrin producing strain, the subsequent disruption of this gene and the exchange of the chromosomal chloroperoxidase gene against the inactivated copy showed that the non-heme chloroperoxidase was not needed for pyrrolnitrin biosynthesis and thus did neither catalyze the chlorination of monodechloroaminopyrrolnitrin nor the oxidation of aminopyrrolnitrin *in vivo* (Kirner *et al.*, 1996).

Dairi *et al.* (1995) had cloned the gene responsible for the chlorination step in 7-chlorotetracycline biosynthesis via complementation of a mutant blocked in this chlorination step. Sequencing of this gene showed that it did not have any homology to a known haloperoxidase. As they did not have the natural substrate for this enzyme they could not measure any halogenating activity.

Surprisingly non-heme haloperoxidases were also isolated from bacteria not known to produce halogenated metabolites (Bantleon *et al.*, 1994; Burd *et al.*, 1995).

All the above mentioned results made it very unlikely that any of the known haloperoxidases could be involved in the biosynthesis of bacterial halometabolites, however, no other halogenating enzymes other than haloperoxidases were known to catalyze the halogenation of organic substrates with more

than a single carbon atom. Nevertheless it became quite clear that specific halogenating enzymes must exist.

The search for novel halogenating enzymes

Probably the most crucial point in the search for specific halogenating enzymes is the use of the right organic substrate. Using the unnatural organic substrate monochlorodimedone results in the selection of unspecific enzymes. When monochlorodimedone is used together with hydrogen peroxide unspecific haloperoxidases will be selected. As hydrogen peroxide is a strong oxidizing agent it inhibits many enzymes and thus the addition of hydrogen peroxide to an assay for halogenating activity could prevent the detection of hydrogen peroxide-independent halogenating activity, even when the natural organic substrate is used.

Another important consideration is the possible reaction mechanism. So far it was generally accepted that enzymatic halogenation proceeds via the oxidation of halide ions (Franssen, 1994). However, the incorporation of fluoride ions could never be explained by the haloperoxidase mechanism. If fluorination has to proceed differently, why should not enzymatic chlorination or bromination follow the same mechanism as fluorination, and what kind of mechanism could this be? Could the halogen be incorporated as halide ion with halogenation being a nucleophilic rather than an electrophilic reaction?

To answer this question it is necessary to know the structure of the natural substrate for the enzyme in question. However, only one natural substrate for a halogenating enzyme from bacteria was known so far and this compound, monodechloroaminopyrrolnitrin, was not easily available (van Pée *et al.*, 1980). Another possible substrate that would be easily available is tryptophan, but for a long time it was not clear whether tryptophan is actually a substrate for a halogenating enzyme.

The recent isolation of 7-chloro-L-tryptophan together with the earlier finding that 7-chlorotryptophan was converted to pyrrolnitrin (van Pée *et al.*, 1980) proved that L-tryptophan was actually the natural substrate for the halogenase catalyzing the first step in pyrrolnitrin biosynthesis (Fig. 2). Using L-tryptophan and monodechloroaminopyrrolnitrin as the organic substrates and chloride as the halide source we searched for the specific halogenases chlorinating these compounds in crude extracts of pyrrolnitrin-producing pseudomonas strains.

As neither the UV-spectra of tryptophan and 7-chlorotryptophan nor those of monodechloroaminopyrrolnitrin and aminopyrrolnitrin are substantially different, it was not possible to develope a spectrophotometric assay. Therefore the reaction mixtures had to be assayed by HPLC. In the case of monodechloroaminopyrrolnitrin halogenase (mcap hal) the assay mixture was extracted with ethylacetate to get rid of the proteins; with tryptophan halogenase (trp hal) proteins could be denatured by boiling.

Another major problem to solve was the question what kind of cofactors or metal ions were needed by these specific halogenases. Of the cofactors added to

A

Trp hal:	5 IKNIVIVGGGTAGWMAASYLVRALQQQ
Hi ndh:	1 MKNVVIVGGGAGGIELATFLGNKLGRQ
Sc trd:	4 VRNVIIIGSGPAGYTAALYTARASLQ
Ec ndh:	5 LKKIVIVGGGAGGLEMATQL
con:	+ ++I+G G +G A

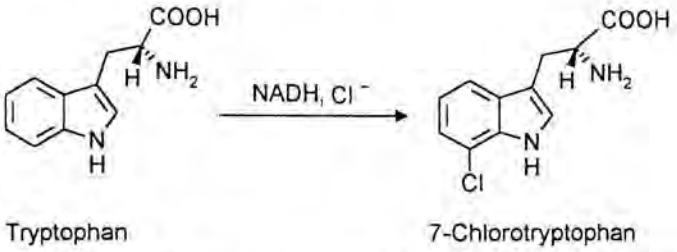
B

Mcap hal:	12 SNHFCVIILGSGMSGTQMGAILAKQQFRVLIIEESSHP
Bc sox:	2 STHFDVIVVGAGSMGMAAGYQLAKQGVKTLLVDAFDPP
Bj fix:	6 FDAIVVGAGMAGNAAALTMAKQGMKVLQLERGEYP
con:	FD+I++G G G ± AKQ ++L ++ P

Fig. 4. Alignment of the amino acid sequences of (A) tryptophan halogenase (trp hal) and of (B) monodechloroaminopyrrolnitrin halogenase (mcap hal) with proteins from the databanks that are similar. Consensus sequences are shown below the alignments. The NAD-binding motifs of Hi nhd, NADH dehydrogenase from *H. influenza*; Ec nhd, NADH dehydrogenase from *E. coli*; Sc trd, thioredoxin reductase from *S clavuligerus*; Bc sox, sarcosine oxidase from *Bacillus sp.* and Bj fix, FixC protein from *B. japonicum* are underlined.

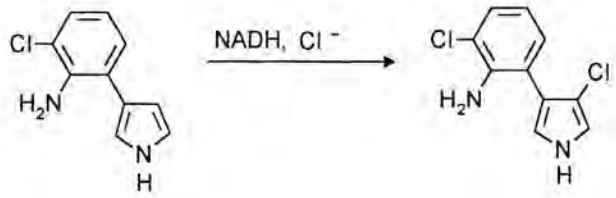
the assay mixture only NADH/NADPH resulted in halogenase activity. As no hydrogen peroxide was needed for halogenating activity, the new halogenases are definitely not haloperoxidases, and thus constitute a novel class of halogenating enzymes.

The NADH-dependency of tryptophan halogenase and monodechloroamonpyrrolnitrin halogenase was confirmed by the presence of NADH-binding sites found in the amino acid sequences derived from the DNA sequences of the corresponding genes (Fig. 4). Surprisingly, the two enzymes have no homology to one another (Hammer et al., unpublished results). When the amino acid sequences of the two novel halogenases were compared with that of the halogenase involved in 7-chlorotetracycline biosynthesis, it was found that this enzyme showed considerable homology to monodechloroaminopyrrolnitrin halogenase, but none to tryptophan halogenase.



Tryptophan 7-Chlorotryptophan

Fig. 5. Chlorination of tryptophan to 7-chlorotryptophan by NADH-dependent tryptophan halogenase (trp hal).



Monodechloroaminopyrrolnitrin Aminopyrrolnitrin

Fig. 6. Chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin by NADH-dependent monodechloroaminopyrrolnitrin halogenase (mcap hal).

Tryptophan halogenase (Fig. 5) consists of an unknown number of identical subunits with a molecular weight of about 61,000. The native molecular weight is not known yet. D-Tryptophan is a better substrate for the enzyme compared to L-tryptophan. When chloride is substituted by bromide in the enzyme assay, no product formation was detected. As no metabolic studies have been done with *P. fluorescens* strain 915 regarding the production of the bromo derivatives of pyrrolnitrin, it is not known whether this strain can produce brominated pyrrolnitrin derivatives. From *P. aureofaciens* it is known that this strain produces brominated pyrrolnitrin derivatives and thus tryptophan halogenase from *P. aureofaciens* should also accept bromide (van Pée *et al.*, 1983). The pH optimum for the chlorination of tryptophan is at pH 6.5 and the optimal chloride concentration is between 50–100 mM. With NADPH as the cosubstrate only 65%of halogenating activity are detectable.

Monodechloroaminopyrolnitrin halogenase (Fig. 6) consists of an unknown number of identical subunits with a molecular weight of about 66,000. The enzyme catalyzes the chlorination and the bromination of monodechloroaminopyrrolnitrin with a pH optimum at pH 7.0. Optimal chloride concentration is between 20–50 mM; chloride concentrations above 75 mM strongly inhibit the reaction. Halogenating activity is lower (about 45%) with NADPH as the cosubstrate.

Further purification of the two enzymes and the search for similar enzymes in other bacteria will show, whether NADH-dependent halogenases are also involved in the biosynthesis of other bacterial halometabolites like chloramphenicol or fluoroacetic acid. These novel halogenases could prove as useful tools for the biotechnological production of halogenated compounds.

Acknowledgements

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Alison Butler and Richard A. Tschirret-Guth

On the Selectivity of Vanadium Bromoperoxidase

Introduction

Haloperoxidases catalyze the oxidation of a halide (e.g., chloride, bromide or iodide) by hydrogen peroxide, which can result in halogenation of organic substrates, i.e., R-H in Equation 1,

$$Br^- + H_2O_2 + R-H + H^+ \rightarrow Br-R + 2H_2O \qquad \text{Eqn. 1}$$

the production of dioxygen through subsequent oxidation of a second equivalent of hydrogen peroxide, or the production of hypohalous acid (i.e., HOCl), depending on the identity of the haloperoxidase. Vanadium haloperoxidases and FeHeme haloperoxidases are two general types of haloperoxidases. Vanadium bromoperoxidase has been isolated primarily from marine algae (for a review see Butler and Walker, 1993). Because of the abundance of halogenated marine natural products, and because V-BrPO can catalyze halogenation reactions, the physiological role of V-BrPO is thought to be in the biosynthesis of the halogenated marine natural products. These natural products range from relatively simple volatile halogenated hydrocarbons (e.g., $CHBr_3$, CH_2Br_2 $CHBr_2Cl$ (Gschwend *et al.*, 1985; Walter and Ballschmiter, 1991; Manley *et al.*, 1992)) to more complex compounds, such as halogenated indoles and terpenes among others (Faulkner, 1993), which may play a defensive role for the host organism (Figure 1).

Some haloperoxidases are isolated from organisms or cells for which halogenated natural products have not been identified, including V-ClPO from the terrestrial fungus *Curvularia inaequalis* and other dematiaceous hyphomycetes (Vollenbroek *et al.*, 1995). V-ClPO is secreted from these fungi and produces hypochlorous acid (HOCl) (van Schijndel *et al.*, 1994). HOCl is a strong bactericidal agent which may be produced as a defense mechanism or as an attack mechanism in the invasion of the plant cell wall of the fungi's host.

Vanadium bromoperoxidase catalyzes peroxidative halogenation reactions (e.g., equation 1 for bromide) (Wever *et al.*, 1985) and the halide-assisted disproportionation of hydrogen peroxide, producing dioxygen (Everett and Butler, 1989; Everett *et al.*, 1990a&b; Soedjak and Butler, 1991; Soedjak *et al.*, 1995). The catalytic mechanism involves first coordination of hydrogen peroxide to the vanadium(V) center followed by a two-electron oxidation of the halide, produc-

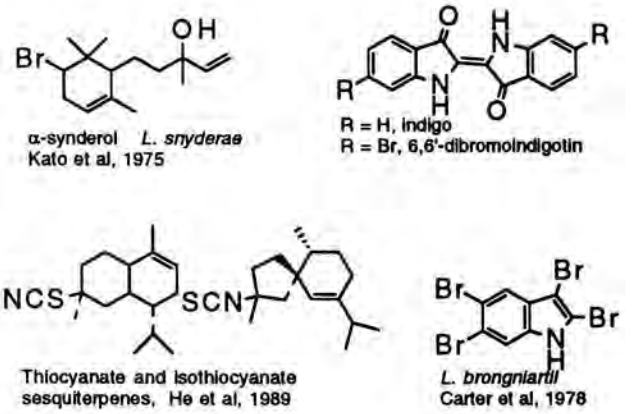


Fig. 1. Selected marine natural products

ing the oxidized halogen intermediate (Tromp *et al.*, 1990). The nature of this intermediate, such as hypobromous acid (HOBr), bromine (Br_2), tribromide (Br_3^-), or an enzyme-trapped bromonium ion equivalent (e.g., Enz-Br, Enz-OBr^-, Enz-HOBr, etc), in the case of bromide, has been the subject of much speculation (de Boer & Wever, 1988; Tschirret-Guth and Butler, 1994, Itoh *et al.*, 1987). Under certain specific conditions (e.g., 300 μM H_2O_2, 100 mM KBr, 130 nM V-BrPO, 100 mM Na_2SO_4, 100 mM acetate buffer, pH 5.0), Br_3^- has been detected (de Boer & Wever, 1988). Under the optimum catalytic conditions for V-BrPO (*A. nodosum*) (i.e., pH 6.5, 2 mM H_2O_2, 0.1 M KBr, 50 μM MCD), an intermediate cannot be observed, because the halogenation of the substrate or the oxidation of a second equivalent of hydrogen peroxide by the oxidized halogen intermediate is very fast (Everett and Butler, 1989; Everett *et al.*, 1990a&b).

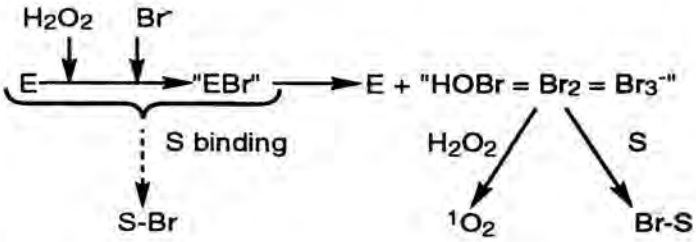
Through competitive kinetic studies comparing the reactivity of the V-BrPO/H_2O_2/KBr system with HOBr, we have recently demonstrated that the nature of the halogenating species produced by V-BrPO depends on the nature of the organic substrate (Tschirret-Guth and Butler, 1994). We found that V-BrPO does not release an oxidized bromine species (e.g. HOBr, Br_2, Br_3^-) during turnover of certain indole derivatives, because these indoles bind to V-BrPO (Tschirret-Guth and Butler, 1994). Our conclusions were based on the following results:

Firstly, V-BrPO preferentially brominates 2-methylindole (forming 3-bromo-2-methylindole) over phenol red (forming tetrabromophenol blue), as shown by a lag phase in the appearance of bromophenol blue. The lag phase is proportional to the concentration of 2-methylindole, although after the lag phase, the rate of bromination of phenol red is independent of the 2-methylindole concentration. By comparison, a lag phase is not observed in the competitive bromination of 2-methylindole and phenol red by HOBr; under these conditions, bromination of 2-methylindole and phenol red occur concurrently and an

increase in the 2-methylindole concentration leads to a decrease in the appearance of bromophenol blue. This differential reactivity between V-BrPO and HOBr suggests that released HOBr is not the active brominating species in the V-BrPO-catalyzed reactions of 2-methylindole, a situation arising from indole binding to V-BrPO. Fluorescence quenching of 2-phenylindole by V-BrPO established that the indole binds to V-BrPO (Tschirret-Guth and Butler, 1994).

Further evidence that the enzyme-catalyzed bromination of indoles is not mediated by enzyme-released HOBr was established from comparison of the rate of V-BrPO-catalyzed bromide-assisted disproportionation of H_2O_2 (forming O_2) in the presence and absence of 2-methylindole *versus* the rate of oxidation of H_2O_2 by HOBr (forming O_2) in the presence and absence of 2-methylindole (Tschirret-Guth and Butler, 1994). In the enzyme reaction, indole bromination is favored over H_2O_2 oxidation, whereas in the non enzymatic reaction ($HOBr + H_2O_2 \pm$ indole), H_2O_2 was preferentially oxidized by HOBr, forming O_2 (see Figure 3 in Tschirret-Guth and Butler, 1994).

A mechanistic scheme involving substrate binding is shown in Scheme 1 (Tschirret-Guth and Butler, 1994):



Scheme 1.

V-BrPO binds H_2O_2 and Br^- leading to a putative 'enzyme-bound' or 'active-site trapped' brominating moiety, 'E-Br', which in the absence of an indole may release HOBr (or other bromine species, e.g., Br_2, Br_3^-). When indole is present, it binds to V-BrPO, preventing release of an oxidized bromine species and leading to indole bromination.

We are interested in the relative reactivity of V-BrPO towards other organic substrates, including substituted indole derivatives. We report below on further competitive kinetic studies with consideration of the nature of the oxidized bromine intermediate as released from or trapped by V-BrPO.

Materials and Methods

Materials.

V-BrPO was purified from the marine brown alga, *Ascophyllum nodosum* as described previously (Butler and Walker, 1993). Protein concentrations were determined using the bicinchononic acid protein assay (Pierce). The concentra-

tion of hydrogen peroxide was determined spectrophotometrically by the formation of triiodide (Cotton & Dunford, 1973). 2-Phenylindole and 2-*tert*-butylindole were synthesized by the method of Houlihan *et al.* (1981). 3-Phenylindole was synthesized by Fisher indole synthesis. 3-*tert*-Butylindole and 1,3-di-*tert*-butylindole were synthesized by the procedure of Smith and Water (1961). All other reagents were purchased from Aldrich. Spectrophotometric measurements were collected either on a Kontron double-beam Uvikon 860 spectrophotometer or on a Hewlett-Packard diode-array HP8452 spectrophotometer.

Hypobromite Solutions.

Hypobromite solutions were prepared by dilution of bromine vapors into 0.07 N sodium hydroxide. The final concentration of hypobromite in solution was determined spectrophotometrically by the oxidation of iodide to triiodide: λ_{max} 353 nm; ε 26,000 $M^{-1}cm^{-1}$. Aliquots of the standard hypobromite solution were diluted into 100 mM potassium iodide in 100 mM acetate buffer, pH 4.5.

Substrate Specificity.

The relative specificity of V-BrPO was determined using monochlorodimedone (MCD) as a reference substrate. The initial change in absorbance at 290 nm (ΔA) was monitored for the reaction of V-BrPO with a) MCD alone (ΔA_a), b) the competing substrate alone (ΔA_b), c) an equimolar mixture of MCD and the competiting substrate (ΔA_c). The relative percent of MCD being brominated during the competitive reaction was estimated by the following equation:

$$MCD(\%) = \frac{\Delta A_c - \Delta A_b}{\Delta A_a - \Delta A_b} \times 100 \qquad \text{Eqn. 2}$$

Dioxygen Measurements.

Rates of dioxygen formation were measured with a Yellow Springs Instrument (YSI, Yellow Springs, OH) oxygen probe (YSI 5331) and monitor (YSI 5300). The reaction mixtures were sparged with nitrogen gas prior to initiation. The reactions were initiated by addition of V-BrPO or by introduction of HOBr via a syringe fitted on a syringe pump. The errors on dioxygen evolution measurements can be estimated to be no more than 5%.

Substituent Effect on the Rate of Indoles Bromination.

The rates of bromination of 3-methylindole, 3-phenylindole, 3-tert-butylindole, 2-tert-butylindole, and 1,3-di-tert-butylindole were determined by following the loss in absorbance at 281 nm, 267 nm, 280 nm, 272 nm, and 291 nm, respectively, and using experimentally determined molar absorption coefficients of

4037 $M^{-1}cm^{-1}$, 8687 $M^{-1}cm^{-1}$, 3336 $M^{-1}cm^{-1}$, 5602 $M^{-1}cm^{-1}$, and 3194 $M^{-1}cm^{-1}$, respectively.

Data Fitting.

All data fitting was performed with GRAPHER™ for Windows or IGORpro™ for Macintosh.

Results and Discussion

Substrate Specificity.

Monochlorodimedone (MCD) is the clasic substrate for the characterization of haloperoxidase activity (Hager, 1966). It has been used widely in the kinetic and mechanistic investigations of V-BrPO (e.g., de Boer & Wever, 1988; Everett *et al.*, 1990a&b). Because of the preferential bromination of 2-methylindole over phenol red (Tschirret-Guth and Butler, 1994), we were interested in the relative reactivity of V-BrPO (*A. nodosum*) towards MCD, phenol red and 2-methylindole. The bromination of MCD in the absence or presence of phenol red and in the absence or presence of 2-methylindole is shown in Figure 2. The presence of

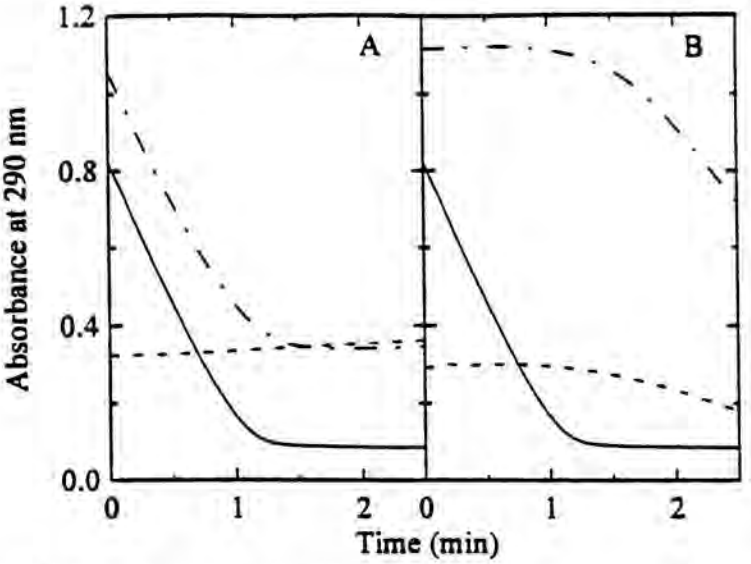


Fig. 2. Effect of phenol red (A) and 2-methylindole (B) on the bromination of monochlorodimedone (MCD) by V-BrPO. Reactions conditions: 970 μM H_2O_2, 50 mM KBr, 4.3 nM V-BrPO in 100 mM sodium phosphate pH 6.50 with 10% ethanol, and with the following substrate concentration: Panel A: (___) 50 μM MCD, (---) 50 μM phenol red, (_._.) 50 μM MCD and 50 μM phenol red; Panel B: (___) 50 μM MCD, (---) 50 μM 2-methylindole, (_._.) 50 μM MCD and 50 μM 2-methylindole.

phenol red does not affect the bromination of MCD and the rate of MCD bromination in the absence and presence of phenol red are almost equal, indicating that MCD is preferentially brominated by V-BrPO (Figure 2A). 2-Methylindole, on the other hand, appears to be a much better substrate than MCD (Figure 2B), since MCD is not brominated in the presence of an equimolar amount of 2-methylindole.

The extent of MCD bromination by V-BrPO in the presence of an equimolar competing substrate is given in Table 1. Most of the indoles examined were brominated preferentially over MCD, with the exception of indole-3-acrylic acid, 5-amino-indole and indoxyl-*K*-D-glucoside. Cytosine, trans-cinnamic acid and phenol red were found to be very poor substrates when compared to MCD, which is supported by the observation of concomitant formation of O_2 in the case of cytosine and phenol red (Soedjak and Butler, unpublished results).

Substituent Effect on the Rate of Bromination.

The rate of bromination of the indole derivatives was investigated to determine the effect of substituents at the C-2 and C-3 positions. We originally anticipated that as the bulkiness of substitutents increased, that indole binding to V-BrPO would decrease and that the competing O_2 formation reaction would increase, because the indole derivative would have been too large to fit in the active site channel. The initial rate (i.e., at <1 min) of the indole bromination was found

Table 1. Substrate specificity of V-BrPO.[a, b]

Competing substrate[c]	MCD reacted (%)	Competing substrate	MCD reacted (%)
cytosine	99.0	indole-3-acetic acid	47.2
trans-cinnamic acid	98.9	farnesol	46.6
phenol red	97.4	3-methylindole	32.9
indole-3-acrylic acid	62.0	5-hydroxyindole	23.8
5-aminoindole	60.1	2-phenylindole	18.9
indoxyl-β-D-glucoside	59.8	2-*tert*-butylindole	5.8
1,3,5-trimethoxybenzene	57.0	2-methylindole	0.5
indole-3-methanol	47.6		

[a] Reaction conditions: 970 μM H_2O_2, 50 mM KBr, 50 μM MCD, 50 μM competing substrate and 2 nM V-BrPO in 100 mM sodium phosphate buffer, pH 6.5 with 10% ethanol.
[b] As determined according to equation 2 (see 'Materials and Methods').
[c] The products of the V-BrPO catalyzed reaction of 2-phenylindole, 2-methylindole, and 3-methylindole are 3-bromo-2-phenylindole, 3-bromo-2-methylindole, and 3-methyl-2-oxindole, respectively (Tschirret-Guth, 1996; Tschirret-Guth and Butler, 1994). Cytosine and 1,3,5-trimethoxybenzene are brominated to 5-bromocytosine and 2-bromo-1,3,5-trimethoxybenzene (Soedjak & Butler, 1990). Farnesol is converted to the terminal bromohydrin (Butler and McAdara,unpublished results). The products and 2-*tert*-butylindole, trans-cinnamic acid, indole-3-acrylic acid, 5-aminoindole, indoxyl-β-D-glucoside, indole-3-methanol, and indole-3-acetic acid have not been characterized yet.

to decrease as a function of the substituent, in the following order: 3-phenylindole ~ 3-*tert*-butylindole > 3-methylindole > 1,3-di-*tert*-butylindole ≫ 2-*tert*-butylindole (Table 2). A lag phase is observed for 2-*tert*-butylindole and, to a lesser extent, 1,3-di-*tert*-butylindole (Figure 3). The cause of this lag phase is not known, however it be could due to substrate inhibition: as the substrate is consumed, the concentration of inhibitor would decrease, and an increase in the rate of the reaction would be observed.

Concomitant dioxygen formation is not observed during the bromination of all the indoles in Table 2. Surprisingly, the rates of bromination of the indoles do not match the rate of dioxygen production in the absence of any substrate. Under the reaction conditions, the rate of the bromide-assisted disproportionation of hydrogen peroxide forming dioxygen is 10.1 ± 0.6 μM/min. In the case of 3-phenylindole, 3-*tert*-butylindole, and 3-methylindole, the rate of substrate bromination is faster than the rate of dioxygen production in the absence of substrate (Table 2). The rate of 2-*tert*-butylindole is, however, slower than the rate of dioxygen production in absence of substrate. 2-*tert*-Butylindole is completely consumed forming a single, but as yet unidentified product using only one equivalent of H_2O_2; thus no side reactions are occurring. Thus the oxidized bromine intermediate in the reaction with 2-*tert*-butylindole must differ from that in the dioxygen formation reaction.

With MCD bromination, the rate of bromination of MCD (at >75 μM MCD) and the rate of dioxygen formation (in the absence of MCD) are the same (Everett and Butler, 1989). The enzyme kinetics suggest the intermediate is the same in both reactions (Everett *et al.*, 1990b). If the MCD concentration is decreased, then both MCD bromination and dioxygen formation occur simultaneously. Under these conditions, the sum of the rates of dioxygen formation and MCD bromination is equal to the rate of of dioxygen formation in the absence of MCD (Soedjak *et al.*, 1995), which further suggests that the intermediate is the same in the MCD bromination and dioxygen formation reactions and formed in a rate limiting step. The same intermediate cannot be formed with 2-*tert*-butylindole because the rate of bromination of 2-*tert*-butylindole is slower than the rate of dioxygen formation

Table 2. Rate of bromination of substituted indole by V-BrPO.

Substrate	Initial rate of substrate bromination[a] (μM/min)	Initial rate of dioxygen production[b] (μM/min)
3-phenylindole	17.7	0.03
3-*tert*-butylindole	17.4	0
3-methylindole	14.9	0
1,3-*di*-*tert*-butylindole	9.5	0
2-*tert*-butylindole	2.7	0

[a] Calculated from the data in Figure 3 at $t < 1$ min.
[b] The rate of dioxygen production in the absence of substrate is 10.1 ± 0.6 μM/min.

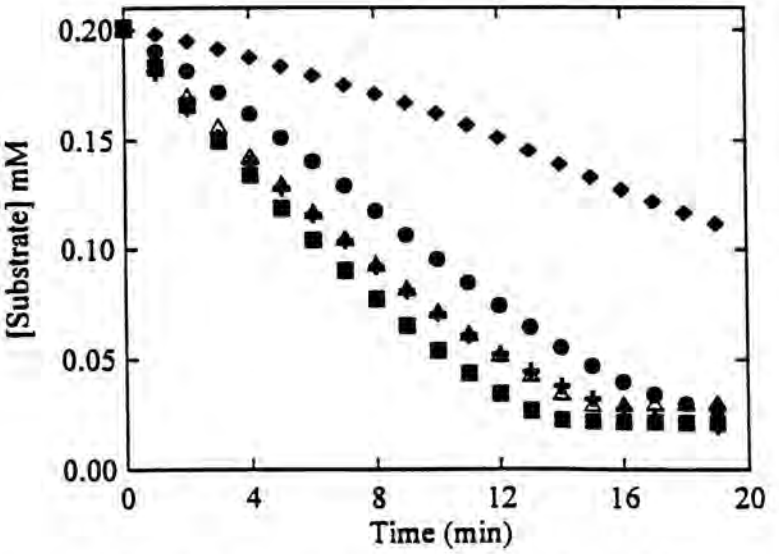


Fig. 3. Bromination of substituted indoles by V-BrPO. (✚), 3-phenylindole; (△), 3-methylindole; (■), 3-*tert*-butylindole; (●), 1,3-di-*tert*-butylindole (◆), 2-*tert*-butylindole. Conditions: 0.22 mM H_2O_2, 50 mM KBr, 0.20 mM substrate, and 2 nM V-BrPO in 100 mM sodium phosphate, pH 6.50 with 20% ethanol.

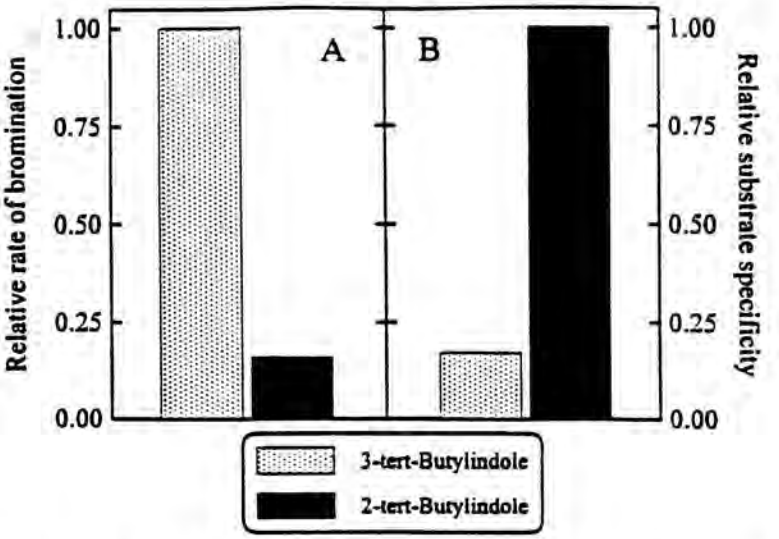


Fig. 4. Comparison of the reactivity of V-BrPO with 2-*tert*-butylindole and 3-*tert*-butylindole. Panel A: Relative rates of substrate bromination measured in separate assays (Data from Table 2). Panel B: Relative amount of substrate brominated in a competition experiment: Conditions: 200 mM H_2O_2, 50 mM KBr, 208 mM 2-*tert*-butylindole, and 204 mM 3-*tert*-butylindole, 2.0 nM V-BrPO in 100 mM sodium phosphate, pH 6.5 with 20% ethanol.

The initial rate of bromination of 2-*tert*-butylindole is only 15% of the initial rate of bromination of 3-*tert*-butylindole (Figure 4A). It is expected in a competition experiment with both substrates present in equimolar amounts, that 3-*tert*-butylindole would be favorably brominated over 2-*tert*-butylindole. However, the opposite result occurred (Figure 4B): 2-*tert*-butylindole is preferentially brominated over 3-*tert*-butylindole. This result is consistent with binding of 2-*tert*-butylindole to V-BrPO, blocking the release of an oxidized halogen intermediate.

Inhibition of V-BrPO by Organic Compounds.

We have observed that some indoles appear to inhibit V-BrPO at high substrate concentrations. Accordingly, it was of interest to investigate whether V-BrPO could be inhibited by organic compounds structurally related to indole. The rate of dioxygen production observed in the presence of different compounds that are not brominated by V-BrPO is shown in Table 3. 3-Nitroindole and benzimidazole do not inhibit V-BrPO whereas benzofuran and 2-*tert*-butylbenzofuran clearly inhibit dioxygen production. Inhibition by 3-*tert*-butylbenzofuran appears to be weaker than the two other benzofurans tested.

Inhibition of dioxygen production by 2-*tert*-butylbenzofuran and 3-*tert*-butylbenzofuran could not be investigated in greater detail due to the poor miscibility of those compounds with water. Only the inhibition by benzofuran was further studied. The double reciprocal plot of the rate of dioxygen formation versus the hydrogen peroxide concentration at various benzofuran concentrations is shown in Figure 5. The effect of benzofuran generates a competitive pattern with respect to hydrogen peroxide. However, the plot of the slope of each linear fit as a function of the corresponding benzofuran concentration does not produce

Table 3. Effect of 3-nitroindole, benzofurans, and benzimidazole on the production of dioxygen by V-BrPO.

Compound tested	Rate of O_2 production in the absence of the compound μM/min[a]	(Compound) mM	Rate of O_2 production in the presence of the compound μM/min
3-Nitroindole	9.4	0.22	9.4
Benzofuran	11.7	1.35	9.2
3-*tert*-Butyl-benzofuran	11.7	1.34	10.5
2-*tert*-Butyl-benzofuran	11.7	1.28	8.5
Benzimidazole	11.7	1.13	10.3
		5.44	10.7

Reaction conditions: 0.1 mM H_2O_2, 50 mM potassium bromide, 2 nM V-BrPO and the specific compound in 100 mM sodium phosphate, pH 5.95 with 10% ethanol.
[a] The variations observed in this column are due to the fact that different batches of V-BrPO were used.

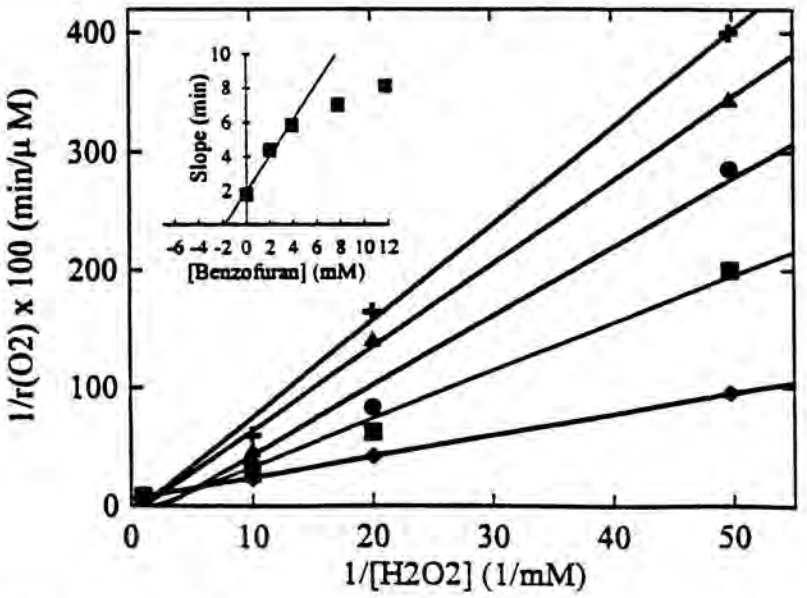


Fig. 5. Inhibition of dioxygen production by benzofuran. Benzofuran: (◆), 0 mM; (■), 2.0 mM; (●), 3.9 mM; (▲), 7.8 mM; (✚), 11.8 mM. Conditions: 50 mM KBr and 2.0 nM-BrPO in 100 mM sodium phosphate pH 6.5 with 20% ethanol.

a straight line (Figure 5, insert). The departure from the generally observed linear behavior is most likely due to the poor miscibility of benzofuran in 20% ethanol at concentrations above 4 mM. A value of the inhibition constant (K_i) for benzofuran was estimated to be ca.1.4 mM by using only the first three data points and calculating the x-axis intercept of the corresponding linear fit. The fact that benzofuran is a competitive inhibitor with respect to hydrogen peroxide suggests that binding of benzofuran must occur near to the active site of V-BrPO and in so doing prevent access of hydrogen peroxide to the vanadium center.

Conclusions

In addition to 2-methylindole (Tschirret-Guth and Butler, 1994), we have shown that other substituted indole substrates, and indole-like compounds (e.g., benzofuran) can bind to V-BrPO blocking the release an oxidized halogen intermediate. The nature of the binding of the indoles to V-BrPO (*A. nodosum*) cannot be immediately determine, because the structure of this enzyme has not been reported yet.

Recently, the crystal structure (2.11 Å resolution) of vanadium chloroperoxidase isolated from *C. inaequalis* was reported by Messerschmidt and

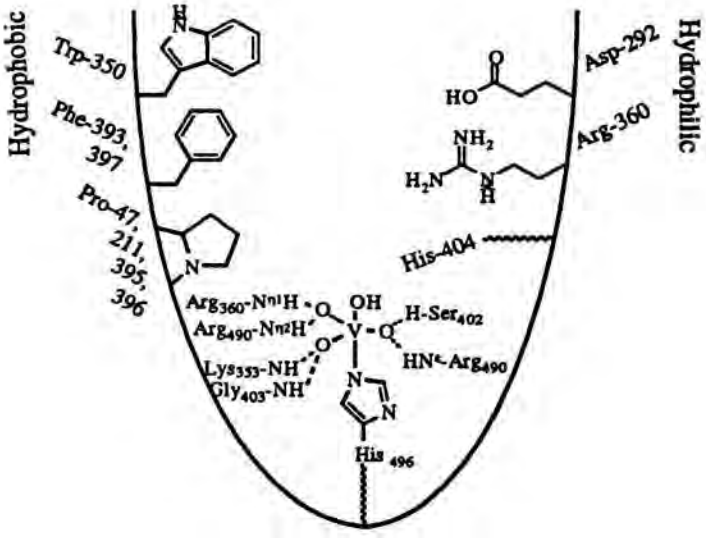


Fig. 6.

Wever (1996). The main structural motif is α-helical, with two four-helix bundles. Vanadate is coordinated at the top of one of these bundles in a broad channel which is lined on one half with predominantly polar residues including an ion-pair between Arg-360 and Asp-292 and several main chain carbonyl oxygens (Figure 6). The other half of the channel is hydrophobic, containing Pro-47, Pro-211, Try-350, Phe-393, Pro-395, Pro-396, and Phe-397.

Vanadate is bound in a pentagonal bipyramidal geometry ligated by four non-protein oxygen atoms, and histidine-496. Vanadium coordination to the protein is stabilized by multiple hydrogen bonding between the vanadate oxygen atoms and the positively charged protein residues Lys-353, Arg-360, Arg-390, and Ser-402, as well as the amide nitrogen proton of Gly-403. Messerschmidt and Wever (1996) propose that the hydrophobic residues Trp-350 and Phe-397 form a chloride binding site along with His-404; a hydrophobic binding site for halides is observed in other proteins such as haloalkane dehalogenase (Verschueren *et al.*, 1993) and certain amylases (Machius *et al.*, 1995). His 404, which is present in the active site channel, must be deprotonated for H_2O_2 to bind to V-ClPO (van Schijndel *et al.*, 1994), and thus it is thought to function in acid-base catalysis.

The full sequence of V-BrPO (*A. nodosum*) has also not been reported, but from what is known (Vilter 1995) sequence similarity is found between V-ClPO (*C. inaequalis*) and V-BrPO (*A. nodosum*), particularly in the active site region. The similarities include regions containing four of the five amino acid residues which hydrogen bond to the vanadate oxygens (i.e. Arg-360, Ser-402, Gly-403, and Arg-490), the histidine ligand (His-496), and the acid-base histidine (His-404) (Simons *et al.*, 1995; Vilter, 1995; Messerschmidt and Wever, 1996). In the proposed halide binding site (Messerschmidt and Wever, 1996), Trp-350 is

present in both V-BrPO and V-ClPO, but Phe-397 is replaced by a histidine residue in V-BrPO, raising questions about the basis of halide specificity of each enzyme. Given the hydrophobic nature of the indole derivatives used in this study and the hydrophobic portion of the active site channel, one can better understand the binding of indoles to V-BrPO.

Acknowledgements

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Bromoperoxidase from a Marine Red Macro-alga, *Corallina pilulifera*

Abstract

We studied the distribution of bromoperoxidase in marine algae. As a result, we selected the red alga, *Corallina pilulifera*, as a high producer of the enzyme. The enzyme was purified to homogeneity and characterized. The enzyme had a molecular mass of 790 kDa and was composed of 12 subunits which gave hexagonal molecular shapes of the enzyme. The enzyme was the non-heme haloperoxidase and contained a small amount of vanadium (4 moles V/mole of enzyme) as well as iron (III) and magnesium. When vanadate (VO_4^{3-}) was added to the dialyzed enzyme which had lost 77% of its brominating activity, the activity increased proportionally to the added amounts of vanadate. EPR analysis revealed that the enzyme as isolated was found to contain vanadium. From these results, it was concluded that vanadium was a prosthetic group of the enzyme. We also studied the application of the enzyme in the production of various halogenated compounds, such as the conversion of phenol to tribromophenol, anisole to *o*- and *p*-bromoanisoles, 1-methoxynaphthalene to 1-methoxy-4-bromonaphthalene and thiophene to 2-bromothiophene.

Introduction

Many physiologically active, halogenated compounds have been found in marine algae. Haloperoxidases, which catalyze the following halogenating reaction: $AH + H_2O_2 + H^+ + X^- \rightarrow AX + 2H_2O$ (where AH is a nucleophilic substrate, and X = Cl, Br, I), are known to be involved in the biosynthesis of these halometabolites. Presently marine biotechnology is attracting considerable attention from both applied and basic sciences. Therefore, we have studied the distribution of haloperoxidases in marine macro-algae and purified and characterized the bromoperoxidase from a red alga, *Corallina pilulifera*.

Distribution of bromoperoxidase activities in marine macro-algae

Bromoperoxidase activities were measured for over 60 macro-algal samples, including 34 genera of Rhodophyta, Chlorophyta and Phaeophyta, which were

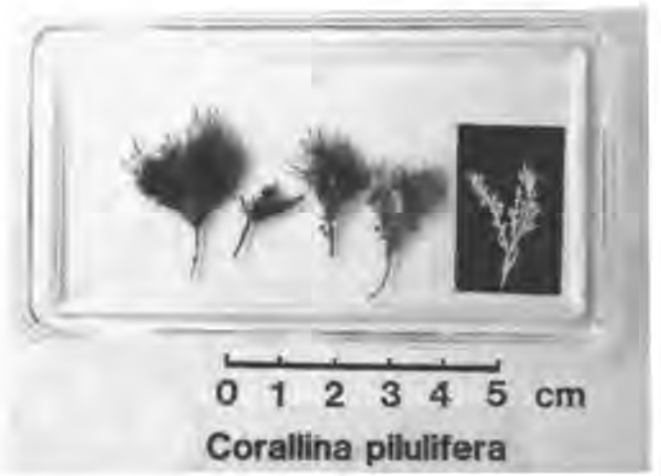


Fig. 1. The marine red macro-alga, *Corallina pilulifera*, the highest bromoperoxidase producer

collected from the seasides of Japan (Yamada *et al.*, 1985; Itoh *et al.*, 1987b). All the coralline algae (8 species) tested showed relatively high enzyme activities. We selected *Corallina pilulifera* for the following experiments not only because this alga was widely found on seahores of any place in Japan but also because it could be collected abundantly and easily (Fig. 1).

Purification and characterization of bromoperoxidase from *C. pilulifera*

Bromoperoxidase was purified from the crude extract of *C. pilulifera* to complete homogeneity (Itoh *et al.*, 1985). From the result that the overall purification was 36-fold, the content of the enzyme in the alga was found to be as much as 3% of the total protein of the crude extract. The enzyme had a molecular mass of about 790 kDa and was composed of twelve subunits of identical molecular mass of 64 kDa. A hexagonal molecular shape of the enzyme was observed by electron microscopy (Itoh *et al.*, 1986) (Fig. 2a) and the complete structure of the enzyme was concluded to be a dodecad aggregate composed of two hexagons face to face, as schematically illustrated in Fig. 2b.
Table 2 summarizes the properties of the enzyme (Itoh *et al.*, 1986; Krenn *et al.*, 1989). The enzyme was specific for I^- and Br^-, and inactive toward Cl^- and F^-. The enzyme was found to contain Fe^{3+}, Mg^{2+} and V5+ (Fig. 3), among which V^{5+} (vanadate, VO_4^{3-}) played a role as a prosthetic group. As shown in Table 3, there was a good correlation between vanadium content and specific activity, whereas no correlation between iron content and specific activity was observed (Krenn *et al.*, 1989). Ferric ion markedly shortened the time required for the full activation of the apo-enzyme by vanadate (Izumi *et al.*, 1992). We also studied the application of the enzyme in the production of various halo-

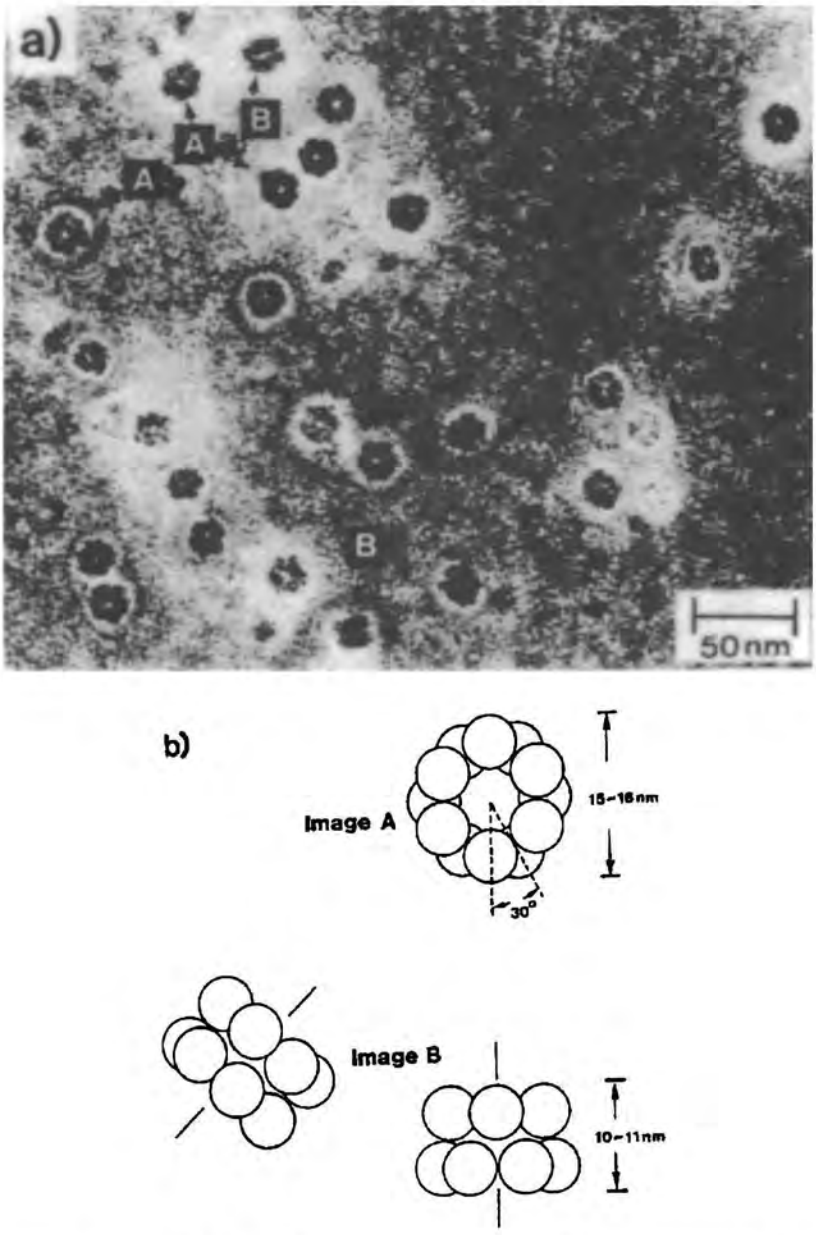


Fig. 2. Electron micrograph (a) and schematic model of the subunit structure of the bromoperoxidase of *Corallina pilulifera*

Table 1. Purification of bromoperoxidase from *Corallina pilulifera*.

Procedure	Protein (mg)	Total activity[a] (Units)	Total activity[b] (Units)	Specific activity[a] (U/mg)	Specific activity[b] (U/mg)
Crude extract	5,933	4,158	7.954	0.7	1.3
Ammonium sulphate (80%)	2,754	1,356	5,965	0.5	2.2
DEAE column (1)	378	973	5,481	1.9	10.7
DEAE column (2)	189	348	5,250	1.8	27.8
Sepharose 6B column	96	317	4,347	3.3	45.2
Cellulofine GC-700	41	149	1,930	3.6	47.1

The enzyme activity in the various fractions was assayed directly (a) and after incubation with vanadate (1 mM, 25°C) (b).

Table 2. Properties of the bromoperoxidase from *Corallina pilulifera*.

Molecular mass	790 kDa
Subunit structure	64 kDa × 12
Isoelectric point	3.0
Carbohydrate residue	–
Halide specificity	Br^-, I^-
Optimum pH	6.0
Km for H_2O_2	9.2×10^{-5} M
Km for Br^-	1.1×10^{-2} M
Catalase activity	+ (halide-dependent)
Peroxidase activity (*o*-dianisidine)	–
Prosthetic group	vanadium (V^{5+})
Metal ions	Fe^{3+}, Mg^{2+}

Table 3. Metal analysis and specific activity of *Corallina pilulifera*.

	Before dialysis	After dialysis[a]
Specific activity (U/mg protein)	20.6	4.8
Vanadium content (mol/mol enzyme)	4.0	0.9
Iron content (mol/mol enzyme)	22	13

[a]dialysis against citrate/phosphate buffer (pH 3.8) containing 1 mM EDTA

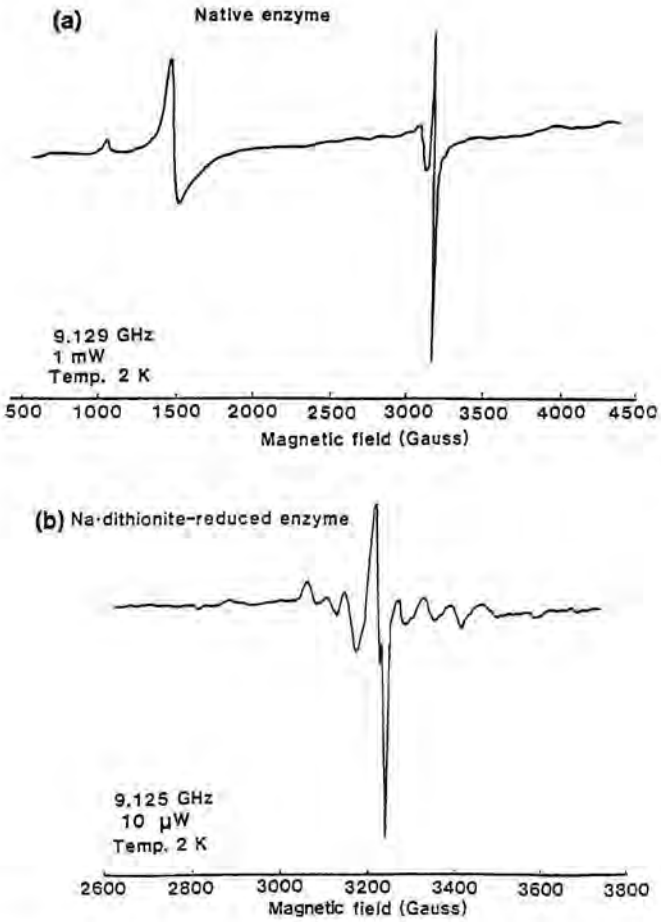
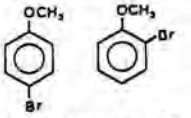
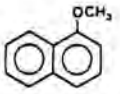
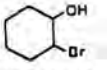
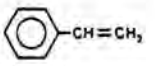
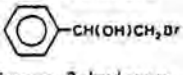
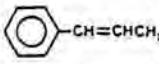
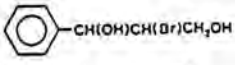
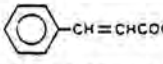
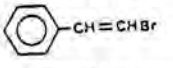
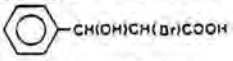


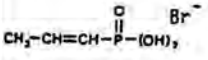
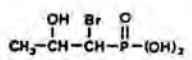
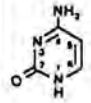
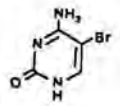
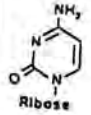
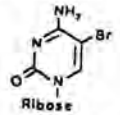
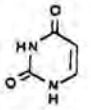
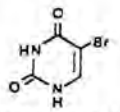
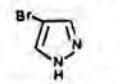
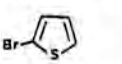
Fig. 3. EPR spectra of bromoperoxidase before (a) and after reduction (b) by dithonite.

genated aromatic compounds, such as the conversion of phenol to tribromophenol, anisole to *o*- and *p*-bromo-anisoles, 1-methoxynaphthalene to 1-methoxy-4-bromonaphthalene and thiophene to 2-bromothiophene as shown in Table 4 (Itoh *et al.*, 1987d, 1988; Izumi *et al.*, 1989). The immobilization techniques using DEAE-Cellulofine and ENT-200 were also found to be suitable for the bromoperoxidase reaction (Itoh *et al.*, 1987a). The immobilized enzyme on DEAE-Cellulofine showed a half-life of 45 days when it was used for the conversion of uracil to 5-bromo-uracil. Thus, the reactor system using the bromoperoxidase could also be used to convert the substrates shown in Table 4 with minor changes of the reaction conditions.

We have recently succeeded in cloning the gene of the enzyme, and the molecular genetical studies of the enzyme will be described elsewhere.

Table 4. Production of various halogenated compounds by bromoperoxidase.

Substrate	Halide	Product
 phenol	Br^-	 2,4,6-tribromophenol
 o-hydroxybenzyl alcohol	Br^-	 2,4,6-tribromophenol
 anisole	Br^-	 p- and o-bromoanisole
 1-methoxy-naphthalene	Br^-	 1-methoxy-4-bromo-naphthalene
 cyclohexene	Br^-	 trans-1-hydroxy-2-bromocyclohexane
 styrene	Br^-	 DL-1-bromo-2-hydroxy-2-phenylethane
 trans-cinnamyl alcohol	Br^-	 (±)-1,3-dihydroxy-2-bromo-3-phenylpropane
 trans-cinnamic acid	Br^-	 trans-β-bromostyrene
		 (±)-erythro-2-bromo-3-hydroxy-3-phenylpropionic acid

Substrate	Halide	Product
 cis-propenyl-phosphonic acid	Br^-	 (±)-threo-1-bromo-2-hydroxypropylphosphonic acid
 cytosine	Br^-	 5-bromocytosine
 cytidine	Br^-	 5-bromocytidine
 uracil	Br^- (I^-)	 5-bromouracil (5-iodouracil)
 pyrazole	Br^- (I^-)	 4-bromopyrazole (4-iodopyrazole)
 thiophene	Br^-	 2-bromothiophene

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A. Messerschmidt, L. Prade and R. Wever

X-Ray Structures of the Native and Peroxide Form of the Vanadium-Containing Enyme Chloroperoxidase from the Fungus *Curvularia inaequalis*

Abstract

The crystal structures of the native and peroxide forms of a chloroperoxidase from the fungus *Curvularia inaequalis* have been solved by difference Fourier techniques using the atomic model of the azide chloroperoxidase complex. The 2.03 Å crystal structure (R = 19.7%) of the native enzyme reveals the geometry of the intact catalytic vanadium center. The vanadium is coordinated by four non-protein oxygen atoms and one nitrogen (NE2) atom from histidine 404 in a trigonal bipyramidal fashion. Three oxygens are in the equatorial plane and the fourth oxygen and the nitrogen are at the apexes of the bipyramid. In the 2.24 Å crystal structure (R = 17.7%) of the peroxide derivate the peroxide is bound side-on to the vanadium after the release of the apical oxygen ligand. The vanadium is coordinated by 4 non-protein oxygen atoms and one nitrogen (NE2) from histidine 496. The coordination geometry around the vanadium is that of a distorted tetragonal pyramid with the two peroxide oxygens, one oxygen and the nitrogen in the basal plane and one oxygen in the apical position. A mechanism for the catalytic cycle has been proposed based on these x-ray structures and kinetic data.

Introduction

Haloperoxidases form a class of enzymes that are able to oxidize halides (Cl^-, Br^-, I^-) in the presence of hydrogen peroxide to the corresponding hypohalous acids according to:

$$H_2O_2 + X^- + H^+ \rightarrow H_2O + HOX.$$

If a convenient nucleophilic acceptor is present, a reaction will occur with HOX to form a diversity of halogenated reaction products. Many of these organohalogens have biocidal effects and thus may provide defense functions.

As has only recently discovered (Vilter, 1984; de Boer *et al.*, 1986), some of the haloperoxidases contain vanadium in the active site. These enzymes are

widespread in the marine environment and have been found in a variety of seaweeds (Wever *et al.*, 1991).

Recently it has been shown (van Schijndel *et al.*, 1993) that the chloroperoxidase (CPO) secreted by the fungus *Curvularia inaequalis* is also a vanadium enzyme with properties similar to the bromoperoxidase. This CPO forms HOCl as a product (van Schijndel *et al.*, 1994), which is a strongly bactericidal and oxidizing agent. The fungus belongs to the group of dematiaceous hyphomycetes, which are pathogenic towards plants and/or are saprophytes, and it has been suggested (Simons *et al.*, 1995) that the enzyme and its product are used in a mechanism to oxidize plant cell walls to facilitate penetration of the fungus into the host.

The vanadium bromoperoxidases have been studied in great detail using a variety of biophysical techniques (Wever and Kustin, 1990) including extended x-ray absorption fine structure (Arber *et al.*, 1989; Carrano *et al.*, 1994) and spin echo envelope modulation (de Boer *et al.*, 1988a; de Boer *et al.*, 1988b). Vanadium(IV) or (III) states have not been observed by EPR or K-edge x-ray studies in the presence of substrates or during turnover, and the reduced enzyme is inactive. Apparently the redox state of the metal does not change during catalytic turnover, and a model has been suggested (de Boer *et al.*, 1988b) in which the vanadium site functions by binding hydrogen peroxide to yield an activated peroxo intermediate, which is able to react with bromide to produce HOBr. Similar models have been proposed (Meister and Butler, 1994; Espenson *et al.*, 1994) for a number of metal-catalyzed oxidations of bromide by hydrogen peroxide.

A detailed kinetic study of the formation of HOCl by the chloroperoxidase (van Schijndel *et al.*, 1994) revealed many similarities with the kinetics of the vanadium bromoperoxidase.

The crystal structure of the vanadium CPO from *Curvularia inaequalis* has been solved recently (Messerschmidt and Wever, 1996) and modelled as azide chloroperoxidase complex. Here we report the crystal structures of the native and peroxide form of the enzyme.

Crystal Structure Determinations

The crystal structure analysis of the azide complex of CPO has been described in detail (Messerschmidt and Wever, 1996). Briefly, the structure has been solved by the method of multiple isomorphous replacement and refined to a crystallographic R-value of 20% at a resolution of 2.1 Å. Crystals from the native form have been obtained by using an azide-free buffer. The other crystallization conditions were the same as for the azide complex. The peroxide complex was produced by soaking the crystals in mother liquor (2.0 M $(NH_4)_2SO_4$, in 0.1 M Tris-H_2SO_4, pH 8.0), containing 20 mM H_2O_2, for two hours. The x-ray measurements were done on a Hendrix/Lentfer x-ray image-plate system

(Mar-Research, Hamburg, Germany) mounted on a Rigaku rotating anode generator operated at 5.4 kW ($\lambda = CuK_\alpha = 1.5418$ Å). The native crystals were measured at 16°C and the peroxide at 100 K. For the cryo measurement the peroxide soaked crystals were brought into a cryo buffer (20 mM peroxide, 1.7 M $(NH_4)_2SO_4$, in 0.1 M Tris-H_2SO_4, pH 8.0, 30.1% glycerol), transferred into a cryo-loop, shock frozen and kept in a stream of evaporating liquid

Data collection and refinement statistics.

Parameter	NAT2	PER3
A. Crystal parameters		
Space group	R3	R3
Unit cell constant		
a(Å)	131.72	129.95
b(Å)	131.72	129.95
c(Å)	112.46	111.71
α(°)	90	90
β(°)	90	90
γ(°)	120	120
Molecules/asymm.unit	1	1
B. Diffraction data		
Resolution (Å)	2.03	2.24
Total observations	128,746	102,166
Unique reflections	45,791	29,948
Completeness (%)*	97.5/80.4	83.8/40.2
R_{merge} §. *	0.104/0.391	0.118/0.404
C. Refinement		
Resolution range (Å)	6.0 – 2.03/2.09 – 2.03	8.0 – 2.24/2.34 – 2.24
No. of reflections in this range	44,007/2838 ($F_O > 2.0(F_O)$)	29,291/3685 (no σ-cutoff)
$R = \sum F_O\text{-}F_C / \sum F_O$ (%)	19.7/32.8	17.7/29.7
No. of atoms:		
All non-hydrogen atoms	4,940	5,097
Non-hydrogen protein atoms	4,479	4,488
Solvent	456	604
Vanadium	1	1
Oxygen vanadium site	4	4
Average temperature factor (Å^2)		
All atoms	30.85	22.17
Protein atoms	29.37	20.41
Solvent	45.58	35.27
Ligand	16.21	14.78
r.m.s deviations from standard geometries:		
Bonds (Å)	0.011	0.011
Angles (°)	1.66	1.61
Mean coordinate error (Å)§	0.23	0.23
σ of mean coordinate error (Å)§	0.01	0.04

*15.60 – 2.03/2.10 – 2.03 for NAT2 and 33.8 – 2.24/2.28 – 2.24 for PER3; §$R_{merge} = \sum\sum/I(h)_i - \langle I(h)\rangle // \sum\sum I(h)_i$, where $I(h)_i$ is the observed intensity in the *i*th source and $\langle I(h)\rangle$ is the mean intensity of reflection h over all measurements of I(h); §determined from Luzzati-plot.

nitrogen using a cryo device (Vectotherm, Karlsruhe) mounted on the image-plate system. The x-ray intensities were processed with MOSFLM (Leslie, 1990) and programs from the CCP4 suite (CCP4, 1994). Crystal parameters and data collection statistics for both structures are given in Table 1. The native form diffracts to 2.03 Å resolution with a very good data completeness whereas the data of the peroxide derivative extend to 2.24 Å resolution with satisfying data completeness (83.8%). Difference Fourier maps were calculated for both structures refining the azide complex model against the relevant observed structure factors omitting the vanadate azide group and the solvent molecules additionally in the peroxide form. The model was refined by energy-restrained crystallographic refinement with XPLOR (Bruenger, 1992), and the parameters were derived by Engh and Huber (1991). The refinement statistics of the current models are given in Table 1. Both structures refine to crystallographic R-factors below 20%. The models maintain strict geometry with deviations from ideal values for bond lenghts and angles of 0.011 Å and 1.7°, respectively. For the native form, residues 1-3 and 578-609 and for the peroxide form, residues 1-2 and 579-609 are not contained in the model because they are not defined by electron density and are probably mobile or disordered. The occupancies of residues 118-127 were set to zero for the same reasons as in the azide complex (see Messerschmidt and Wever, 1996).

Results and Discussion

The vanadium site of the native form

The difference electron density map of the native form at the vanadium site (Fig. 1) obtained by refining the model of the azide form without the vanadate and azide groups against the observed structure factors of the native form shows the vanadium bound to 4 oxygen atoms forming an orthovanadate group and to the NE2 nitrogen atom of histidine 496. The vanadium coordination geometry is trigonal bipyramidal with 3 oxygen atoms in the equatorial plane (bond lengths about 1.65 Å), one apical oxygen atom (bond length 1.93 Å) and the other apical nitrogen atom of histidine 496 (bond length 1.96 Å). Fig. 2 shows the final $2F_O$-F_C electron density map demonstrating the good quality of the electron density map. Further it displays three well resolved water molecules (1262, 1449 and 1482) in the active site. Fig. 3 presents the hydrogen bonding pattern around the vanadium active site. The bond length of the apical oxygen OV4 to the vanadium is with 1.93 Å in the range of an OH ligand indicating that the VO_4 group is bound as hydrogen vanadate(V). The negative charge of the hydrogen vanadate(V) group is compensated by hydrogen bonds to surrounding positively charged or hydrophilic protein functions. The apical oxygen OV4 forms three hydrogen bonds, two to water molecules (SOL 1440, 2.59 Å; SOL 1262, 2.92 Å), one to nitrogen ND1 of histidine 404 (2.97 Å). The latter one seems to be important because histidine 404 has been identified as necessary for

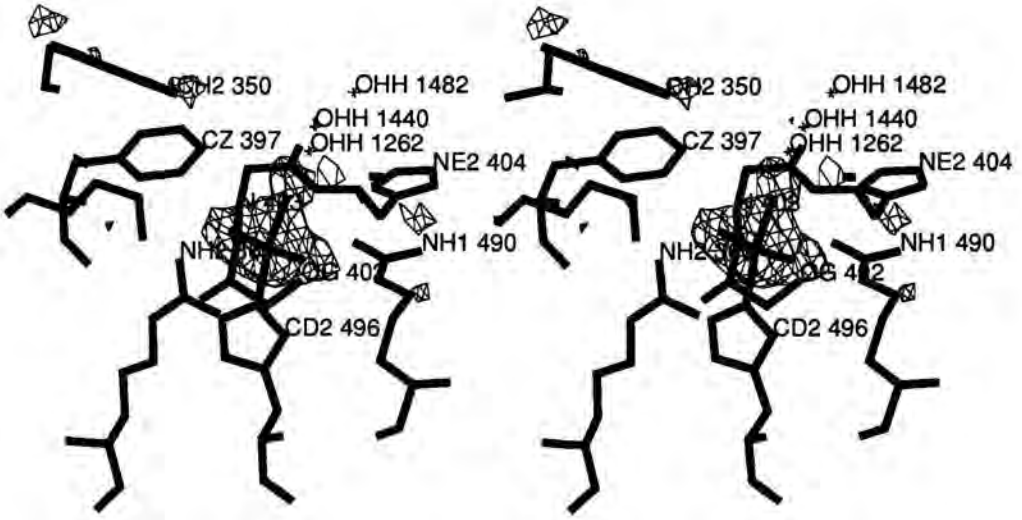

Fig. 1. Difference electron density map of the native form of CPO around the vanadium-binding site contoured at 3.0 σ. The difference electron density for the hydrogen orthovanadate(V) is the strongest feature in the whole map.

the catalytic activity (see Messerschmidt and Wever, 1996). Binding of peroxide is inhibited when histidine 404 is doubly protonated.

The vanadium site of the peroxide form

The difference electron density map of the peroxide form at the vanadium site is shown in Fig. 4. The phases used for the calculation of this map have been obtained from an atomic model which did not contain the vanadate group and the two water molecules 1420 and 1377. The solvent structure (data set measured at 100 K) had been generated by several cycles of model building and crystallographic refinement. The difference electron density demonstrates that the peroxide has reacted with the vanadate group. The apical oxygen OV4 has been released and the peroxide binds side-on in the equatorial plane to the vanadium. Positive electron density is visible for two additional water molecules in the active site (1165, 1420). The vanadium peroxide complex has 5 direct ligands only. The coordination geometry is that of a distorted tetragonal pyramid. The apical ligand is oxygen OV3 (bond length about 1.60 Å) identifying this as a V = O bond. The two peroxide oxygens OV2 and OV4 (bond lengths: V-OV2 and V-OV4, about 1.87 Å; OV2-OV4, 1.47 Å), oxygen OV3 (bond length 1.93 Å), and nitrogen NE2 from histidine 496 (bond length 2.19 Å) constitute the basal plane. One empty apical coordination site at the vanadium is sterically blocked by the side chain of arginine 360. The empty coordination site generated by the release of OV4 seems to be predestinated to accept the chloride ion during continuation of turnover. The bound peroxo species may be

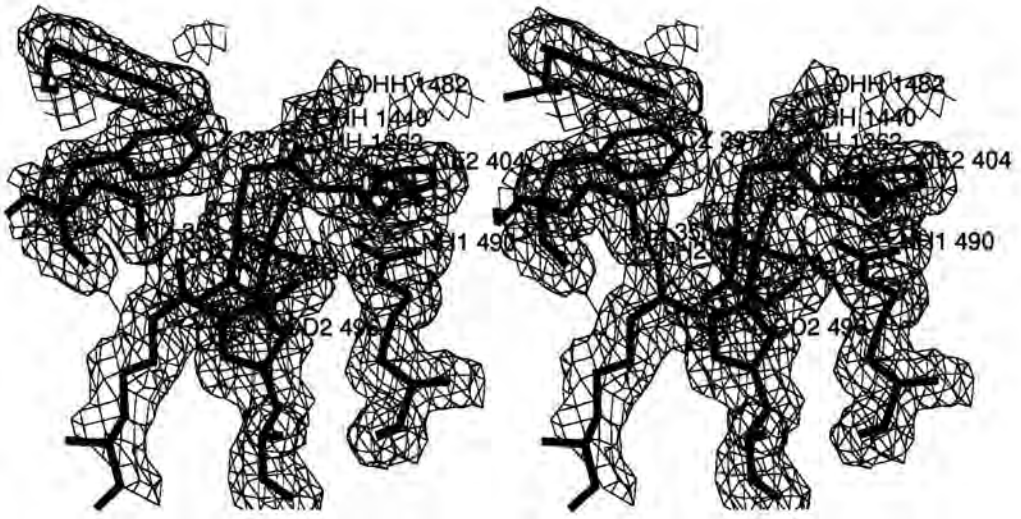

Fig. 2. Final $2F_O$-F_C electron density map of the native form of CPO around the vanadium-binding site contoured at 1.0 σ.

addressed as a monoperoxo-metavanadate(V). The final $2F_O$-F_C electron density map of the peroxide derivative around the vanadium site (Fig 5.) documents the good quality of the electron density map. The hydrogen bonding network

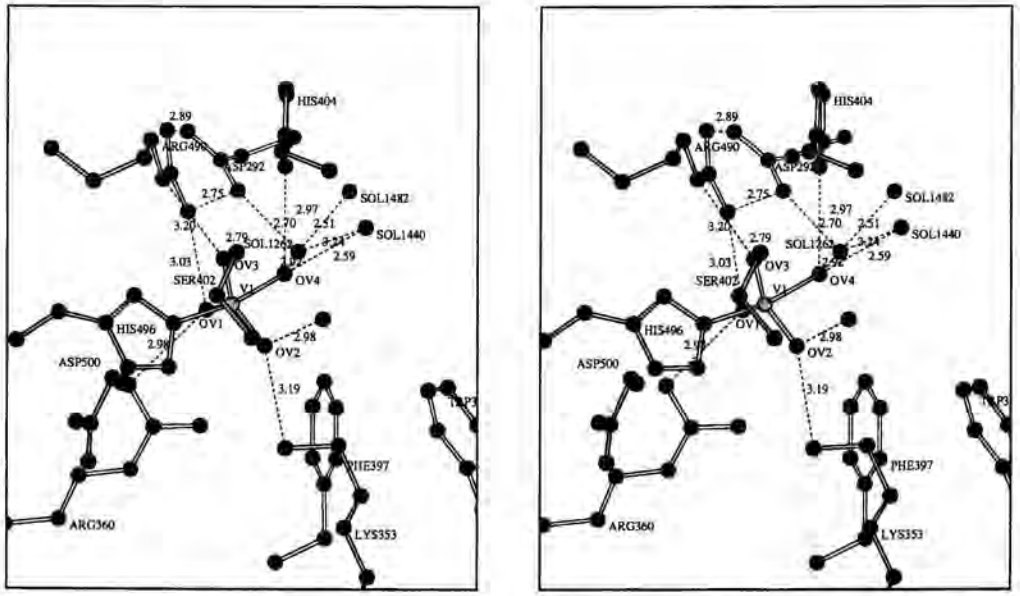

Fig. 3. Hydrogen-bond pattern of the native form of CPO around the vanadium-binding site. The figure has been made with MOLSCRIPT (Kraulis, 1991).

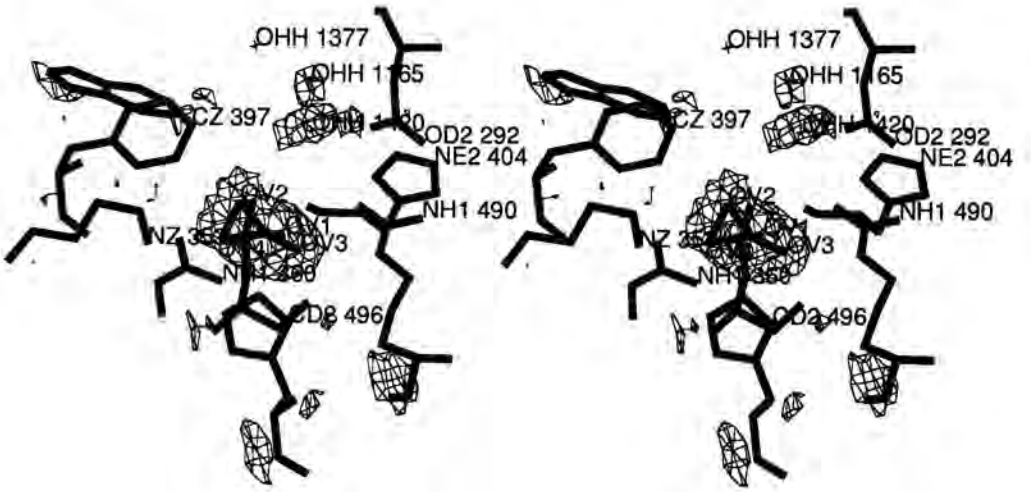

Fig. 4. Difference electron density map of the peroxide form of CPO around the vanadium-binding site contoured at 3.0 σ. The difference electron density for the peroxide metavanadate(V) is the strongest feature in the whole map.

around the vanadium site of the peroxide derivative is depicted in Fig. 6. Histidine 404 is no longer hydrogen-bonded to any oxygen function of the vanadium group. It forms a hydrogen bond to water molecule 1420. OV4 of the

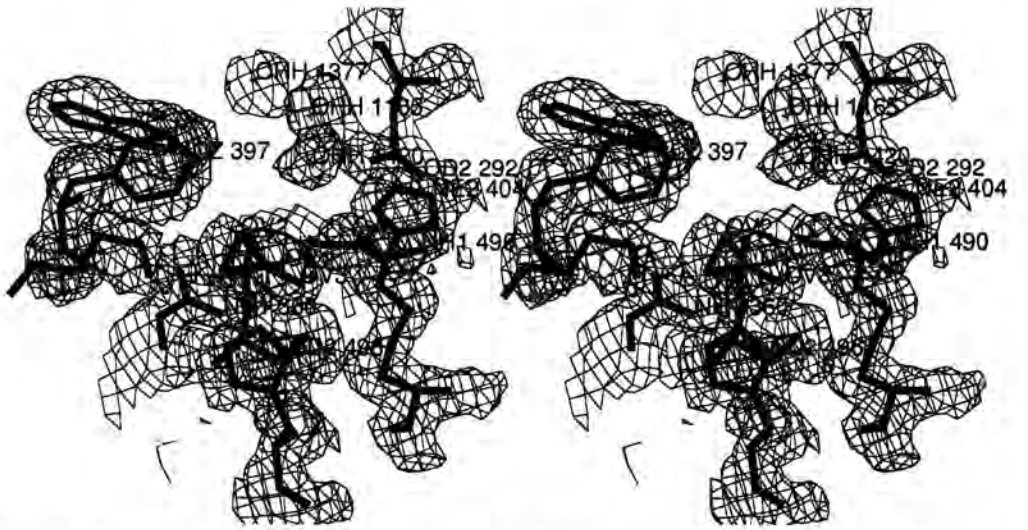

Fig. 5. Final $2F_O$-F_C electron density map of the peroxide form of CPO around the vanadium-binding site contoured at 1.0 σ.

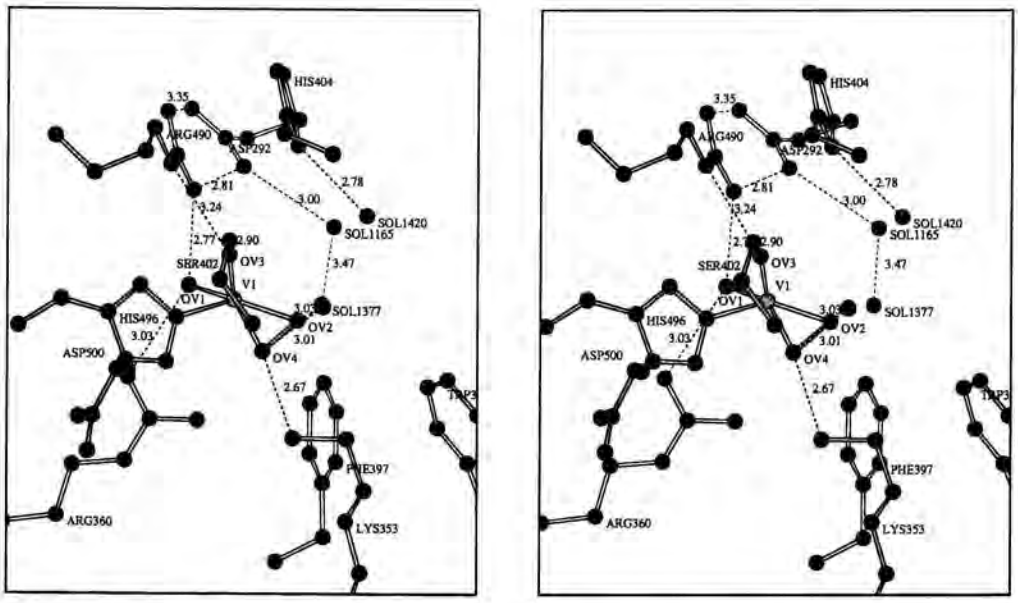

Fig. 6. Hydrogen-bonding pattern of the peroxide form of CPO around the vanadium-binding site. The figure has been made with MOLSCRIPT (Kraulis, 1991).

peroxide group is hydrogen-bonded to NZ of lysine 353 and to the amide nitrogen of glycine 403. OV2 of the peroxide group is linked via a hydrogen-bond to the same amide nitrogen. OV3 makes hydrogen-bonds to OG of serine 402 and NE of arginine 490 and OV1 to NH1 of arginine 360 and NH2 of arginine 490. The empty vanadium coordination site generated by the release of OV4 is directed towards the active site pocket supplying good access for the second substrate from the solvent.

Proposal for the catalytic mechanism

The determination of the native state and the peroxide intermediate by x-ray structure analysis makes it possible to propose a catalytic reaction scheme. Steady state kinetic studies on CPO (van Schijndel *et al.*, 1994) had shown that in the pH range between 6 and 7 the enzyme mechanism is a ping-pong type. It has also been demonstrated that peroxide binds first. The proposed catalytic mechanism is displayed in Fig. 7. We start from the native enzyme (panel 1). The apical oxygen is hydrogen bonded to His 404. This hydrogen-bond makes the apical OH-group more nucleophilic than a normally bound OH. The peroxide molecule approaches this apical OH and gets singly deprotonated. The generated apical water molecule is a weak vanadium ligand only and may leave the vanadium coordination sphere (panel 2). The hydroperoxide coordinates to the vanadium at this empty coordination site (panel 3) and the more nucleophilic oxygen OV2 abstracts the proton from the peroxide. The OH ligand is

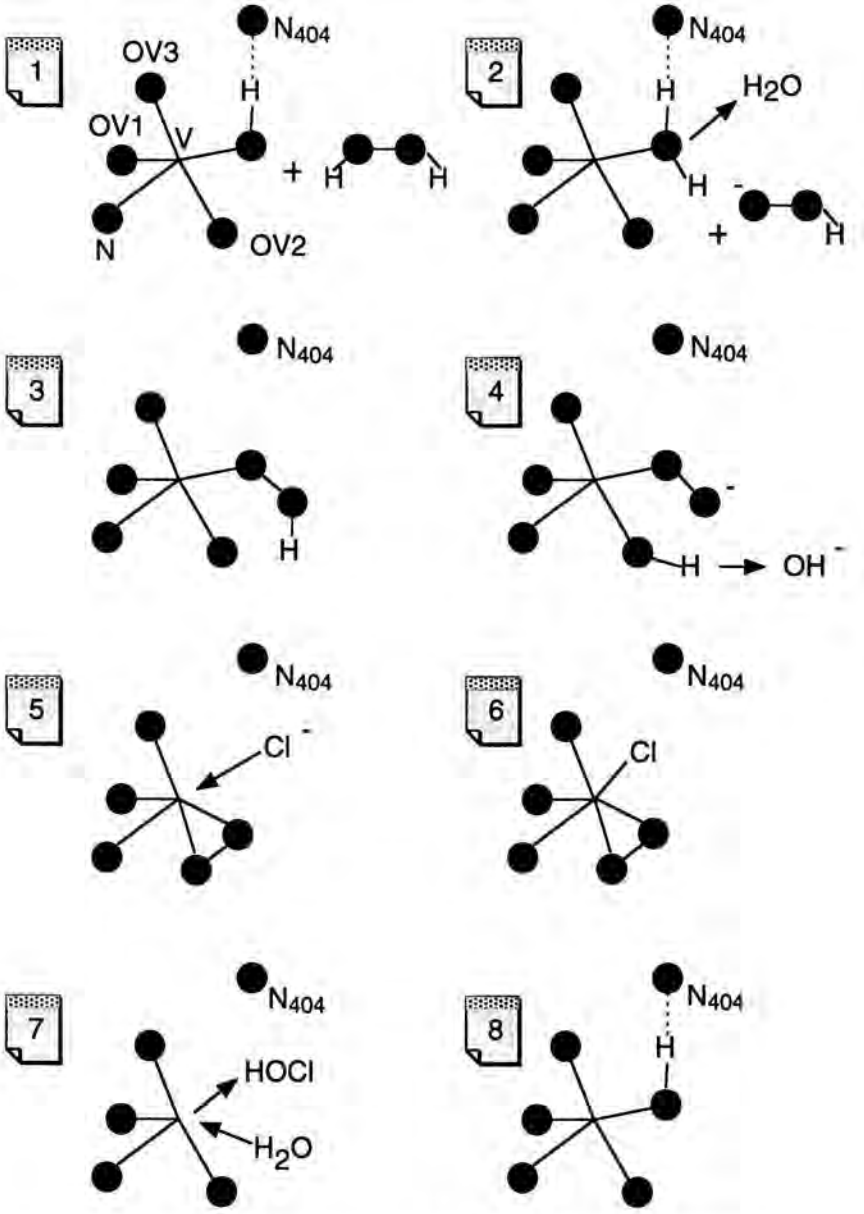

Fig. 7. Proposal for the catalytic mechanism of CPO from *Curvularia inaequalis*

displaced by the now more nucleophilic negatively charged peroxide oxygen (panel 4). The peroxide is now bound as in the peroxide complex which is observed in the crystal structure. At this stage the chloride ion binds to the empty vanadium coordination site (panel 5 and 6). The bound peroxide is an oxidizing species and accepts two electrons from the chloride. The peroxide bond will be broken after the acceptance of the first electron and the O-Cl bond will be formed and the V-O bond of the upper peroxide oxygen will be broken after uptake of the second electron. The OCl^- molecule will take up a proton of a surrounding water molecule and will leave the active site as HOCl (panel 7). The generated OH^- will coordinate to the vanadium site to rebuild the native state (panel 8). The formation of the peroxide intermediate should be impossible when His 404 is doubly protonated. In this case the apical OH group could no longer form a hydrogen bond to His 404 and would lose its ability to activate the peroxide by its deprotonation.

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R. Wever

Applications for (Halo)peroxidases

Abstract

Peroxidases are enzymes which are able to catalyse a variety of reactions. These include the classical peroxidase reaction that is the one-electron oxidation of organic aromatic compounds, the oxidation of halide ions to the corresponding hypohalous acids and as has been more recently discovered enantio-selective oxygen-transfer reactions resembling those catalysed by cytochrome P450. In this overview the potential applications of peroxidases in a variety of products and processes are discussed together with a discussion of some problems to be expected upon introduction of these enzymes on the market.

Introduction

Many enzymes have found their application in daily life although this is mainly restricted to enzymes used in detergent such as proteases, lipases, amylases and cellulases. In addition, the food industry and biotechnological fermentation industry uses a variety of other enzymes which account for more than 50% of the current applications of industrial enzymes (Hodgson, 1994). A few examples are glucose isomerase in the conversion of glucose into fructose, pectolytic enzymes in the treatment of fruit juices to allow easier filtrations, clarification and concentration of the juice and chymosin for cheese making. The use of new enzymes in other processes is finding its way also thanks to the development of gene technology which allows us to produce enzymes in question in sufficient quantities. Research activities are now also directed to the application of oxido-reductases. In particular peroxidases using hydrogen peroxide as a cheap and clean oxidant, are a point of focus of current industrial interest. For application on an industrial scale a number of requirements have to be met which may seriously delay the introduction of such enzymes on a market. Some of these are obvious, other requirements are not realized in particular by those working in academic institutions with no direct links to industry.

Important factors which determine whether or not an enzyme will penetrate a certain market is that its application in a process should be competitive towards existing processes, be cheaper or more selective and preferably produces

less environmental stress. Also, the enzymes should be stable and resistant towards oxidative inactivation, they should have large turnover number in terms of product produced or converted per unit time per molecule of enzyme and they should have a long life time and operational stability.

Another important factor is that large, preferably kilogram quantities should be available at low cost and this will in most cases rule out standard time-consuming laboratory isolation procedures from unprocessed biological materials. There are a number of sources or approaches which have advantages or are being used as an alternative to produce more of an enzyme and these will be discussed shortly.

Enzyme production

One of the methods to produce desired enzymes is the use of cultured plant cells in which the enzyme is secreted into the culture medium. Several peroxidases may be produced in this way (Maldanoldo and Van Huystee,1980, Kwak *et al.*, 1995). To culture animal cells is another option but it is very unlikely to be applicable on a larger scale than a few mg of protein. The major problem is that Chinese hamster ovarian and other cell lines which are used as producer for recombinant enzymes or proteins grow only slowly and expensive growth media are required. Furthermore, these cells secrete only small quantities of desired enzyme and this makes them unsuitable as a source for most applications. The same arguments hold for the application of *Xenopus* oocytes. Also transgenic plants may be used (Gazarin, 1995) but growing plants is a relatively slow process and the amount of enzyme produced per biomass (typically 50 mg/kilo plant) may be too low to be economical. However, an exception may be when the enzyme is localized and concentrated in a seed or fruit and which may be harvested easily. Introduction of non-natural genes into animals is now also possible, forcing the transgenic animal to produce a desired enzyme or protein (e.g. lactoferrine) in its milk. Since there are in general viable alternatives for the use of transgenic animals a strong consumer and public opposition exists in some countries.

Gene amplification or gene introduction for novel enzymes into production hosts has boosted the introduction of recombinant enzyme systems and engineered micro-organisms as filamentous fungi, yeasts, bacteria or viruses are used as sources for industrial enzymes. In particular, filamentous fungi have been successfully employed as hosts in the industrial production of extracellular (glyco)proteins. Certain industrial strains are capable of secreting gram quantities of these proteins per litre medium. In addition, filamentous fungi are able to correctly perform post-translational modifications of eukaryotic proteins and many strains have U.S. Food and Drug Administration approval. Furthermore, large scale fermentation technology and downstream processing experience is available and the cost price may be as low as 2000 $ a kilo. Thus, nowadays it

is possible to produce enzymes in bulk. This holds for simple enzymes containing no prosthetic groups. If a prosthetic group is normally present in the enzyme, the recombinant enzymes are sometimes secreted into an inactive apoform which has to be reactivated by addition of the prosthetic group. This additional reactivation step may substantially increase the costs of the enzyme.

Considering all the arguments, it is likely that that we will see other enzyme systems appearing on the market and in the production of consumables. This in particular holds for peroxidases. In this contribution some of the proposed applications of these enzymes will be discussed in terms of their properties. This review will not cover all aspects rather a selection is made which is mainly based on the more recent patent literature. The reader is also referred the overview by Neidleman and Geigert (1986) which covers the literature until 1986 and to the review by Franssen, 1997 in these Proceedings on the potential application of haloperoxidases in organic chemistry.

Use of peroxidases to label proteins with radio-isotopes

In the field of nuclear medicine, there is considerable interest in the use of radioactively labelled proteins. Iodination of a protein is most commonly used. For his application in which a peroxidase is used to iodinate proteins, commercial kits are available, e.g. that from Biorad which uses lactoperoxidase and glucose oxidase. Since this method is mild and results in a stable attachment of the label it is particular useful to label monoclonal antibodies. However, there are some disadvantages of using ^{125}I and ^{131}I in particular for patient application. Bromination with ^{77}Br is another option, an advantage is that the binding strength of the C-Br bond is stronger than that of the C-I bond and this bond strength is an important factor for the in vitro stability of the radiolabelled compound. Again, catalytic halogenation by a haloperoxidase at neutral pH may be used and a procedure has been developed using the heme-containing bromoperoxidase from the algae *Penicillus capitatus* (Manthey *et al.*, 1984). Care must be taken to control the hydrogen peroxide concentration since otherwise a sharply decreased activity is found. An alternative (Lambert and Slegers, 1994) is bromination by the vanadium bromoperoxidase from *Ascophylum nodosum*. This enzyme has also a pH optimum around neutral but its is considerably more stable (De Boer *et al.*, 1987) than its heme-containing counterpart.

Analytical applications

Peroxidases are used these days in large number of diagnostics to determine the concentration of organic metabolites. The metabolite is converted by a specific enzyme to another product in a reaction in which hydrogen peroxide is generated in a stoichiometric amount. The peroxide is quantitatively determined using a peroxidase which in most cases is horseradish peroxidase. The metabolites may be sugars (glucose, galactose, lactose, maltose, saccharose) or other

compounds such as phenol, cholesterol, urate, acetate, lactate, pyruvate and L- and D-amino acids.

The peroxidase may also be used in immuno-assays (ELISA's, monoclonal antibodies) to quantitatively detect and determine a certain antigen. In these cases the peroxidase is coupled to the antibody which will have a specific interaction with the antigen and which may be detected now by a colour reaction or chemiluminescence which is highly sensitive. A patent (Wever *et al.*, 1995) using the vanadium chloroperoxidase to determine Cl^- in a liquid has also appeared. In the method the halide is oxidized in the presence of hydrogen peroxide to hypochlorous acid which is detected colorimetrically. Since the vanadium peroxidase has a very high affinity for halides the assay allows detection of Cl^- in the μM concentration and is more sensitive than existing methods. The amounts of peroxidases which are needed in these diagnostics are relatively small and are estimated to be in the order of 50 kilo/year world wide. Thus, most industrial companies will produce these enzymes using classical large scale purification methods.

Peroxidases as a pharmaceutical/antimicrobicidal agent

It is well established (Klebanoff, 1968, Albrich *et al.*, 1981, Weiss, 1989) that the products of haloperoxidases which are formed by oxidation of a halide or a pseudohalide (Cl^-, Br^-, I^-, SCN^-) are bactericidal. This bactericidal effect may be direct but it may also be mediated by singlet oxygen formation (Kanovsky, 1984).

$$H_2O_2 + X^- + H^+ \longrightarrow \underset{\text{bactericidal}}{HOX} + H_2O \qquad (1)$$

$$H_2O_2 + HOX \longrightarrow \underset{\text{bactericidal}}{{}^1O_2} + H^+ + Cl^- + H_2O \qquad (2)$$

Several patents have appeared now in which applications of peroxidases are claimed. Most of these patents deal with mammalian peroxidases (myeloperoxidase, eosinophil peroxidase and lactoperoxidase) since these enzymes catalyse the formation of bactericidal products in vivo and are implicated in defense systems of the host. One of the first patents in this area was that by Kessler and Rosenbaum (1984) in which the peroxidases were used for killing bacteria in the treatment of dental diseases in situ such as in the oral cavity or as a denture cleaner (mouthwashes, toothpastes). Montgomery (1994) propose a similar system: an orally activated antimicrobial dentifrice that includes SCN^- and lactoperoxidase. These inventions are based upon the natural system present in

saliva and in which the salivary peroxidase generates HOSCN using hydrogen peroxide formed by metabolic active bacteria in the oral cavity.

$$\underset{\text{(generation by bacteria)}}{H_2O_2} + \underset{\text{(saliva)}}{HSCN} \xrightarrow{\text{salivary peroxidase}} \underset{\text{(inhibitory)}}{HOSCN} + H_2O \qquad (3)$$

During glycolysis of bacteria and in particular of *Streptococcus mutants* which is present in tooth plaque, acid is generated and as a consequence of the pH drop decay of teeth occurs. Hypothiocyanate (HOSCN) formed by the peroxidase inhibits this decay process by inhibiting bacterial glycolysis. There is indeed a toothpaste on the market (Zendium) in the Netherlands which contains glucose oxidase to increase the generation of hydrogen peroxide from carbohydrates and reinforces the natural system. A more recent version of the toothpaste does also contain lactoperoxidase. Other oral administrations are mouthwash or lozenge.

There is also a claim that some of these products which have antibacterial properties may be used to attack human tumour cells, notably in bone marrow which has been removed from a patient undergoing radiotherapy (Beggs and Davis, 1991, Beggs *et al.*, 1991). It is possible to attach a (halo)peroxidase to an antibody which is capable of binding to the target site (tumour cell) and as a result hypohalite may be generated at the target site resulting in specific killing of the target cell.

Along the same lines Allen (1992) proposed a method and compositions for the treatment of infection and control of bacterial flora using haloperoxidases. These enzymes will selectively bind to and inhibit the growth of microbes. According to the patent the methods and composition are highly useful in the therapeutic or prophylactic antiseptic treatment of human or animal subjects since their use can be designed to be highly effective in combating bacterial or fungal infections. The ability to selectively inhibit the growth of target microbes results from the fact that the haloperoxidases selectively bind to microbes. The target bound haloperoxidase catalyses halide oxidation and facilitates the disproportionation of peroxide to singlet molecular oxygen at the surface of the target microbe. This results in selective killing of the microbe with a minimum of damage to other systems. The binding of the peroxidase to the target may be related to the hydrophobic properties and the strong positive charge of some of the peroxidases. According to this patent suitable haloperoxidases are myeloperoxidase, eosinophil peroxidase, lactoperoxidase, chloroperoxidase and the heme-containing chloroperoxidase from the fungus *Caldariomyces fumago.*

A patent has also appeared directed on prophylactic and therapeutic applications of peroxidases (M. Pourtois *et al.*, 1992) for the manufacture of medicaments for the prevention and treatment of enveloped virus infections and, in particular, of herpes simplex and human immunodeficiency virus infections. Peroxidases of the medicaments include horseradish peroxidase, lactoperoxidase and myeloperoxidase. The authors claim also that the medicament is a topical

medicament (cream, gel, bandage, pad), an oral dentifrice or an injectable composition.

A problem with all these applications is that expression of the mammalian peroxidases in suitable hosts is difficult and only possible at high costs. Expression of active myeloperoxidase has been reported to occur only in Chinese hamster ovarian cell line (Jacquet *et al.*, 1991) and the procedure is probably too expensive for a realistic application. It is possible to express both the heme-containing chloroperoxidase from the fungus *Caldariomyces fumago* and horseradish peroxidase. However, reactivation of the inactive apoprotein and incorporation of the prosthetic group in a peroxidase is not straightforward (Tam and Welinder,1996). An option is to use lactoperoxidase which as a side product of cheese production (whey) is relatively cheap.

A major problem in using heme-containing peroxidases is that they are not stable towards oxidative inactivation by elevated concentration of hydrogen peroxide or their products. Also during catalysis inactive intermediates may accumulate inhibiting the action of these enzymes. A solution to this is to keep the concentration of hydrogen peroxide at a low level. However, this requires careful control of the reaction conditions and this limits the usefulness of heme-containing haloperoxidases in most applications. Accordingly, sources were screened for other haloperoxidases whose activity is substantially unaffected by relatively high concentrations of either hydrogen peroxide or hypohalous acid (Geigert *et al.*, 1990). Indeed, these investigators discovered that dematiaceous hyphomyctes secrete non-heme haloperoxidases which were reported to be very stable (Hunter *et al.*, 1990). For some of the fungi reported it was shown later that secreted peroxidases belonged to the class of vanadium peroxidases (Van Schijndel *et al.*, 1993, Vollenbroek *et al.*, 1995). Subsequently a patent (P. Barnett *et al.*, 1995) was filed in which these exceptionally stable haloperoxidases were used in a antimicrobial composition, comprising a vanadium haloperoxidase. In the patent it is claimed that the antimicrobial compositions of the invention may be employed to provide hygiene benefits for hard surface cleaning and fabric washings, but also to provide hygiene and cleaning in industrial/institutional applications such as in hospitals. The antimicrobial compositions can also be successfully used in deodorants in view of their ability to combat bacteria which cause malodour.

An entirely different application has been proposed by Wever *et al.* (1995) and this is the use of haloperoxidases as an additive in antifouling paint. In the patent it is claimed that the peroxidase will inhibit the growth of organisms on the paint and thus acts as an antifouling agent on ship or yachts. The principle of the invention is that sea water contains about 1 mM Br^- and 500 mM Cl^- and if sufficient peroxide is present the antifouling paint will continuously generate HOX as a bactericidal agent. A limiting factor is the concentration of H_2O_2 in sea water which is about 1 μMM.The peroxide is generated by photo-oxidation processes of water initiated by the UV light of the sun. Also peroxide may be generated as a result of biological activity. This idea is actually borrowed from nature. In some seaweeds a vanadium bromoperoxidase is pre-

sent on the surface of the plant (Wever *et al.*, 1991) which may be present to prevent bacterial growth on the surface by generating the bactericidal HOBr. Another application of the vanadium haloperoxidases suggested is use as a paint preservative in water based paints.

Application in bleaching/detergents

Industrial enzymes in detergent formulations make up a substantial part of the biocatalysts market and it is likely that peroxidases with their degrading and bleaching properties will also be used in detergents in the future. Several patent applications have appeared mainly generated by research groups within Novo-Nordisk A/S and there considerable activity in this area. One of the first patent patent in which this novel lead has been disclosed is that by Kirk *et al.* (1989). The claim is made that an enzyme exhibiting peroxidase activity is capable of exerting a bleaching effect on fabrics. The main advantage would be that by using the detergent additives of the invention amounts of hydrogen peroxide or its precursors can be reduced and yet provide a satisfactory bleaching effect. Thus, the amount of unspent bleaching agents released into the environment is reduced and further the inventors claim that the overall performance of detergents compositions in which the bleaching agents are included is improved. A more specific application (Damhus *et al.*, 1991 and Pederson, 1992) is that of the inhibition of dye transfer during washing or rinsing of fabrics by addition of enzyme exhibiting peroxidase activity to the wash liquor. Since the peroxidase will oxidize or bleach the bleeding dye during the wash the peroxidase will inhibit the transfer of a textile dye from a dyed fabric to another fabric when these fabrics are washed an/or rinsed together in wash liquor. This problem is most noticeable when white or light coloured fabrics are washed together with fabric with a more intense colour from which the dye is leached during washing. Peroxidases are derived from a strain of *Coprinus* or *Bacillus pumilus*. Also a microperoxidase which is a hemopeptide derived from cytochrome *c* may be used for this application (Pedersen *et al.*, 1993). Similarly, these new hemopeptides with peroxidase activity may be used for waste water treatment and for paper pulp bleaching.

A related application is the use of peroxidases in removing excess dye from newly dyed or printed textiles by using wash liquor containing oxidase or peroxidase and suitable oxidant, to reduce back-staining and to improve waste water quality (Pedersen and Schmidt, 1992). The advantage of this treatment is that it bleaches any dye leaching from the material so it prevents redeposition of dye (backstaining) even when relatively small amounts of water are used. It reduces time, energy and water, produces less polluted wash water and improves dye fastness. Bleaching of foodstuffs is also a possibility and there is a patent on the decolourisation of fish roe by treatment with hydrogen peroxide and peroxidase (Novo Nordisk, 1994).

Some of the fungal and bacterial peroxidase have been sequenced and cloned and expression systems have been developed. The tertiary structure of one of these enzymes (*Coprinus cinereus*) has been determined (Petersen *et al.*, 1994) and protein engineering has become a major tool now in developing new variants of *Coprineus cinereus* peroxidase. The native *Coprinus* peroxidase is susceptible towards high concentrations of hydrogen peroxide, resulting in the formation of compound III and inactivation. This problem has been tackled and Pedersen *et al.* (1995) have produced some variants with improved hydrogen peroxide stability at alkaline conditions (pH 7–10). Methods to express the mutants in filamentous fungi especially *Aspergillus oryzae* and *A. niger* (Welinder and Andersen, 1993) are also available. Presently there seems to be no major obstacle for introducing these peroxidases in detergent formulations. However, despite all the research efforts by the industrial enzyme producers and the scientific progress made, a washing powder containing a peroxidase has not yet appeared on the market.

Use of peroxidases in polymerisation and depolymerisation processes

Peroxidases are able to oxidize a large number of organic compounds by one-electron oxidation steps. The radicals produced may dimerise or polymerise and the products formed are in general much less soluble in water. This property may be used to remove carcinogenic aromatic amines and phenols from industrial aqueous effluent. This idea was put forward by Klibanov *et al.* (1983) using horseradish peroxidase that will oxidize phenols to phenoxy radicals. These radicals will react with other aromatic rings causing enzymatic precipitation of aromatic organic material. Peroxidases may also be used to remove colour from bleach plant effluent (Paice and Jurase, 1984). Instead of horseradish peroxidase chloroperoxidase (Pickard *et al.*, 1991) may also be used. Also degradation by peroxidases of environmental pollutants is a suggested use.

Other applications in which a peroxidase is involved in polymerisation processes are the preparation of a glue or binder from lignine sulphonate (Yde, 1994) using a a peroxidase and hydrogen peroxide at alkaline pH to give high molecular weight polymers which may be used in the manufacture of wood composites (wood fibre boards, plywood and laminated wood beams). Its main advantage is that very high molecular weight lignine is prepared without the use of organic solvents. Similarly, the treatment of pulp with a phenol oxidizing enzyme as derived from *Coprinus* or *Bacillus pumillus* after completion of grinding and refining of logs gives paper or paperboard of improved strength (Hansen and Nielsen, 1993). Peroxidases may also be used in the tanning of hides resulting in an increased degree of fixation (Ingvorsen *et al.*, 1993). A process somewhat related to this is the use (Maat and Roza, 1995) of a peroxidase in dough to improve specific volume, staling resistance and crumb structure of bread. Oxidative coupling may also be used in colouring process of hair exposing it to a solution containing soybean peroxidase, hydrogen peroxide and a aromatic

compound (Procter & Gamble, 1975). Finally, by treating aqueous coffee with a peroxidase enzyme in the presence of a peroxide an instant coffee with an improved quality is obtained (Unilever, 1989). By this treatment the coffee beverage has obtained a pleasant, mild aroma with a hint of Mocha. These examples show that it just a matter of time and peroxidases will have found their way in daily life.

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Potential Application of Haloperoxidases in Organic Chemistry

Abstract

Haloperoxidases are enzymes which are able to chlorinate, brominate or iodinate organic compounds at the expense of the corresponding halide ion and hydrogen peroxide. All haloperoxidases isolated thus far produce hypohalous acid (HOX), which is the actual halogenating agent. Therefore, no regio- or stereoselective halogenation reactions have been observed with these enzymes. The nonheme haloperoxidases are extremely stable towards hydrogen peroxide, HOX, elevated temperatures and organic solvents. The heme enzymes are much more labile, but show significant selectivity in the halide-independent oxidation of sulphides to sulphoxides, and alkenes to epoxides. As soon as enzymes are isolated or developed which combine the selectivity of heme-containing peroxidases with the stability of nonheme haloperoxidases, large-scale applications will follow very soon.

Introduction

The use of enzymes as catalysts in organic reactions has become common practice in chemical laboratories nowadays and is also gaining increasing industrial interest (Poppe and Novak, 1992; Faber and Franssen, 1993; Faber, 1995). In order to be useful to the organic chemist, enzymes have to meet the following criteria:

1. They have to be regio- and/or stereoselective;
2. They should be stable for a prolonged period under storage conditions and under turnover conditions, even at elevated temperatures or in the presence of organic solvents;
3. They should display high turnover (fast reactions);
4. They should be easily available, either commercially, or through a microorganism that is easy to cultivate.

Many hydrolases, like proteases, lipases and esterases fulfil these requirements and are therefore the enzymes which are applied most frequently at the moment.

For example, the kinetic resolution of the C3-isomers of methyl 2-methoxytetrahydrofuran-3-carboxylate (**1**, see Scheme 1) has been performed on a preparative scale using the commercially available lipase of *Candida rugosa*. The enzyme accepts only the 3S-isomers of the mixture and the product is formed with an e.e. of more than 98% (Franssen *et al.*, 1996). The reaction was run in octane as a solvent. Since a very crude lipase preparation is used, the reaction rate per mg biocatalyst is rather low but this is compensated for by the low price of the enzyme. Furthermore, the enzyme can be used repetitively under these conditions (Franssen *et al.*, 1992). Compound **2** is a building block for some naturally occurring insect antifeedants.

Enzymes which would be able to incorporate halogen atoms in organic compounds, using halide ions as the source of F, Cl, Br and I, would definitely be of interest to the organic chemist. The reasons for this are threefold:

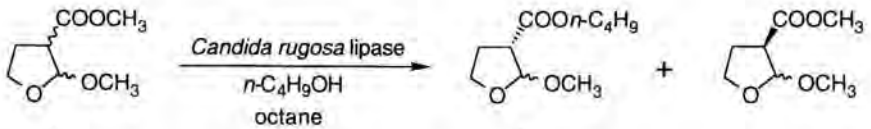
1. Halogen atoms are mostly easy to replace by nucleophiles, making halogen-containing compounds useful intermediates in synthetic routes;
2. Halogenated compounds have, in many cases, enhanced physiological activity when compared to their parent compounds;
3. The use of fluoride, chloride, bromide and iodide ions is strongly preferred above molecular fluorine, chlorine, bromine and iodine, both from a technical and from an environmental point of view.

Three kinds of halogenating enzymes have been described thus far: an S-adenosyl methionine: halide methyl transferase (Wuosmaa and Hager, 1990; Harper, these proceedings), haloperoxidases (Neidleman and Geigert, 1986; Franssen and van der Plas, 1992; Franssen, 1994) and NADH-dependent halogenating enzymes (Van Pée, these proceedings).

The first enzyme catalyses the transfer of a methyl group of SAM to chloride, bromide or iodide ions. It has been isolated from fungi and terrestial plants. The enzyme reacts according to the equation (1):

$$SAM + X^- \rightarrow SAHC + CH_3X \qquad (1)$$

This enzyme is only able to produce methyl halides and is therefore of very limited preparative value. Since the NADH-dependent halogenating biocatalysts will be dealt with elsewhere, the remainder of this paper will be devoted to haloperoxidases.



1 2

Scheme 1. Example of a lipase-mediated preparation of a chiral building block for natural products.

Haloperoxidases

Haloperoxidases are enzymes which are able to halogenate organic compounds using halide ions and hydrogen peroxide as substrates. The general reaction equation reads as follows:

$$AH + H_2O_2 + H^+ + X^- \rightarrow AX + 2H_2O \quad (2)$$

In eq. (2), AH stands for an organic substrate and AX for the halogenated product. X^- can be chloride, bromide or iodide, depending on the enzyme; fluoride is never accepted as a substrate. The enzymes are called chloro-, bromo- or iodoperoxidases, depending on the smallest halide ion they are able to oxidise.

Haloperoxidases are ubiquitous enzymes: they have been found in many different classes of organisms, as can be concluded from the brief overview given in Table 1 (Franssen, 1994).

The wide occurrence of haloperoxidases in Nature indicates that these enzymes are very important, if not essential, for life on Earth. However, the exact role of many haloperoxidases is unknown. Most probably, these enzymes are involved in the defence mechanism of their owners, since a large number of living species produce halogenated compounds (so-called halometabolites) which are more or less toxic. Alternatively, the mammalian chloroperoxidases called myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are involved in the production of the antimicrobial agents hypohalous acid (acc. to eq. (3)) and singlet oxygen (acc. to eq. (4)):

Table 1. Some sources of haloperoxidases.

name	source		prosthetic group
chloroperoxidases			
chloroperoxidase	*Caldariomyces fumago*	(mold)	heme[1]
"	*Curvularia inaequalis*	(mold)	vanadium
"	*Pseudomonas pyrrocinia*	(bacterium)	none[2]
myeloperoxidase	white blood cells	(vertebrates)	heme
bromoperoxidases			
bromoperoxidase	*Streptomyces aureofaciens*	(bacterium)	none[2]
"	*Penicillus capitatus*	(green alga)	heme
"	*Corallina pilulifera*	(red alga)	vanadium
"	*Ascophyllum nodosum*	(brown alga)	vanadium
"	*Xanthoria parietina*	(lichen)	vanadium
lactoperoxidase	milk, saliva, tears	(mammals)	heme
iodoperoxidases			
horseradish peroxidase	horseradish roots	(higher plant)	heme
thyroid peroxidase	thyroid glands	(vertebrates)	heme

[1]heme = ferriprotoporphyrin IX; [2]the enzyme contains a catalytic triad

$$H_2O_2 + X^- + H^+ \xrightarrow{\text{MPO, EPO}} HOX + H_2O \quad (3)$$

$$HOX + H_2O_2 \xrightarrow{\text{spontaneous}} {}^1O_2 + H_2O + H^+ + X^- \quad (4)$$

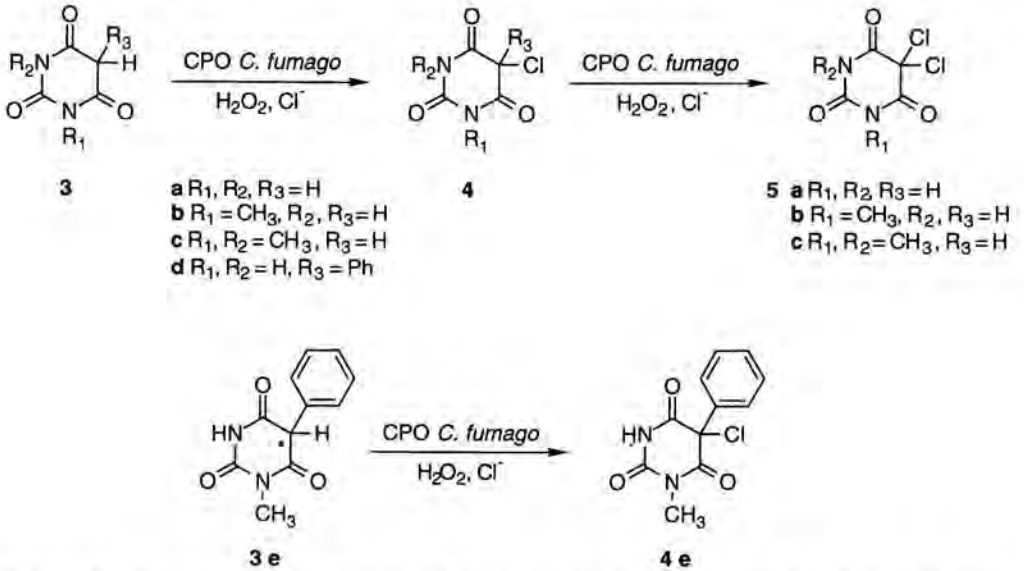
Haloperoxidases may also have other physiological roles, e.g. thyroid peroxidase is involved in the biosynthesis of the iodinated hormone thyroxin.

Of course, the lack of knowledge of the natural function of most haloperoxidases does not preclude their application in organic synthesis, for many enzymes in industrial or university laboratories are being used for the conversion of non-natural compounds. In the following sections, the state of affairs concerning the application of haloperoxidases as catalysts in organic synthesis will be presented. The examples are clustered by the prosthetic group of the enzymes.

Halogenation reactions

Heme enzymes

Halogenation reactions with heme-containing haloperoxidases have mainly been carried out using the CPO[1] from the mould *Caldariomyces fumago*, although

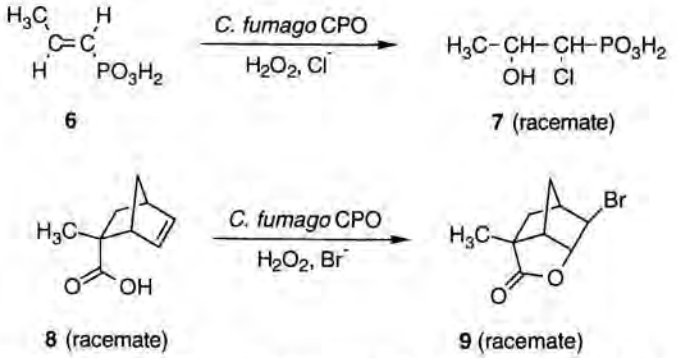


Scheme 2. Conversions of barbituric acid derivatives by the chloroperoxidase from *C. fumago* (Franssen and van der Plas, 1987).

[1] CPO = chloroperoxidase, BPO = bromoperoxidase, HRP = horseradish peroxidase

recently some interesting work using lactoperoxidase, a mammalian bromoperoxidase, has been published (Ishihara *et al.*, 1995). The *C. fumago* CPO is commercially available, though expensive. However, *C. fumago* is easy to cultivate, it produces the enzyme in copious amounts and a reliable prescription for the isolation of enzyme has been published (van Deurzen *et al.*, 1994).

A large number of halogenation reactions using the *C. fumago* CPO have been published (see for reviews: Neidleman and Geigert, 1986; Franssen and van der Plas, 1992; Franssen, 1994). During the 1980s, we have been working on the CPO-mediated chlorination of barbituric acid and its derivatives (**3**, see Scheme 2). These compounds are smoothly converted into their monochloro derivatives (**4**) and, in case of **4a–c**, into their dichloro derivatives **5a–c**, in high yields. In order to investigate the stereochemical course of this reaction, racemic **3e** was subjected to CPO-mediated chlorination. However, the product **4e** did not show any optical rotation, indicating that the enzymatic reaction was not stereoselective (Franssen and van der Plas, 1987). The same conclusion was reached by Kollonitsch *et al.* (1970) for the formation of the chlorohydrin of propenylphosphonic acid (**6**, see Scheme 3) and by Ramakrishnan *et al.* (1983) for the bromolactonisation of the bicyclic acid **8**.



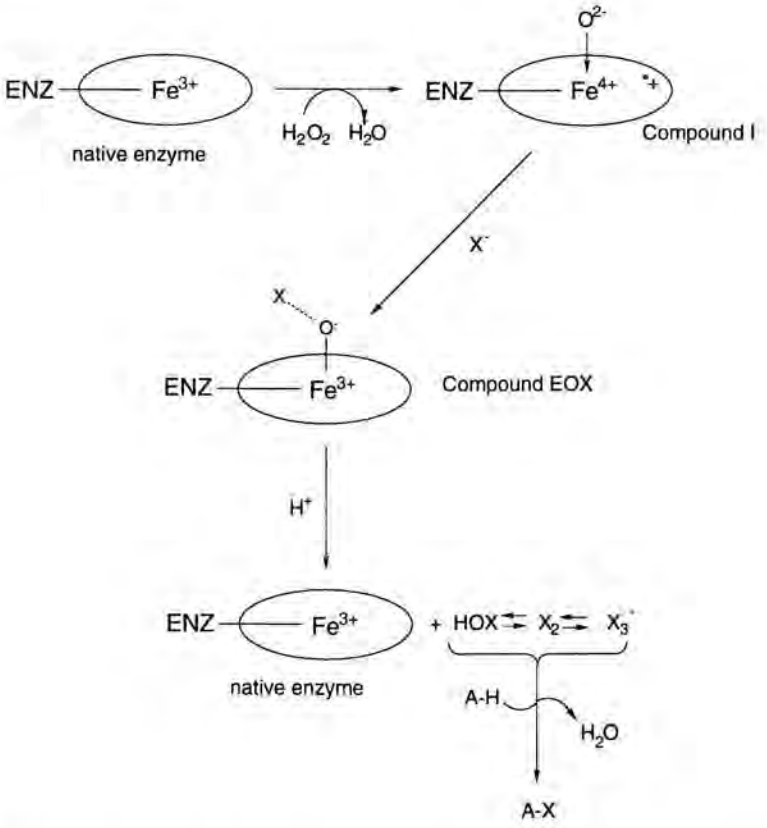
6 **7** (racemate)

8 (racemate) **9** (racemate)

Scheme 3. Examples of (unselective) halogenation reactions, catalysed by the chloroperoxidase from *C. fumago*.

The background of this lack of selectivity was unraveled by Libby *et al.* (1989, 1992), who showed by careful kinetic studies that the *C. fumago* CPO produces HOX/X_2 as the ultimate product. The complete reaction scheme for the halogenation of organic compounds by this enzyme (and other heme-containing haloperoxidases) is shown in Scheme 4.

Despite the lack of selectivity, heme-containing haloperoxidases can still be useful to the organic chemist. These enzymes produce the very reactive species HOX in a relatively slow manner, in a homogeneous, diluted solution. Therefore, yields obtained in haloperoxidase-mediated halogenation reactions are often higher than halogenation reactions where diluted HOX is added to the substrate. Compare, for example, the reaction of *C. fumago* CPO with pyrazole and two derivatives thereof (**10**) to the chlorination of these compounds by

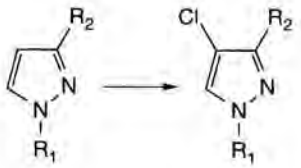


Scheme 4. Halogenation mechanism of heme-containing haloperoxidases. The protein part of the enzyme is represented by ENZ, the heme group is depicted as Fe^{x+} (x = 3 or 4) in the center of an ellipse, for reasons of clarity. Compound I contains an Fe^{4+} species and a radical cation which is situated in the heme moiety. A-H = organic substrate, A-X = halogenated product, X = Cl, Br or I.

adding diluted HOCl (Franssen *et al.*, 1987). As can be concluded from the data in Table 2, the yields of the CPO-mediated reactions are significantly higher.

It should be stressed here that the *only* way to find whether a haloperoxidase-mediated reaction is regio- or stereoselective, is to compare the product outcome to the products obtained by very slow addition of 1-2 eq. of HOX to the substrate *under the same reaction conditions*, i.e. in the same buffer, using the same concentration of substrates (including H_2O_2), and at the same temperature. The yield of the product(s) might differ from the enzymatic reaction, as shown above. However, *the structure of the product(s) in the enzymatic reaction should be different from that of the HOX-mediated reaction in order to make a claim for a selective haloperoxidase-mediated reaction justified.* Comparing the enzymatic reaction to reactions of HOX or other halogenating agents in pure water, organic solvents or mixtures thereof (Coughlin *et al.*, 1993) does not make sense,

Table 2. Yields of the chlorination reaction of pyrazole (**10a**) and its 1- and 3-methyl derivatives (**10b,c**), mediated by *C. fumago*

CPO or by HOCl[a].	substrate	yield (%) CPO, H_2O_2, Cl^-	HOCl
 **10** **11**	**10a** (R_1, R_2 = H)	68	20
	10b (R_1 = CH_3, R_2 = H)	83	68
	10c (R_1 = H, R_2 = CH_3)	91	67

[a]Taken from Franssen *et al.*, 1987.

since the reaction pattern of HOX is strongly dependent on the solvent (M.C.R. Franssen, unpublished results).

A disadvantage of heme-containing haloperoxidases is their limited stability towards elevated temperatures, towards their substrate hydrogen peroxide and product HOX, and towards organic solvents. Especially the vulnerability towards H_2O_2 and HOX poses serious problems; e.g., in case of *C. fumago* CPO the concentration of H_2O_2 should be kept below ± 2 mM in order to prevent inactivation of the enzyme due to bleaching of the heme group (Itoh *et al.*, 1987; Liu *et al.*, 1987). Care should be taken that there is always enough organic substrate present to react with the HOX as soon as it is formed. The optimum pH for this enzyme is 2.7, which might be a problem for the conversion of acid-labile substrates. Water-miscible organic cosolvents can be used without problems only up to 10% v/v in case of methanol and DMSO (Cooney and Hueter, 1974), although halide-independent oxidation reactions can be run in 30% *t*-butyl alcohol (van Deurzen *et al.*, 1994).

In summary, heme-containing haloperoxidases possess the following features:

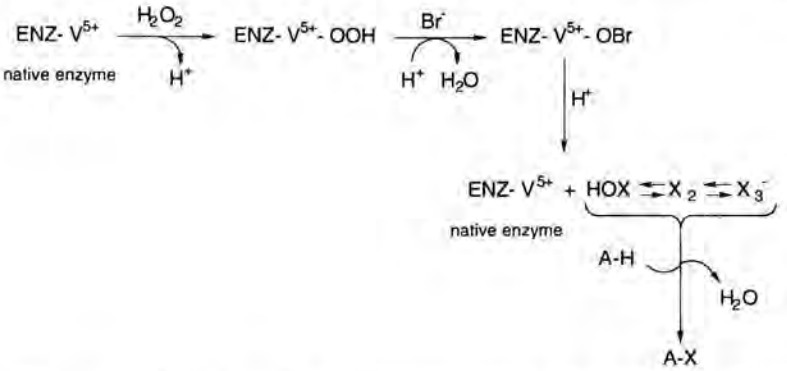
1. They catalyse the smooth, yet unselective halogenation of organic compounds, producing hypohalous acid (HOX) as the active halogenating species;
2. Their rate of reaction is moderate, except for the CPO from *C. fumago*, which is an extremely active enzyme ($k_{cat} = \pm 1000\ s^{-1}$);
3. They are not stable against ambient concentrations of their substrate (H_2O_2) and their product (HOX), as well against elevated temperatures and higher concentrations of organic solvents;

4. Several enzymes are commercially available, although they are rather expensive. However, *C. fumago* is easy to cultivate and its CPO is easy to isolate.

Vanadium enzymes

As pointed out in Table 1, many haloperoxidases are known to date which possess vanadium as the prosthetic group. The BPO from the brown alga *Ascophyllum nodosum* was the first vanadium-containing haloperoxidase which was purified to homogeneity (De Boer *et al.*, 1986). This enzyme has been tested with the barbituric acids which were already listed in Scheme 2. Just as with the CPO from *C. fumago*, 1-methyl-5-phenylbarbituric acid (**3e**) was not brominated in a stereoselective way (Franssen *et al.*, 1988). From this work and the extensive kinetic investigations in the group of Wever (De Boer and Wever, 1988; van Schijndel *et al.*, 1994) it became clear that vanadium haloperoxidases also produce HOX as the active halogenating agent, which precludes any regio- or stereoselective reaction of these enzymes. The simplified reaction sequence is depicted in Scheme 5.

Although the vanadium enzymes are just as unselective in halogenation reactions as the heme haloperoxidases, they have one distinct advantage: they are, without exception, extraordinary stable. E.g., incubation of the CPO from the fungus *Curvularia inaequalis* with 0.5 mM HOCl for 2 min still gave 90% residual activity, whereas the *C. fumago* CPO was completely inactivated in that period under the same conditions. The *Curv. inaequalis* enzyme does not loose any activity when treated with 200 mM H_2O_2 for 25 h; the *C. fumago* CPO is already completely inactive after 2 min. under those conditions (Liu *et al.*, 1987). Incubation of this *Curv. inaequalis* vanadium CPO at 80°C resulted in an initial decrease in activity of about 20% after which the enzyme remains stable for 6.5 h. Enzyme activity is not inhibited by guanidine/HCl until 2 M, and the



Scheme 5. Halogenation mechanism of vanadium-containing haloperoxidases. The protein part of the enzyme is represented by ENZ, for reasons of clarity. A-H = organic substrate, A-X = halogenated product, X = Cl, Br or I.

enzyme activity also remains constant when organic cosolvents like methanol are added until 40% v/v (Van Schijndel *et al.*, 1994). The same phenomena were observed with the vanadium-containing CPO from the red seaweed *Corallina officinalis* (Sheffield *et al.*, 1992; Rush *et al.*, 1995). The turnover of these enzymes is quite acceptable.

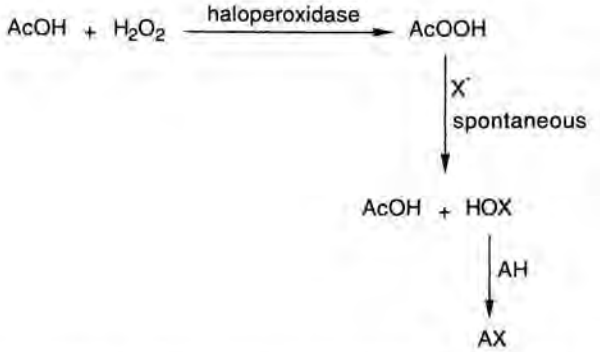
Unfortunately, only one of these interesting enzymes is commercially available (for an outrageous price), namely the above-mentioned CPO from *Corallina officinalis*. Vanadium haloperoxidases are not difficult to isolate, but definitely not so easy as the *C. fumago* CPO.

Summarising, the characteristics of vanadium-containing haloperoxidases are:

1. They catalyse the smooth, yet unselective halogenation of organic compounds, *via* enzymatically produced HOX;
2. Their turnover is rather high, k_{cat} ranging from 10–50 s^{-1};
3. They possess excellent stability towards H_2O_2, HOX, organic solvents and high temperatures;
4. Only one vanadium-containing haloperoxidase is commercially available, and very expensive; isolation of these enzymes from their natural sources is not difficult.

Nonheme, nonvanadium enzymes

Several bacterial haloperoxidases have been isolated, which do not possess heme or vanadium as an essential constituent. Very recently, it was shown that the BPOs from *Streptomyces aureofaciens* strains ATCC10762 and Tü24, as well the CPOs from *S. lividans*, *Pseudomonas pyrrocinia* and *P. fluorescens* possess a catalytic triad (Pelletier *et al.*, 1995). For all these enzymes, acetate is essential for their halogenating activity, which brought the authors to the tentative reaction mechanism as depicted in Scheme 6. The catalytic triad in the enzyme active



Scheme 6. Tentative reaction mechanism for the halogenation of organic compounds as catalysed by the nonheme, nonvanadium haloperoxidases. A-H = organic substrate, A-X = halogenated product, X = Cl or Br.

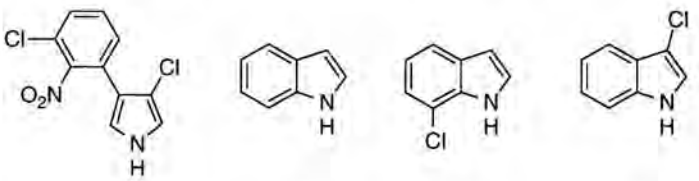
site is responsible for the reaction of acetic acid with hydrogen peroxide, forming peracetic acid. This reaction is well known for lipases (Björkling *et al.*, 1990). The peracetic acid on its turn reacts with halide ions, presumably in a spontaneous reaction, outside the enzyme active site. This would, again, yield HOX as the active halogenating agent.

These findings shed new light on the papers which have appeared on the chlorination of indole by the CPO from the bacterium *Pseudomonas pyrrocinia*. This bacterium produces pyrrolnitrin (**12**, see Scheme 7), and it was thought that this CPO was involved in its biosynthesis. According to two papers from the group of van Pée (Wiesner *et al.*, 1986; van Pée, 1990), the *P. pyrrocinia* CPO converts indole (**13**) into 7-chloroindole (**14**) upon incubation with H_2O_2 and chloride ions. This would be a regioselective reaction, since the pyrrole ring in indole is much more reactive than the benzene ring. However, recent investigations have shown that not 7-chloro-, but *3*-chloroindole (**15**) is the actual reaction product (K.-H. van Pée, personal communication), which is in line with the production of HOCl by this CPO as described above. The actual enzyme which is involved in the conversion of tryptophane into 7-chloro-tryptophane, the first step in the biosynthesis of pyrrolnitrin (**12**), appears not to be a haloperoxidase (K.-H. van Pée, these proceedings).

Nonheme nonvanadium enzymes are just as stable as their vanadium counterparts, but their activity is low (1–5 s^{-1} for standard substrates, $\pm$ 0.2 s^{-1} for indole). None of these enzymes is yet commercially available, but that will change in the near future (K.-H. van Pée, personal communication).

In summary, the following can be said about the nonheme, nonvanadium enzymes:

1. Most likely, they produce HOX as the active halogenating species, just like all the other haloperoxidases. However, the mechanism by which they produce HOX is most extraordinary;
2. Their reaction rates are lower than those of other haloperoxidases;
3. Their stability is excellent, comparable to the vanadium enzymes;
4. None of these enzymes is commercially available yet.



12 **13** **14** **15**

Scheme 7. The chemical structures of pyrrolnitrin (**12**), indole (**13**), 7-chloroindole (**14**) and 3-chloroindole (**15**).

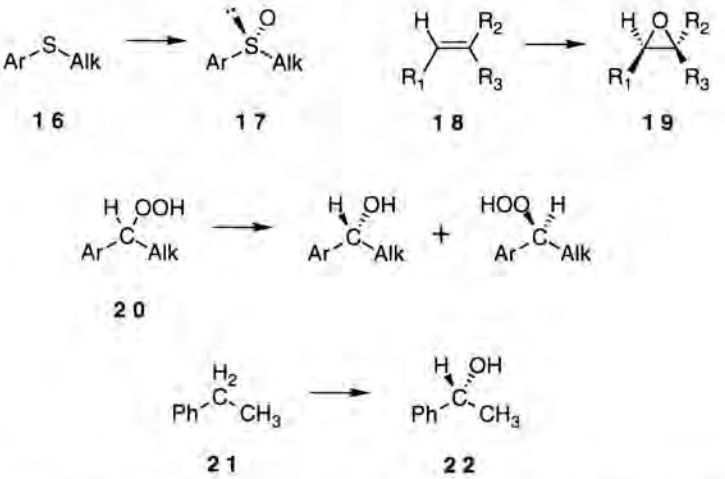
Oxidation reactions

One might get the impression that haloperoxidases are completely unselective enzymes and therefore of no commercial use. This is not true, as it has been pointed out above that they can be beneficial as mild alternatives of molecular halogens (X_2) or HOX. Besides this, there are some very selective *oxidative* reactions known of these enzymes. These reactions are examples of the so-called "classical" oxidation reactions (oxidation of X^- to X^+-species being nonclassical oxidation), which are restricted to the heme-containing haloperoxidases like horseradish peroxidase (HRP) and, in particular, the *C. fumago* CPO. Recently, a truncated cytochrome c known as microperoxidase-11, was also shown to be able to carry out stereoselective oxidation reactions (Colonna *et al.*, 1994).

These oxidation reactions comprise the direct, stereoselective transfer of the ferryl-oxygen atom in Compound I (see Scheme 4) to the organic substrate. Very high enantiomeric excesses have been obtained in the conversion of aryl alkyl sulphides (**16**, see Scheme 8) to chiral sulphoxides (**17**) (Colonna *et al.*, 1992; van Deurzen *et al.*, 1994), the epoxidation of alkenes (**18** → **19**) (Allain *et al.*, 1993; Dexter *et al.*, 1995; Lakner and Hager, 1996), the kinetic resolution of hydroperoxides (**20**) (Fu *et al.*, 1992; Hoeft *et al.*, 1995) and the stereoselective hydroxylation of alkylbenzenes (**21** → **22**) (Zaks and Dodds, 1995).

In general, the heme-containing haloperoxidases possess the following features when used as *oxidation* catalysts:

1. They are highly selective catalysts for the preparation of homochiral sulphoxides, epoxides, hydroperoxides and benzylic alcohols, the CPO from *C. fumago* being the best;
2. The reactions are catalysed in a rather slow manner (k_{cat} is ± 0.5–$1\ s^{-1}$);



Scheme 8. Examples of stereoselective oxidation reactions displayed by heme peroxidases.

3. Enzyme stability is low, as pointed out before. This is especially a problem in these oxidation reactions, since high substrate concentrations are needed in order to achieve acceptable reaction rates. Irreversible inactivation by H_2O_2 is frequently encountered;

4. The enzymes are expensive, although the *C. fumago* CPO is easy to obtain from its culture broth, as mentioned earlier.

General conclusions and outlook

We have seen in this review that there are two different combinations of properties in haloperoxidase-catalysed reactions:

1. There are *very stable* enzymes (vanadium haloperoxidases) which carry out the smooth, yet *unselective halogenation* of a large variety of organic substrates;
2. There are relatively *unstable* enzymes (heme peroxidases) which perform the *very selective oxidation* of organic substrates.

The question now arises: how can we combine the stability of the nonheme enzymes with the selectivity of the heme enzymes, in other words: how can we obtain stable enzymes which catalyse selective halogenation reactions?

In my opinion, there are three options to do this.

1. Increase the selectivity of the stable, active, vanadium haloperoxidases;
2. Increase the stability of heme-containing haloperoxidases;
3. Continue the screening for novel halogenating enzymes which are both stable and selective.

These options are discussed below.

1. Increase the selectivity

Increasing the selectivity of haloperoxidases implies, in practice, either completely changing the catalytic mechanism, or preventing the release of free HOX into the reaction medium. The goal of both strategies is to keep the active, electron-deficient halogen species inside the active site, preferably bound to an amino acid residue, because it is only in this chiral environment that stereoselectivity is to be expected.

There is some evidence for binding of organic substrates to the active site of *Ascophyllum nodosum* BPO (Tschirret-Guth and Butler, 1994). When this binding occurs before or during the formation of the active halogenating agent, some selectivity might be expected (Butler, these proceedings).

Another option is changing the structure of the enzyme. Making HRP more selective in sulphoxidation and epoxidation reactions by site-directed mutagenesis has been shown to be fruitful. The phenylalanine residue at position 41

blocks the active site in HRP but is not essential for activity. Substitution of phe-41 by an alanine makes the heme more accessible. As a result thereof, the mutant F41A is able to epoxidise styrene, which native HRP cannot do (Newmyer and Ortiz de Montellano, 1995). F41L increases the enantioselectivity of sulphoxidation (Ozaki and Ortiz de Montellano, 1994) and broadens the scope of the epoxidation reactions (Ozaki and Ortiz de Montellano, 1995).

Although these site-directed mutagenesis studies seem to be very useful to increase the scope and selectivity of peroxidase-catalysed oxidation reactions, it should be stressed that this approach might be less succesful for the halogenation reactions. One could consider to decrease the dimensions of the active site in order to slow down the release of free HOX, but on the other hand the active site should remain accessible to the organic substrate as well.

2. Increase the stability of heme enzymes

The most vulnerable part of the heme-containing haloperoxidases is the heme function itself. Treatment with too high concentrations of hydrogen peroxide or HOX causes complete and irreversible inactivation of the enzyme due to bleaching of the heme. Protecting the heme edge by site-directed mutagenesis could be a solution for this problem, but care should be taken that the ferric ion should remain accessible. It has recently been shown that reconstitution of the *C. fumago* apo-CPO, expressed in *E. coli*, is feasible at high pressure, albeit with low yield (Zong *et al.*, 1995). This might enable the substitution of the protoporphyrin IX of the native enzyme by a more stable heme group.

Increasing the stability of heme enzymes is absolutely needed for an industrial process (Pickard *et al.*, 1991). Maybe the three-dimensional structures of haloperoxidases can learn us which factors are involved in the (in)stability of these enzymes. The X-ray structure of the very active CPO from *C. fumago* has been solved recently (Sundaramoorthy *et al.*, 1995), as well as the structure of the stable V-CPO *Curvularia inaequalis* (Messerschmidt and Wever, 1996; Messerschmidt, these proceedings) and the stable nonheme nonvanadium CPO from *Streptomyces aureofaciens* (Hecht *et al.*, 1994).

3. Further screening

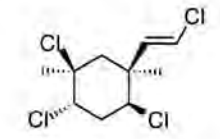
The option which is maybe the least appealing but which might be the most fruitful, is the continued screening for novel halogenating biocatalysts. The background of this option is simple: Nature contains many different halometabolites which contain chlorine or bromine atoms attached to chiral carbon atoms. For some of them, in particular the ones depicted in Scheme 9, it is very difficult to envisage a biosynthetic route *without* the involvement of stereo- and regioselective halogenating enzymes.

If such enzymes really exist, then the question arises why they have not been detected up to now. The reasons may be twofold:

1. The wrong organisms have been taken as a source for halogenating enzymes. E.g., it is known that the brown alga *Ascophyllum nodosum* produces a lot of bromoform (Wever, 1988), which can be easily formed from methyl ketones and HOBr. The formation of bromoform, of course, does not preclude the presence of regio- and/or stereoselective halogenating enzymes but it is does indicate that nonselective enzymes are abundant in this species. Considering the compounds shown in Scheme 9, screening for a halogenating enzyme in *Plocamium* might be much more interesting.

2. The correct organisms have been investigated, but the selective enzymes have not been found because the enzymes were not fed with their natural substrates. It might be that some haloperoxidases react unselectively with artificial substrates like monochlorodimedone and phenol red (De Boer *et al.*, 1987), whereas they react in a selective manner with their natural substrate because they possess a specific binding site for it.

It should be stressed that finding out which enzyme is responsible for the introduction of halogen into halometabolites is not easy, as was demonstrated by the thorough investigations of the group of van Pée on the biosynthesis of chloramphenicol (Facey *et al.*, 1996). Maybe, the selective halogenating enzymes belong to a new, yet undiscovered class of enzymes which do not need hydrogen peroxide as a substrate. An indication for this is presented elsewhere in these proceedings by van Pée.



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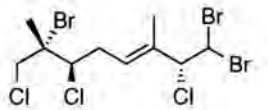

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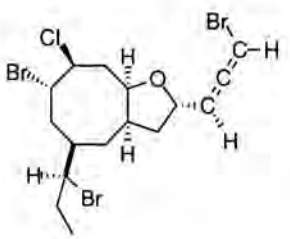

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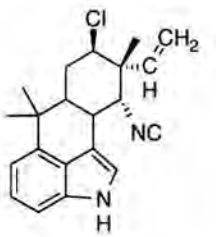

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Scheme 9. Halometabolites which contain chlorine or bromine atoms, attached to chiral carbon atoms. Compound **23** is from *Laurencia implicata* (red alga), **24** from *Plocamium sp.* (red alga), **25** from *Plocamium cartalagineum* (red alga) and hapalindole A (**26**) is from the blue-green alga *Hapalosiphon fontinalis*.

In my opinion, the key for selectivity in enzymatic halogenation resides in the chiral halometabolites. Studies on the biosynthesis of compounds like the ones depicted in Scheme 9 will reveal if stereo- and/or regioselective halogenating biocatalysts really exist in Nature.

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Diversity and Mechanisms of Bacterial Dehalogenation Reactions

Abstract

Halogenated aliphatic compounds occur widespread as environmental pollutants. Since many of these compounds are xenobiotics and show large differences in degradability which can be correlated to critical steps in catabolic pathways, they are suitable for studies on the evolution of dehalogenating pathways. We have investigated the degradation of 1,2-dichloroethane and 1,3-dichloropropene in detail. For both compounds, the initial step is hydrolytic dehalogenation. The 1,2-dichloroethane and 1,3-dichloropropene dehalogenases were found to belong to different groups of identical enzymes detected in bacteria isolated from various sites. Genetic analysis and adaptation experiments indicated that the 1,2-dichloroethane degradation pathway may be of recent evolutionary origin. The large-scale use of 1,3-dichloropropene in agriculture may have contributed to the distribution of genes encoding hydrolytic dehalogenases in the environment.

Introduction

The biodegradation of synthetic chlorinated chemicals that enter the environment is dependent on the capacity of microbial enzymes to recognize these xenobiotic molecules and cleave or labilize carbon-halogen bonds (Janssen *et al.*, 1994). Microbiological studies have led to the isolation of a range of organisms that degrade halogenated aliphatic compounds and use them as a carbon source for growth, and a several dehalogenating enzymes that directly act on carbon-halogen bonds have now been identified (Leisinger and Bader 1993; Janssen *et al.*, 1994; Fetzner and Lingens, 1994).

In a few cases, the carbon-halogen bond is not directly cleaved but labilized by introduction of other functional groups (Ensley, 1991). This way of dechlorination is often observed in organisms that do not utilize chlorinated compounds for growth but are only able to degrade them by fortuitous co-metabolic reactions. From a biotechnological point of view, these cometabolic reactions are less attractive for application since the degradation process does not stimulate the growth of the organisms involved. On the other hand, co-

metabolic transformation provides the only possibility for the aerobic biodegradation of some important compounds, including trichloroethylene.

This paper focusses on organisms that grow on synthetic organochlorine compounds. It is highly unlikely that chemicals such as dichloromethane, 1,2-dichloroethane, and γ-hexachlorohexane did occur on earth in biologically significant concentrations prior to the start of their industrial synthesis at around the beginning of this century. If at all produced by natural processes, this probably did not lead to concentrations in the biosphere which would give a selective advantage to organisms that can degrade them. Yet, the three compounds mentioned are known to support growth of specific pure cultures that produce the required dehalogenating enzymes. This raises the questions by which catalytic mechanisms organochlorine bonds are cleaved and how the genes encoding the enzymes that perform these reactions evolved and spread in the environment.

One possibility is that dehalogenases which convert xenobiotic organohalogens are derived from enzymes that recognized naturally produced halogenated compounds. Possibly, they adapted to xenobiotic compounds by a few additional mutations (see below). To understand this process of evolution of new activities, careful investigation of the biochemistry and genetics of catabolic pathways is necessary.

In this paper, a number of dehalogenation reactions for halogenated aliphatic compounds are reviewed, and experimental results on the distribution and evolution of dehalogenases are discussed.

Use of chlorinated aliphatic compounds for growth

Of the low-molecular weight halogenated aliphatic compounds, a number of environmentally important chemicals have been found to support growth of pure cultures. This includes methylchloride, dichloromethane, 1,2-dichloroethane, 1,3-dichloropropylene, 1-chlorobutane and other primary alkylhalides, and hexachlorocyclohexane. Chloropropanols and epichlorohydrin, several chlorocarboxylic acids and chloroethanol can also serve as a carbon source for specific bacterial cultures. Detailed rules in the sense of structure-degradability relationships are difficult to establish for haloaliphatics, but a few trends can be mentioned (Fig. 1). In many cases, degradation decreases with an increasing degree of chlorine substitution. Thus, of the chloromethanes only methylchloride and dichloromethane have been found to support growth, but not chloroform and carbon tetrachloride. Similarly, of the chloroethanes, only ethylchloride and 1,2-dichloroethane serve as a carbon source for known cultures. This rule holds only for homologous series of compounds, however. When different groups of compounds are compared, the rule does no longer hold. For example, dichloromethane and trichloroacetate, which have two and 1.5 chlorines per carbon,

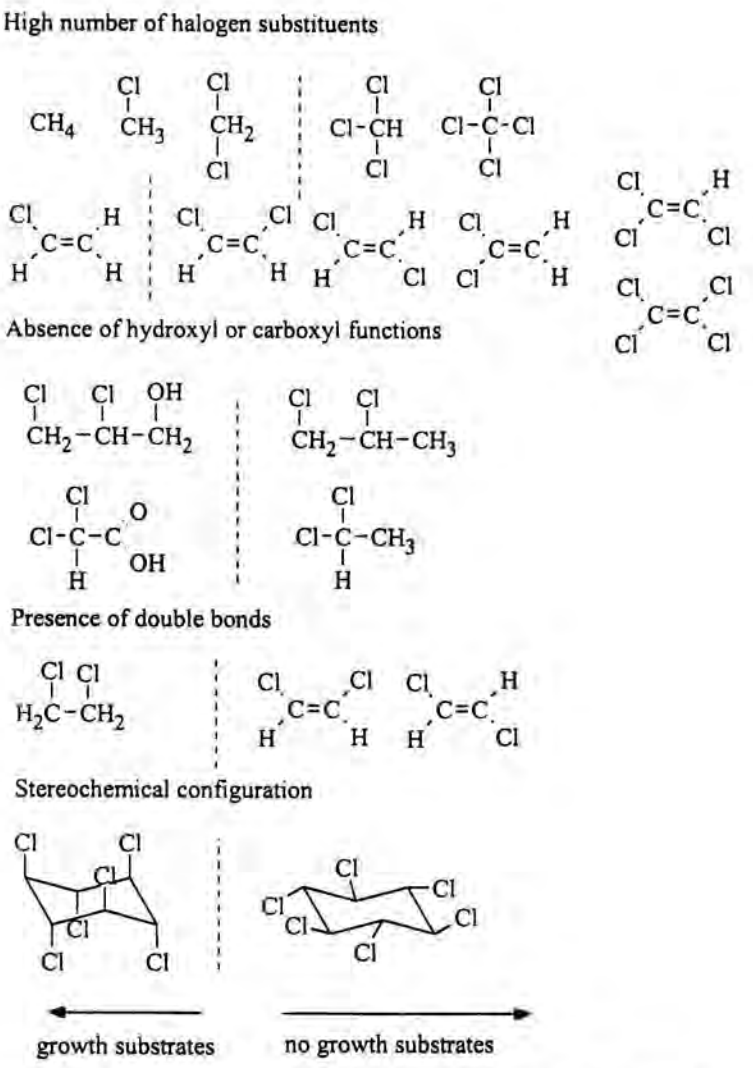

Fig. 1. Factors that limit bacterial growth on halogenated aliphatic compounds.

respectively, are easier to degrade than 1,1-dichloroethane which has only one Cl per C. A factor that increases degradability is the presence of oxo- or hydroxyl groups.

The reason for the lack of general structure-degradability relationships is twofold. First, blocks in a degradation pathway may occur at different steps, which may be catalyzed by enzymes that have completely different preferences concerning the chemical properties and substitution pattern of the substrate. Second, the specificities of dehalogenases often can not be described by simple structural rules.

This can be illustrated with the degradation of 1,2-dichloroethane as compared to 1,2-dibromoethane. The former compound may serve as a growth substrate for specific cultures of *Xanthobacter autotrophicus* and *Ancylobacter aquaticus* that produce a haloalkane dehalogenase, the gene of which is encoded on a large catabolic plasmid (van den Wijngaard *et al.*, 1992; Tardiff *et al.*, 1991). The organisms do not grow on 1,2-dibromoethane, which instead is very toxic for them. We also have not been able to isolate 1,2-dibromoethane degraders from environmental samples that did contain chloroalkane-degrading organisms. Nevertheless, 1,2-dibromoethane is an excellent substrate for the dehalogenase that degrades dichloroethane, as it is for other haloalkane dehalogenases. The recalcitrance of dibromoethane is explained by the poor conversion and high reactivity of the intermediate bromoacetaldehyde (van der Ploeg *et al.*, 1996). Although 1,2-dibromoethane is easier to dehalogenate, it is difficult to mineralize because of a block at a later catabolic step, i.e. dehydrogenation of the aldehyde which is formed as an intermediate.

One of the critical steps in the biodegradation of organochlorine compounds is obviously the cleavage of the carbon-chlorine bonds. An overview of the dehalogenation reactions observed with halogenated aliphatic compounds is

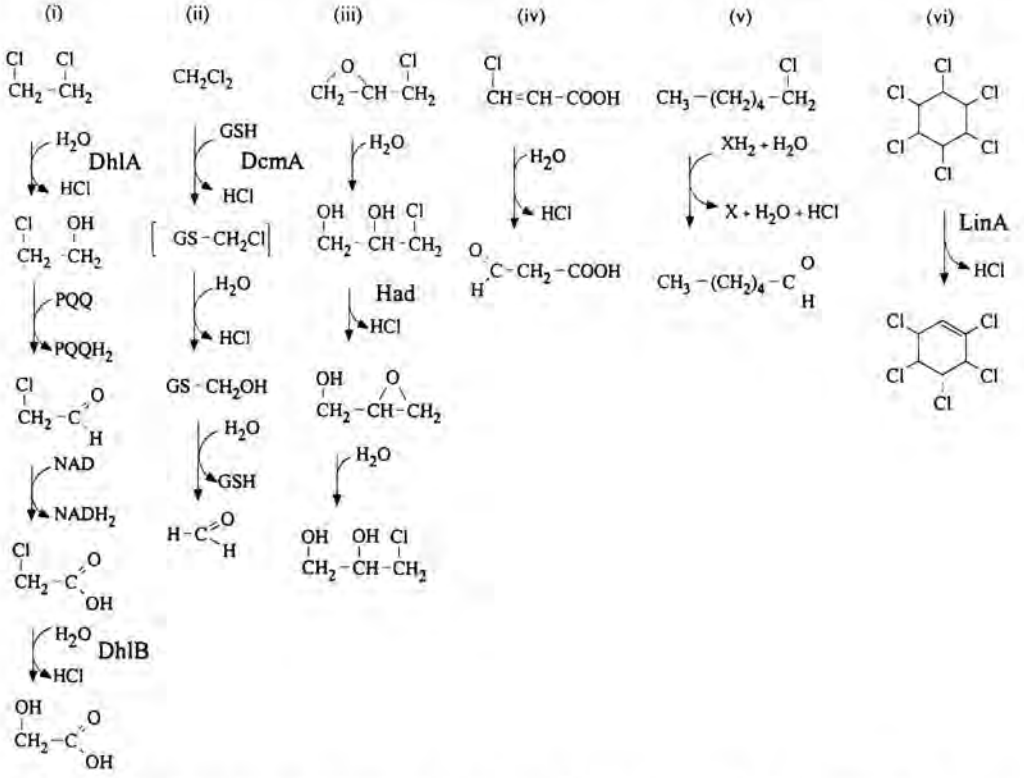

Fig. 2. Dehalogenation mechanisms for chlorinated aliphatic compounds. See text for details.

shown in Fig. 2. Briefly, six different reaction types have been investigated in some detail (Janssen *et al.*, 1994; Fetzner and Lingens, 1994; Nagata *et al.*, 1993): (i) Hydrolytic dehalogenation by dehalogenases (DhlA, DhlB), without the need for additional cofactors or oxygen; (ii) Substitution of a halogen by glutathione, catalyzed by glutathione-S-transferase (DcmA), a reaction analogous to the general detoxification mechanisms observed in mammalian systems; (iii) A lyase type of reaction (Had), proceeding by intramolecular substitution of a hydroxyl oxygen on a neighboring carbon atom carrying a halogen substituent; (iv) Substitution of a halogen on an unsaturated carbon atom, leading to an aldehyde, possibly by a hydratase type of reaction; (v) Oxidative conversion, by action of a monooxygenase in a reduced cosubstrate and oxygen-dependent reaction; (vi) Eliminative dehalogenation or dehydro-dehalogenation (LinA), as observed during the initial step of γ-hexachlorocyclohexane degradation.

The specificity of these dehalogenases may correlate well with the range of compounds that can support growth. Thus, the recalcitrance of compounds such as 1,1-dichloroethane, 1,1,2-trichloroethane, 1,2-dichloropropane and 1,2,3-trichloropropane, and the β- and δ-isomers of hexachlorocyclohexane may be explained by the low dehalogenase activity of the enzymes (DhlA, LinA) that are involved in the degradation of the closely related compounds or isomers (Fig. 2).

Degradation of 1,2-dichloroethane

We have studied the degradation of 1,2-dichloroethane by strains of *A. aquaticus* and *X. autotrophicus* in detail (Pries *et al.*, 1994a). The initial catabolic step is carried out by a haloalkane dehalogenase that converts 1,2-dichloroethane and a number of other chloro- and bromo-compounds. For discussing the possible evolutionary origin of these activities, the following observations are important. First, 1,2-dichloroethane is not known to be a natural compound. It does not (yet) occur on the ever growing list of naturally occurring halogenated compounds. Thus, it is unlikely that organisms that can degrade dichloroethane evolved by selective evolution before the compound was synthesized and introduced in the environment by industrial activities.

If organisms did not specifically adapt to 1,2-dichloroethane before its industrial production started, there are two possibilities for the evolutionary origin of the 1,2-dichloroethane-degrading bacteria. First, there may be organisms that already had a functional 1,2-dichloroethane-degradation pathway formed by a set of enzymes which had evolved for a different function, possibly utilization of a naturally produced organohalogen. Second, industrially emitted 1,2-dichloroethane may have been present at high concentrations in some areas, which provided a niche which stimulated rapid selection of genetically modified strains that degrade dichloroethane. Third, another synthetic compound than 1,2-di-

chloroethane may have played a role in creating selection pressure for dechlorinating activity towards dichloroethane.

We support the second possibility for different reasons. In the first place, the haloalkane dehalogenase gene and the (modified) chloroacetaldehyde dehydrogenase gene are plasmid encoded. Plasmid localization of genes that are under evolutionary pressure is also observed for other systems, for example resistance to antibiotics and heavy metals. The evolution of a catabolic plasmid for the degradation of bromoacetate was recently mimicked in the laboratory. In other strains in which a functional catabolic pathway was obtained under selection pressure, the catabolic genes are also plasmid encoded or located on transposons (van der Meer *et al.* 1992; Thomas *et al.*, 1992).

A second argument for the recent evolutionary origin is the lack of regulation of the synthesis of the 1,2-dichloroethane-degrading haloalkane dehalogenase. The gene encoding this protein is constitutively expressed from consensus *E. coli*-like promoter sequences. Although there is an open reading frame encoding a protein with sequence similarity to the tetracyclin repressor (TetR), this does not seem to be a functional repressor. The development of inducible expression for a catabolic gene requires, in addition to the catabolic enzyme itself, the evolution of a regulatory protein that can recognize and bind the substrate. This apparently has not yet occurred for the regulation of 1,2-dichloroethane degradation. Instead, the function of the repressor may have been lost and the

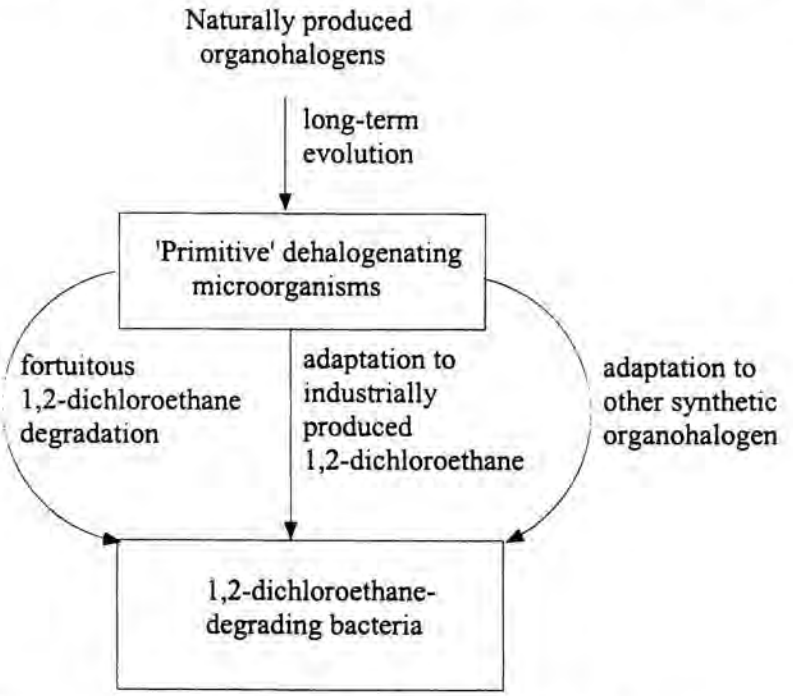

Fig. 3. Possible routes for the evolution of dehalogenating organisms.

dhlA gene is expressed from a constitutive promoter that may have been added during evolution of the pathway.

Other indications that the 1,2-dichloroethane pathway is of recent origin come from structural and biochemical studies on haloalkane dehalogenase itself, discussed below.

Haloalkane dehalogenases: diversity, evolution, and distribution

Haloalkane dehalogenases catalyze the hydrolytic cleavage of carbon-chlorine bonds in a wide range of halogenated alkanes. These enzymes play a role in the degradation of various alkylhalides. Based on the X-ray structure of the haloalkane dehalogenase from *X. autotrophicus*, and the sequence similarity with other hydrolytic enzymes, it has become clear that the haloalkane dehalogenases (Fig. 3, Table 1) belong to a group of hydrolytic proteins called α/β-hydrolase fold enzymes (Ollis *et al.*, 1992). This group also includes various other bacterial proteins involved in the biodegradation of natural and xenobiotic compounds. Details on the reaction mechanism and kinetics of haloalkane dehalogenase can be found elsewhere in this volume.

For reasons outlined above, the dehalogenase must have preexisted as an enzyme with a closely related function in preindustrial times, or it must have adapted to 1,2-dichloroethane during the last century or so from an enzyme that had a different function. We have proposed, on the basis of the sequence of mutant enzymes, that the dehalogenase has recently evolved from a more primitive dehalogenase. The sequence of the dehalogenase gene harbors sequence duplications that are indicative of recent evolutionary changes, since similar duplications arise when the gene is put under selection pressure in the laboratory (Pries *et al.*, 1994b).

Recently, we have sequenced the dehalogenase genes from a number of different 1,2-dichloroethane-degrading bacteria. Surprisingly, the haloalkane dehalogenases present in 10 independently isolated cultures obtained from different locations turned out to be identical, and they all contained the same sequence repetitions in the cap domain. In this respect, this haloalkane dehalogenase clearly distinguishes itself from the enzymes that hydrolyze chloroacetic acid, for which a large diversity has been found. At least 8 homologous genes have been sequenced, including the chloroacetate-converting dehalogenase of the dichloroethane catabolic pathway (van der Ploeg *et al.*, 1991). Chloroacetate, however, is a naturally occurring compound, and bacteria that have the capacity to degrade it are easily obtained from soil and water samples.

The lack of evolutionary divergence between the 1,2-dichloroethane dehalogenases isolated from different organisms again indicates that the enzyme is of recent evolutionary origin, but also immediately raises the question of how these genes then were distributed to different geographic locations. One possibility is the widespread use of the chlorinated aliphatic 1,3-dichloropropene as a nematocide in agriculture. It has been suggested that a gene similar to the 1,2-dichloro-

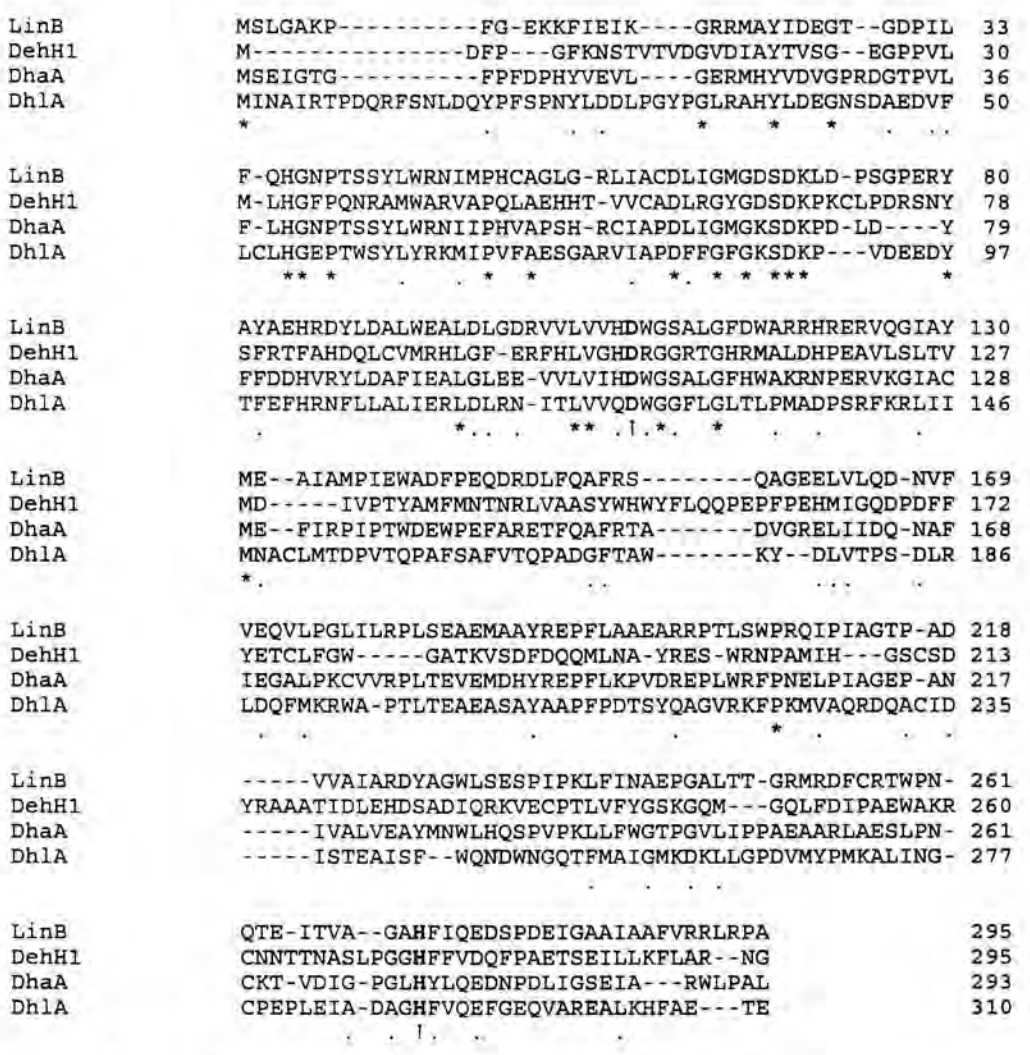

Fig. 4. Sequence alignment of hydrolytic dehalogenases (Table 1). LinB, tetrachorocyclohexadiene dehalogenase; DehH1, fluoroacetate dehalogenase; DhaA, chlorobutane dehalogenase; DhlA, haloalkane dehalogenase.

ethane dehalogenase gene of *X. autotrophicus* GJ10 occurs in a gram-negative organism that was selected from soil that shows a higher rate of 1,3-dichloropropene disappearance (Verhagen *et al.*, 1995), but this has not yet been confirmed by complete sequencing of the dehalogenase gene.

Recently, we have investigated in more detail the catabolic pathway and dehalogenases in a *Pseudomonas cichorii* strain that slowly utilizes 1,3-dichloropropene. The first step is hydrolysis of 1,3-dichloropropene to 3-chloroallylalcohol by a haloalkane dehalogenase. Further metabolism proceeds

Table 1. Dehalogenases of the α/β-hydrolase fold type that have been detected in bacteria that utilize halogenated aliphatics as a carbon source.

	DhlA	LinB	DehHI	DhaA
Organism	*Xanthobacter*, *Ancylobacter*, *Pseudomonas*	*Pseudomonas paucimobilis*	*Moraxella* sp. strain B	*Rhodococcus*, *Pseudomonas cichorii*
"Natural" substrate	1,2-dichloroethane	tetrachloro-cyclohexadiene	fluoroacetate	1-chlorobutane
Substrates	C-1 to C-4 1-chloro-n-alkanes, C-1 to C-12 1-bromo-n-alkanes, C-2 to C-3 α,ω-dihalo-n-alkanes	a number of mono-chlorinated alkanes, 1,2-di-bromoethane	halogenated acetates	C-2 to C-10 1-chloro-n-alkanes, C-1 to C-16 1-bromo-n-alkanes, C-2 to C-9 α,ω-dihalo-n-alkanes
No. of amino acids	310	295	294	293
Catalytic triad[1)] Halide binding	Asp-His-Asp Trp-Trp	Asp-His-Glu Trp-?	Asp-His-Asp Arg(?)-Trp(?)	Asp-His-Glu Trp-?
Molecular mass	35,143	33,050	33,307	33,248
References	Keuning *et al.*, 1985; Janssen *et al.*, 1989; Verschueren *et al.*, 1993	Nagata *et al.*, 1993	Kawasaki *et al.*, 1992	Curragh *et al.*, 1994; Kulakova *et al.*, 1997

[1)]Based on sequence similarity to haloalkane dehalogenase, in which a halide-binding residue is located immediately distal to the nucleophile. In three cases, this is a Trp. The Arg residue in DehH1 may be involved in carboxylate or fluorine binding.

via 3-chloroacrylic acid, and a dehalogenase for the *trans*-isomer of this compound is also present. The haloalkane dehalogenase was sequenced and appeared to be identical to the haloalkane dehalogenase from a *Rhodococcus* (Kulakova *et al.*, 1997). This enzyme belongs to the same group of α/β-hydrolase fold enzymes as the *X. autotrophicus* GJ10 1,2-dichloroethane dehalogenase (Janssen *et al.*, 1994), the fluoroacetate dehalogenase of *Moraxella* (Kawasaki *et al.*, 1992), and the tetrachlorocyclohexadiene hydrolase of lindane-utilizing bacteria (Nagata *et al.*, 1993) (Fig. 3).

These results are in agreement with the hypothesis that the large-scale use of the nematocide 1,3-dichloropropene has stimulated the evolution and spread of haloalkane dehalogenase producing organisms. It also suggests that during the evolution of 1,3-dichloropropene degrading bacteria, a haloalkane dehalogenase gene has been recruited by *P. cichorii* from gram-positive organisms that degrade chlorobutane or related chloroalkanes. The genetic organization of

the dichloropropene degradation genes and the exchange processes that led to the evolution of a 1,3-dichloropropene catabolic pathway are currently under study.

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Kinetics and Specificity of Haloalkane Dehalogenase

Abstract

This paper summarizes our recent efforts to study the structural basis of the kinetics and specificity of haloalkane dehalogenase. Release of the charged halide ion, which is already cleaved-off from the substrate in the first step of the reaction, was found to occur after a rate-limiting conformational change. The X-ray structure of a Phe172Trp dehalogenase mutant indicated that a helix-loop-helix region that covers the active site of the enzyme is involved in this conformational change. Cleavage of the carbon-halogen bond was much faster than halide release, however. Protein engineering resulted in dehalogenase mutants with an improved catalytic activity for 1,2-dibromoethane, but not for 1,2-dichloroethane. This indicates that the enzyme is optimized for its natural substrate 1,2-dichloroethane.

Introduction

Haloalkane dehalogenase from the bacterium *Xanthobacter autotrophicus* GJ10 converts haloalkanes to their corresponding alcohols (Keuning *et al.*, 1985). The dehalogenase is the first enzyme in the degradation route of 1,2-dichloroethane, and is essential for growth of bacteria on this substrate as the sole source of carbon and energy (Janssen *et al.*, 1985; van den Wijngaard, 1992). The three-dimensional structure and reaction mechanism of the enzyme are known (Verschueren *et al.*, 1993a, b, c; Pries *et al.*, 1994a, 1995).

The reaction proceeds via the formation of a covalent alkyl-enzyme which is subsequently hydrolyzed by activated water. Two tryptophans stabilize the chlorine leaving group and histidine 289 acts as a general base to activate a water molecule that hydrolyzes the alkyl-enzyme intermediate (Figure 1).

Although the dehalogenase is one of the best studied enzymes involved in biodegradation, little is known about the kinetics and specificity of the conversion of halogenated alkanes by this enzyme. The small hydrophobic active site cavity (37 $Å^3$, Verschueren *et al.*, 1993a) predicts a low affinity for polar and large halogenated compounds. However, considerable activities for the large dehalogenase substrate 1-bromohexane and for the polar substrate 2-bromoethanol were found (Pries *et al.*, 1994b; van den Wijngaard *et al.*, 1992).

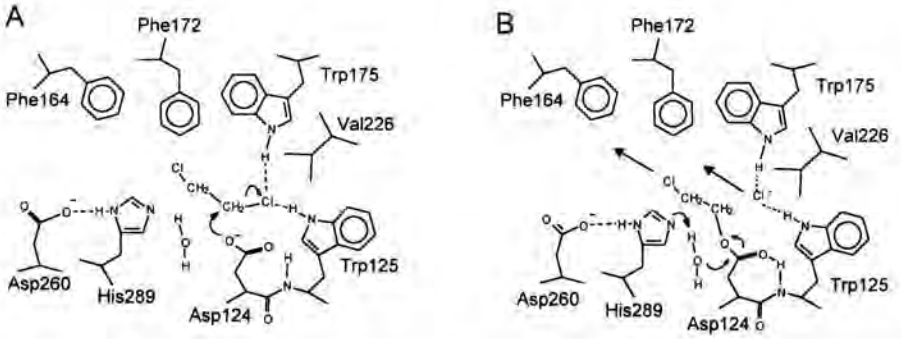


Fig. 1. Proposed reaction mechanism of haloalkane dehalogenase. A) Nucleophilic attack of Asp124 on the C_α of the substrate 1,2-dichloroethane, leading to carbon-halogen bond cleavage and formation of the covalent alkyl-enzyme intermediate. B) His289-catalyzed hydrolysis of the intermediate.

Furthermore, the steady-state kinetics of 1,2-dichloroethane and 1,2-dibromoethane conversion do not reflect the kinetics of carbon-halogen bond cleavage. The k_{cat} values for 1,2-dichloroethane and 1,2-dibromoethane are similar, while the K_m values for these two substrates differ strongly.

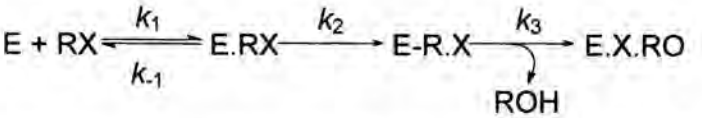
This paper describes the influence of various amino acids on the kinetics and specificity of haloalkane dehalogenase.

The kinetics of substrate conversion

Identification of the rate-limiting step

Conversion of 1,2-dichloroethane and 1,2-dibromoethane by the dehalogenase is sensitive to the use of ^2H_2O as the solvent (Kennes *et al.*, 1995). The effect of ^2H_2O on the k_{cat} and not on the k_{cat}/K_m for both 1,2-dichloroethane and 1,2-dibromethane conversion indicates that for a 3-step mechanism (Scheme I), where $K_m = K_s k_3/(k_2 + k_3)$, $k_{cat} = k_2 k_3/(k_2 + k_3)$ and $k_{cat}/K_m = k_2/K_s$, only k_3 is affected and that step 3 is mainly rate-limiting (Figure 2A).

Hydrolysis of the alkyl-enzyme intermediate to free enzyme and products was also found to be rate-limiting in a stopped-flow fluorescence experiment with enzyme in excess over substrate (Figure 2B). Fitting the fluorescence transient



Scheme I. Simplified scheme summarizing the dehalogenase reaction. E.RX is the Michaelis complex and E-RX is the covalent alkyl-enzyme intermediate.

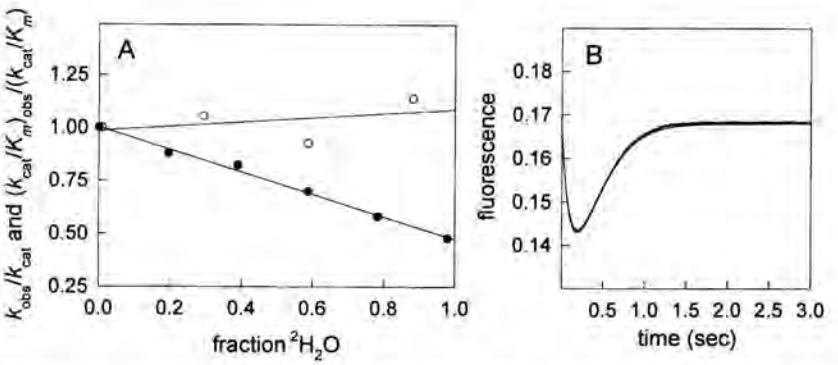


Fig. 2. A) The observed k_{cat} (k_{obs}) and k_{cat}/K_m ($(k_{cat}/K_m)_{obs}$) for 1,2-dibromoethane conversion as the fraction of the k_{cat} and k_{cat}/K_m observed in ^1H_2O was determined at different $^2H_2O/^1H_2O$ ratios; -●-, k_{obs}/k_{cat} and -○-, $(k_{cat}/K_m)_{obs}/(k_{cat}/K_m)$. B) Fluorescence transient obtained after rapid mixing of haloalkane dehalogenase (15 μM) and 1,2-dibromoethane (12.5 μM). The solid line represents the best fit by numerical simulation (Johnson, 1986) of the data to Scheme I. Similar results were obtained for 1,2-dichloroethane conversion (Figures taken from Schanstra and Janssen, 1996a).

with numerical simulation (Frieden, 1994) of Scheme I yielded a slow k_3 (5 s^{-1}). The slow step in the conversion of 1,2-dibromoethane is thus most likely k_3 which can be split up in: 1) hydrolysis of the alkyl-enzyme intermediate, 2) alcohol release, and 3) halide release.

In a rapid-quench-flow experiment with 1,2-dibromoethane in excess over enzyme, a transient burst of alcohol formation was observed (Figure 3A), indicating that all steps including hydrolysis of the alkyl-enzyme intermediate

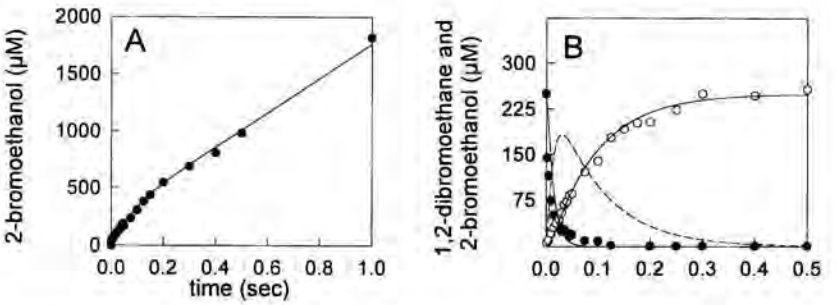


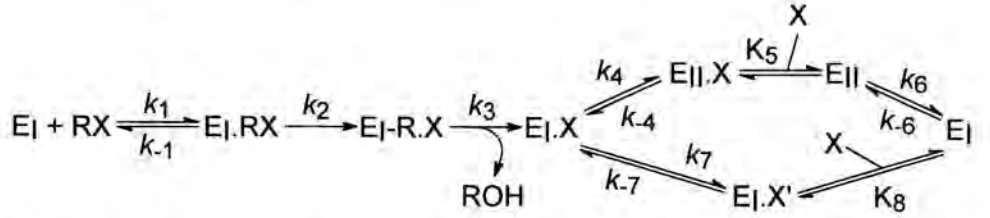
Fig. 3. Rapid-quench-flow analysis of 1,2-dibromoethane conversion. A) Burst experiment with substrate in excess over enzyme. -●- 2-bromoethanol production upon mixing enzyme (550 μM) with excess of 1,2-dibromoethane (10 mM). B) Single turnover of haloalkane dehalogenase. 1,2-dibromoethane decrease (-●-) and 2-bromoethanol production (-○-) after mixing enzyme (445 μM) and 1,2-dibromoethane (250 μM). The solid lines are the best fits of the data by numerical simulation with rate-constants given in Table 1. The dashed line is the simulated concentration of the alkyl-enzyme intermediate (E-R.X) in time. Similar results were obtained for 1,2-dichloroethane (Figures were taken from Schanstra *et al.*, 1996b).

are faster than the steady-state turnover. Thus, hydrolysis of the alkyl-enzyme is not rate-limiting. Alcohol-release is unlikely to be the rate-limiting step since the alcohol was never found back in the X-ray structure (Verschueren *et al.*, 1993b). Furthermore, the enzyme has a very low affinity for the alcohol produced from 1,2-dibromoethane (Schanstra *et al.*, 1996b). Halide release is a good candidate for the rate-determining step since halide ions were observed in the X-ray structure after completion of the chemical part of the reaction (Verschueren *et al.*, 1993b). Therefore, this step was studied separately.

Halide release

Quenching of the intrinsic protein fluorescence of the dehalogenase upon binding of substrates and halide (Verschueren *et al.*, 1993c) was used to study halide binding and release with stopped-flow fluorescence measurements. Under pseudo-first order conditions an unusually complex dependence of the observed binding rate (k_{obs}) on the halide concentration was found. This dependence of the k_{obs} could be explained by the presence of two parallel routes for halide release (Scheme II, from $E_I.X$ to E_I).

Halide release was found to follow mainly the upper route in Scheme II, in which a slow enzyme isomerization (k_4) that could limit the k_{cat} precedes actual halide dissociation (K_5) and another enzyme isomerization (k_6), which are very fast. Furthermore, halide binding and release in ^2H_2O was slower than in ^1H_2O, showing that the previously found solvent kinetic isotope effect on the steady-state k_{cat} for 1,2-dichloroethane and 1,2-dibromoethane conversion could be caused by a lower rate of the enzyme isomerization preceding halide release rather than by slowing down a chemical step (Schanstra and Janssen, 1996a). Halide binding via the lower route in Scheme II (from $E_I.X$ to E_I) was found to occur only at high halide concentrations and is a more common ligand binding mechanism involving the formation of an initial collision complex (Johnson, 1986). Only a small amount of halide will exit via this route.



Scheme II. Haloalkane dehalogenase reaction scheme deduced from pre-steady-state kinetic studies (combined from Schanstra and Janssen 1996a; Schanstra *et al.*, 1996b). The parallel routes for halide release are from $E_I.X$ to E_I. Symbols: E_I, haloalkane dehalogenase; RX, substrate; $E_I.RX$, Michaelis complex; E_I-R.X, covalent alkyl-enzyme intermediate; $E_I.X$, halide bound enzyme; $E_{II}.X$, different conformation of halide bound enzyme; E_{II}, different conformation of free enzyme; $E_I.X'$, initial collision complex.

The rates of the other steps in the dehalogenase reaction

Further kinetic analysis of substrate conversion by rapid-quench-flow (Figure 3) and stopped-flow fluorescence showed that both for 1,2-dibromoethane and 1,2-dichloroethane conversion, hydrolysis of the alkyl-enzyme intermediate (k_3, Scheme II) is only about 2-fold faster than the rate of halide release (Table 1). The rate of cleavage of the carbon-halogen bond (k_2, Scheme II) was found to be much faster than the rate of halide release. In rapid-quench-flow experiments, the rate of hydrolysis of the alkyl-enzyme intermediate was affected by the use of ^2H_2O as the solvent (Table 1). Thus, besides the effect of ^2H_2O found on the rate of halide release (via the enzyme isomerization), ^2H_2O also contributes to the lower steady-state k_{cat} in ^2H_2O by lowering the rate of dealkylation of the covalent intermediate. This is not a surprising observation since activation of a water molecule, by transfer of a proton to His289, is involved in this step (Figure 1).

The main difference between the kinetics of conversion of 1,2-dibromoethane and 1,2-dichloroethane was located at the beginning of the reaction sequence (steps 1 and 2, Scheme I). Both substrate binding and carbon-halogen bond cleavage were found to be slower with 1,2-dichloroethane than with 1,2-dibromoethane. This is in agreement with the observation that the enzyme has a higher affinity for bromide than for chloride (Kennes *et al.*, 1995) and that bromine is a better leaving group.

Mutants of haloalkane dehalogenase with an increased catalytic rate

The properties of in vitro constructed haloalkane dehalogenase mutants Phe172Trp (Schanstra *et al.*, 1996c) and Val226Ala (Schanstra *et al.*, 1996d) confirmed that the observed enzyme isomerization preceding halide release is the main rate-limiting step for 1,2-dibromoethane conversion in the wild type enzyme. The enzyme isomerization preceding actual halide release was 6–9 fold faster in these mutants, resulting in a similar increase in the overall rate of halide

Table 1. Haloalkane dehalogenase kinetic constants for 1,2-dibromoethane and 1,2-dichloroethane conversion obtained by rapid-quench-flow analysis of substrate conversion (taken from Schanstra *et al.*, 1996b).

	k_1 ($\mu M^{-1}.s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k_4[a] (s^{-1})	k_{cat}[b] (s^{-1})	K_m[b] (μM)
1,2-dibromoethane	0.75	>20	>130	10	4	2.8	4.3
1,2-dibromoethane (^2H_2O)	0.75	>20	>130	4	3	1.7	2.6
1,2-dichloroethane	9×10^{-3}	20	50	14	8	4.6	0.72×10^3

[a] k_4 indicates the overall rate of halide release as determined by rapid-quench-flow techniques (thus the rate to go from E_1X to E_1 in Scheme II).

[b] Steady-state kinetic constants calculated from the derived rate constants.

release (Schanstra *et al.*, 1996c, 1997). This resulted in a 2–3 fold higher steady-state k_{cat} for 1,2-dibromoethane (compare Table 1 and Table 2).

Since halide release had become much faster, hydrolysis of the alkyl-enzyme intermediate (k_3) was now rate-limiting for 1,2-dibromoethane conversion in these mutant enzymes. An increase in k_{cat} was not found for 1,2-dichloroethane conversion although the rate of chloride release increased. This was caused by a significantly lower rate of carbon-halogen bond cleavage (k_2, Table 2), which became rate-limiting for the Phe172Trp enzyme, resulting in a steady-state k_{cat} for 1,2-dichloroethane in this mutant that was even lower than with the wild type enzyme. The rate of substrate binding (k_1) was also lower in the mutant enzymes than in the wild type dehalogenase.

The structural origin of the rate-limiting enzyme isomerization

Phenylalanine 172 and valine 226 make up part of the hydrophobic wall lining the active site cavity of the dehalogenase (for a schematic representation see Figure 1). We have proposed that the characteristics of the halide release kinetics found for the Val226Ala enzyme are mainly caused by repositioning of Phe172 by the Val226Ala mutation since in the wild type enzyme these two residues are in close contact (Schanstra *et al.*, 1996d). This is in agreement with the observation that the Val226Ala enzyme and the Phe172Trp enzyme show similar properties with respect to the kinetics of halide release. The X-ray structure of the Phe172Trp dehalogenase shows that the helix (helix 4, 159-161)-loop-helix (helix 5, 171–181) structure, which is part of the cap domain and covers the active site of the dehalogenase, is somewhat different in this mutant from the wild type enzyme (Schanstra *et al.*, 1996c).

The rate of halide release in the dehalogenase mutants increased significantly due to an elevated rate of the enzyme isomerization preceding actual release ($E_I.X \rightarrow E_{II}.X$, Scheme II). We have hypothesized for the wild type enzyme that

Table 2. Haloalkane dehalogenase kinetic constants of Phe172Trp and Val226Ala mutant enzymes (taken from Schanstra *et al.*, 1996c, d).

	k_1 ($\mu M^{-1}.s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k_4 [a] (s^{-1})	k_{cat} [b] (s^{-1})	K_m [b] (μM)
1,2-dibromoethane							
Phe172Trp	0.4	25	30	9	75	6.3	29
Val226Ala	0.41	45	60	12	43	8.1	35
1,2-dichloroethane							
Phe172Trp	3.0×10^{-3}	30	4.5	9.5	>75	2.9	7.5×10^3
Val226Ala	4.5×10^{-3}	25	14	9	50	4.9	3.1×10^3

[a] k_4 indicates the overall of rate of halide release as determined by rapid-quench-flow techniques (thus the rate to go from $E_I.X$ to E_I in Scheme II).

[b] The steady-state k_{cat} and K_m calculated from the derived rate constants.

this isomerization is a step necessary to allow water to enter the buried active site cavity and solvate the charged halide ion (Schanstra and Janssen, 1996a). A possible route could involve a tunnel extending from the active site towards the solvent, as suggested by X-ray crystallography (Verschueren *et al.*, 1993a). This tunnel was also proposed to be used for substrate binding, which we found to be extremely fast, however. The proposed tunnel seems to be blocked by Leu262, and if substrate binding and product release occur via this route, Leu262 has to move away (Verschueren *et al.*, 1993a). However, the kinetics of substrate binding and halide release are fundamentally different. Halide release is much slower, although a halide ion is smaller than the corresponding dihaloethane. Since halide ions cannot easily diffuse through a hydrophobic protein environment and since the process requires a slow enzyme isomerization it is likely that a more extensive motion is involved.

A possible route for halide release could be formed by a conformational change in a part of the cap domain that shields the active site from the solvent. This may expose the active site, allowing water to enter and solvate the halide ion. The effect of the mutations Val226Ala and Phe172Trp was that the rate of the enzyme isomerization increased drastically which suggested that the conformational change could be located in the helix-loop-helix structure covering the active site. The observed difference of this helix-loop-helix structure of the Phe172Trp enzyme supports the idea that motions required for halide release take place there. The effects of the mutations and solvent isotope effects (Schanstra and Janssen, 1996a) also suggest a larger conformational change than the movement of a single residue in the interior of the enzyme such as Leu262. Large conformational changes that accompany substrate binding have also been observed in lipases (Tilbeurgh *et al.*, 1993) and aldose reductase (Rondeau *et al.*, 1992; Grimshaw *et al.*, 1995). However, in these cases the enzyme switches from an open to a closed conformation, which is different from rapid transient events that occur in haloalkane dehalogenase.

Substrate specificity

The substrate range of the dehalogenase was tested for a variety of chlorinated and brominated compounds (Table 3). For all pairs of substrates in this table, the K_m for chlorinated-compounds was significantly higher than for the brominated analogs. This correlates with the lower affinity of the enzyme for chloride than for bromide (Schanstra *et al.*, 1996b). However, the k_{cat} values for most brominated and chlorinated nonbranched substrates with chain lengths up to 4 carbon atoms were comparable, indicating a similar rate-limiting step for such compounds.

Long chain haloalkanes were poor substrates with a high K_m and a low k_{cat}. No solvent kinetic isotope effect of ^2H_2O on 1-chlorohexane conversion was

Table 3. Substrate range of haloalkane dehalogenase (taken from Schanstra *et al.*, 1996b).

	chlorinated			brominated		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
CH_2X-CH_2X	0.53	3.3	6.2×10^3	0.01	3.0	3.0×10^5
CH_3-$(CH_2)_2$-CH_2X	2.2	1.5	6.8×10^2	0.06	0.94	1.6×10^4
CH_3-$(CH_2)_4$-CH_2X	1.4	0.088	63	0.3	0.64	2.1×10^3
CH_2X-CHX-CH_3	13	0.15	12	1.3	2.1	1.6×10^3
CN-CH_2X	6.3	1.5	2.4×10^2	0.49	2.7	5.5×10^3
CH_3-(O)-CH-CH_2X	48	1.8	38	2.2	2.6	1.2×10^3
CH_2OH-CH_2X	>400	-[a]	0.70 [b]	11	2.8	2.5×10^2
CH_2X_2	>100	-	0.70 [b]	2.4	3.9	1.6×10^3
H_2N-CO-CH_2X	>100	-	0.046[b]	20	1.0	5.0×10^1

[a]-, not detectable.
[b]Measured as the first order rate-constant at concentrations of 400 mM CH_2OH-CH_2Cl, 100 mM Cl-CH_2-Cl and 100 mM H_2N-CO-CH_2Cl.

observed, while such an effect was present in the conversion of 1-chlorobutane and 1,2-dichloroethane (Figure 4A) which suggests a different rate-determining step for 1-chlorohexane. The large difference in k_{cat} between the branched substrates 1,2-dichloropropane and 1,2-dibromopropane is probably also accompanied by a different rate-determining step since ^2H_2O did not affect the rate of 1,2-dichloropropane conversion, while the rate of 1,2-dibromopropane conversion was reduced by 50% in ^2H_2O (Figure 4B).

Polar compounds, such as halogenated alcohols and epoxides, were poorer substrates than apolar compounds. The K_m of these compounds was increased compared to their apolar counterparts.

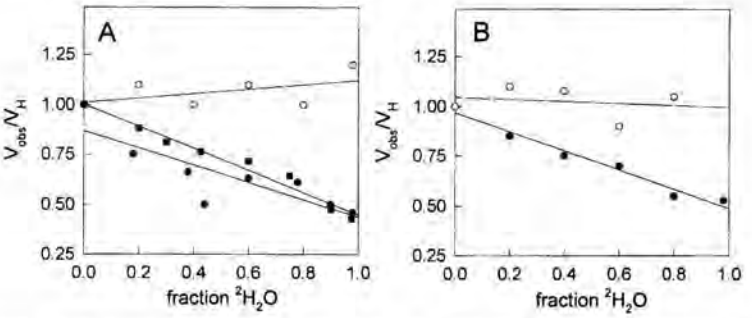


Fig. 4. Solvent ^2H_2O kinetic isotope effects on haloalkane dehalogenase activity. The observed rate (V_{obs}) as a fraction of the rate observed in ^1H_2O (V_H) was determined at different $^2H_2O/^1H_2O$ ratios. A) The V_{obs}/V_H for 1-chlorobutane (5 mM, -●-), 1-chlorohexane (3 mM, -○-) and 1,2-dichloroethane (5 mM, -■-) conversion. B) The V_{obs}/V_H for 1,2-dibromopropane (4 mM, -●-) and 1,2-dichloropropane (25 mM, -○-) conversion (Figures taken from Schanstra *et al.*, 1996b).

Connections between haloalkane dehalogenase reaction steps

The increase in the rate of halide release in the mutant dehalogenases was always accompanied by a decrease in the rate of substrate binding and the rate of cleavage of the carbon-halogen bond. The rate of hydrolysis of the alkyl-enzyme intermediate, however, was hardly affected. This 'uncoupling' between the rates of alkylation and dealkylation in haloalkane dehalogenase is allowed since these two processes are not the reverse of each other, unlike acylation and deacylation in the serine proteases (Stryer, 1988) and in α/β-hydrolase fold enzymes that have a Ser as the nucleophile (Ollis *et al.*, 1992). Alkylation occurs by displacement of the halogen on the C_α of the substrate with a carboxylate oxygen of Asp124, while dealkylation occurs by attack of activated water on the C_γ of alkylated Asp124 (Verschueren *et al.*, 1993b; Pries *et al.*, 1994a). Furthermore, dealkylation is largely determined by the main domain of the enzyme. Activation of a water molecule by the Asp260-His289 couple and stabilization of the tetrahedral intermediate by the oxyanion hole are all functions provided mainly by main domain residues located at the hydrophilic bottom of the active site (Verschueren *et al.*, 1993b) which are not strongly influenced by mutations that affect halide release.

The observed decrease in the rate of substrate binding and carbon-halogen bond cleavage and the increase in the rate of halide release in the mutants suggests that these steps are linked in the dehalogenase reaction. The proposed involvement of the helix-loop-helix structure that covers the active site cavity of the enzyme in these processes can explain this observed coupling. An increase in the flexibility of this region would facilitate the conformational change required for halide release, but destabilize bound substrate and the transition state during carbon-halogen bond cleavage, resulting in a net increase in both k_{cat} and K_m for 1,2-dibromoethane conversion. A rigid hydrophobic active site cavity would stabilize smaller hydrophobic substrates, but also the halide product. Especially for 1,2-dichloroethane the rigidity of the active site of the dehalogenase seems to be important for substrate binding and carbon-halogen bond cleavage. The kinetic mechanism of the wild type enzyme seems to be optimized towards 1,2-dichloroethane conversion in the sense that there is an optimal balance between the rates of steps that cannot be changed independently. This makes elevating the k_{cat} of the dehalogenase towards a compound like 1,2-dichloroethane a difficult task. We found that a significant increase in k_{cat} could still be accomplished for the substrates 1,2-dibromoethane and 1-bromo-2-chloroethane, both 'nonnatural' substrates in which the balance between C-X cleavage and X-release is not optimal.

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D. Dunaway-Mariano

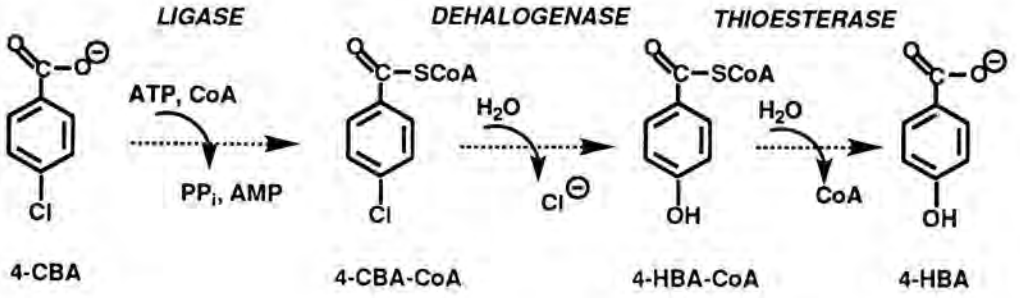
Studies on the Structure, Catalytic Mechanism and Ancestry of the 4-Chlorobenzoate-Coenzyme A Dehalogenase

Abstract

The 4-chlorobenzoate-coenzyme A dehalogenase catalyzes the second step of the 4-chlorobenzoate degrading pathway, the hydrolysis of 4-chlorobenzoate-coenzyme A to 4-hydroxybenzoate-coenzyme A. Mechanistic and structural studies of the dehalogenase from *Pseudomonas* sp. strain CBS3 have demonstrated a specialized mode of catalysis in which the carboxylate side chain of active site aspartate 145 displaces the chloride from the aromatic ring. The ring is activated for nucleophilic attack by two hydrogen bonds donated from the backbone amide hydrogens of glycine 114 and phenylalanine 64 to the benzoyl carbonyl oxygen. The aromatic side chains contributed by tryptophan 137 and 89 and phenylalanine 64 and 82 envelope the benzoyl ring in a hydrophobic environment. The arylated enzyme intermediate is hydrolyzed at the acyl carbon by attack of a water molecule, activated by histidine 90. A hydrogen bond donated by the indole NH of tryptophan 137 is available to stabilize the tetrahedral intermediate formed. The subunit fold, as defined by the X-ray crystal structure of the trimeric enzyme complexed with 4-hydroxybenzoate-coenzyme A, provides a cleft at the subunit interface into which the nucleotide moiety of the coenzyme A binds. The benzoyl moiety binds at a second cleft containing the catalytic residues while the pantothenate and pyrophosphate groups of the coenzyme span the two clefts, at the solvent interface. Interactions between enzyme and substrate ligand responsible for binding and catalytic turnover are examined. In addition, possible connections linking catalysis in the dehalogenase with structurally related enoyl-CoA dependent enzymes are explored.

Introduction

The bioremediation of unnatural substances is but one application of the remarkable capacity for rapid adaptation displayed by prokaryotes. The mechanism by which genes are recruited and retooled to provide new function for adaptation is a fascinating field of study with many important applications. In our laboratory we have focused our study of the evolution of new enzyme function on the investigation of the enzymes of the 4-chlorobenzoate (4-CBA)



Scheme 1.

degrading pathway (Scheme 1) (Scholten *et al.*, 1991). This pathway allows the utilization of 4-CBA as a substrate for oxidative metabolism through its conversion to 4-hydroxybenzoate (4-HBA). 4-HBA feeds into the *ortho* cleavage pathway and hence to the β-ketoadipate pathway and TCA cycle.

The 4-CBA degrading pathway represented in Scheme 1 has been shown to function in several of the special strains of 4-CBA degrading bacteria belonging to the genera *Pseudomonas* (Klages, 1980), *Arthrobacter* (Marks,1984; Müller, 1988; Shimao,1989; Adrians, 1989), *Nocardia* (Klages, 1979), *Corynebacterium* (Zaitsev, 1991; Groenwegen, 1992) and *Alcaligenes* (Van den Tweel, 1986) isolated from soil or sediment samples collected from different 4-CBA/PCB contaminated regions around the world (for a recent review see Dunaway-Mariano, 1994). We wonder how these bacterial strains acquired this pathway. Are the enzymes functioning in the 4-CBA degrading pathways of these different strains of bacteria structurally the same or different? Are these enzymes old or recently retooled from preexisting enzymes performing other essential metabolic functions? To address these questions we have started looking at the similarities and dissimilarities existing between the respective sets of pathway genes recruited by the individual bacterial strains and have begun to examine the genes and gene products of a given 4-CBA degrading pathway in relation to those of preexisting metabolic pathways to obtain clues as to the identities of the progenitor genes. Comparison of the structure and catalytic mechanism of the progenitor enzyme to the 4-CBA pathway enzyme will define the changes made in retooling catalytic function. In this paper the structure and mechanism of the 4-CBA-CoA dehalogenase from *Pseudomonas* sp. strain CBS3 are described as well as the insights that they provide into the ancestry of this unique enzyme.

Results and Discussion

The Three Dimensional Crystal Structure of the 4-CBA-CoA Dehalogenase From Pseudomonas sp. strain CBS3.

The crystal structure of the dehalogenase-4-HBA-CoA complex, determined by Matt Benning and Hazel Holden to 1.8 Å resolution (Benning, 1996), provides

us with a clear picture of the enzyme active site and potential interactions between reactant and active site residues. First, in this section we will look at the global aspects of the protein structure and then in the following sections examine the specific features of the active site in relation to substrate binding and catalysis. The 30 kDa dehalogenase subunit, originally believed to exist in tetrameric form in solution, crystallizes as a trimer with each of the three active sites separated by ca. 42 Å and related by a 3-fold rotational axis of symmetry (Benning, 1996). Each subunit contains two domains, linked by a calcium ion. The larger of the two domains includes residues 1–205 and 263–269 and consists of 8 α-helices and 10 β-sheets which form two layers of mixed sheet, arranged perpendicular with respect to one another. The two layers are flanked on either side by the α-helices.The small domain includes residues 206–262 and consists of 3 amphiphilic α-helices which pack against the large domain of the adjacent subunit. The 4-HBA-CoA ligand is bound in a folded conformation resembling a fish hook where the benzoyl and pantoic acid moieties form the stem, the pyrophosphate moiety the bend and the adenosine moiety (the 3′-phosphate is missing from this structure) forms the tip. The nucleotide region binds in a shallow crevice formed at the subunit interface. The pantoic acid and benzoyl moieties are inserted into a deep crevice formed between two helices. The pyrophosphate moiety binds to the surface of the protein which spans these two crevices and is itself exposed to solvent. Unfortunately, the crystal structures of other enoyl-CoA dependent enzymes with which the dehalogenase shares significant sequence homology have not yet been reported so that a comparison of folds can not be made at this time. A search of the protein structural data banks which include numerous other CoA thioester dependent enzymes failed to identify a protein having a fold similar to that of the dehalogenase (Benning, 1996).

Protein-Ligand Binding Interactions

From the X-ray structure of the dehalogenase-4-HBA-CoA complex the noncovalent binding interactions which contribute favorably to the binding energy can be inferred (see Benning, 1996). At the back of the active site crevice the ligand hydroxybenzoyl ring is bound by a hydrogen bond formed between Asp145 and the benzoyl C(4)OH, and at the mouth of the active site the ligand is anchored by two hydrogen bonds between the amide backbone hydrogens of Gly114 and Phe64 and the benzoyl C=O. The environment surrounding the benzoyl ring is hydrophobic. The side chains of Trp89, Trp137 and Phe64 encircle the benzoyl ring, appearing to participate in perpendicular stacking interactions with it. The pantoic acid moiety is engaged in three hydrogen bonds: one between the cysteine moeity NH and the backbone C=O of Ala62, the second between the adjacent C=O and a bound water molecule and the third between the pantothenate amide NH and the N(7) of the adenine ring. The pyrophosphate unit β-P interacts with the guanidinium group of Arg24 while the α-P interacts with the gaunidinium group of Arg257 from the neighboring subunit. The ribose interacts with the guanidinium of Arg67 while the N(1) of the

adenine ring hydrogen bonds to the back bone amide hydrogen of Leu66 and the C(6)NH hydrogen bonds with the backbone C = O of Phe64. The adenosine portion of the ligand is buried away from solvent.

The contributions made by the noncovalent bonding interactions observed in the crystal structure are currently being examined by altering the structure of the ligand or that of the enzyme residue involved and measuring the ligand binding constant. From the binding constants measured for the truncated forms of the 4-HBA-CoA ligand (Table 1) it is evident that CoA moiety, and in particular, the nucleotide region is crucial to tight binding. The nucleotide or nucleoside alone does not, however, bind strongly. Comparison of the binding constant of 4-HBA-CoA with 4-BA-CoA would suggest that the hydrogen bond between Asp145 and the hydroxybenzoyl C(4)OH contributes at least one order of magnitude to the binding constant, yet the dissociation constant (0.1 μM) of the 4-HBA-CoA-Asp145Ala dehalogenase complex (measured by the spectral titration method described in Taylor, 1995) is not measurably larger than that of the wild-type dehalogenase complex ($K_d = 0.5\,\mu M$), suggesting that an interplay of hydrophobic and electrostatic effects is at work. Indeed, substitution of the aromatic side chains encircling the benzoyl ring with a different hydrophobic side chain does not significantly inhibit 4-HBA-CoA binding but substitution with a polar residue does. Specifically, the K_d values measured for the Phe64Leu, Trp137Phe and Trp89Phe mutants are 0.4, 2, and 3 μM, respectively whereas the K_d of the Trp137Tyr mutant is too large to be measured by spectral titration, i.e., $>50\,\mu M$ (Liu, 1996).

The three Arg residues (R24, R257 & R67) which appear to form ion pairs with the phosphoryl moieties of the CoA unit have been mutated in order to access their contribution to productive substrate binding (Hong, unpublished). The two mutants which have been characterized, Arg24Leu and Arg257Leu, display steady-state turnover rates ($k_{cat} = 0.4\ s^{-1}$ and $0.2\ s^{-1}$, respectively) comparable to that of the wild-type enzyme ($k_{cat} = 0.6\ s^{-1}$) however, the K_m values obtained ($K_m = 30\,\mu M$ and $120\,\mu M$, respectively) are significantly larger than that measured for wild-type ($K_m = 3\,\mu M$). The modest perturbation in binding affinity is in line with the multiple sites of binding interaction suggested by the

Table 1. The inhibition constants (K_i) measured for 4-HBA-CoA structural analogs as competitive inhibitors of wild-type 4-CBA-CoA dehalogenase (from Taylor, 1996).

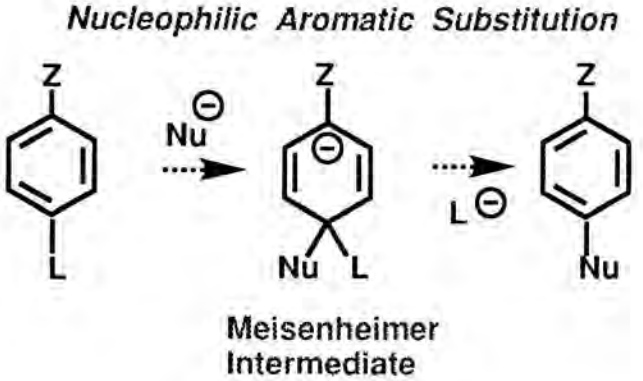
Inhibitor	K_i (μM)
4-HBA-CoA	2.5
BA-CoA	70
CoA	140
4-HBA-Pantothenate	>2000
ADP, 5'-AMP, Ade, PP_i	>5000
3'-AMP	1200

crystal structure of the dehalogenase-4-HBA-CoA complex and the minimal affect on k_{cat} consistent with the fact that these residues reside some distance away from the catalytic site (Benning, 1996).

Catalysis

At the outset of our studies of 4-CBA-CoA dehalogenase catalysis the assumption was that the dehalogenation reaction proceeds by a nucleophilic aromatic substitution pathway in which an anionic intermediate, known as the Meisenheimer complex, is formed (Scheme 2). The rates of such reactions in solution are controlled by the reactivity of the nucleophile-electrophile pair and/or by the dissociation energy of the bond to the leaving group. Given that the Cl^- ion is a good leaving group the need for the enzyme to stabilize the departing chloride ion may not be essential. Nonetheless, studies of the dechlorination reaction catalyzed by the haloalkane dehalogenase have shown that the departure of the chloride ion is assisted by two tryptophan residues (Verschueren, 1993). The nucleophile (H_2O)-electrophile (4-CBA-CoA) pair of the 4-CBA-CoA dehalogenase reaction represents, however, the greatest challenge to the enzyme. Water is such a poor nucleophile that general base catalysis would surely have to be a component of the dehalogenase catalytic mechanism. Likewise, the benzoyl ring of the 4-CBA-CoA is not sufficiently activated by the electron withdrawing substituent for nucleophilic addition to the ring to occur in the absence of electrophilic catalysis. We began devising experiments to test for these forms of catalysis. As it turned out, we found experimental evidence for general base catalysis and electrophilic catalysis in this enzymatic reaction but not until after we discovered something we had not counted on, and that is, nucleophilic catalysis.

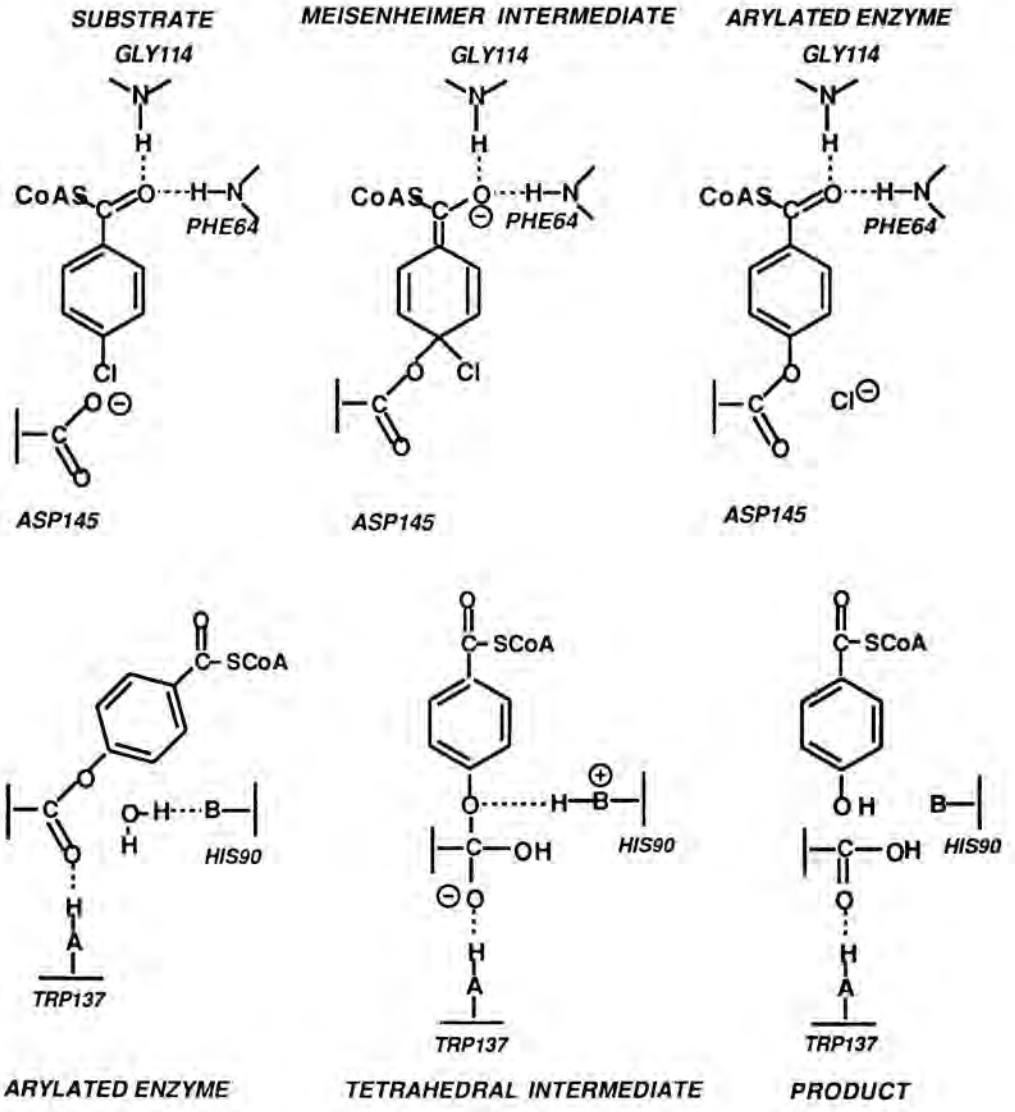
Presteady state kinetic studies of the dehalogenase reaction carried out with equivalent amounts of [^{14}C]4-CBA-CoA and enzyme in a rapid quench apparatus allowed us to detect the formation of a covalent adduct formed



Scheme 2.

between enzyme and reactant during the initial phase of a single turnover on the enzyme (Yang, 1994). The time course for the appearance and disappearance of this adduct compared to those of the substrate and product gave a clear indication that the covalent adduct is a reaction intermediate. The identity of the intermediate as an arylated enzyme was revealed by the product of a single turnover reaction carried out in 98% ^{18}O-enriched H_2O. This product was analyzed for ^{16}O Vs ^{18}O at the benzoyl C(4)OH first converting it to 4-HBA and then to the methyl derivative for analysis by GC-mass spectroscopy. While a single turnover resulted in product having 75% C(4)-^{16}O and 25% C(4)-^{18}O label a 1000-turnover reaction in 93% ^{18}O-enriched H_2O resulted in product having 13% C(4)-^{16}O and 87% C(4)-^{18}O (Yang, 1994). The conclusion drawn from these data is that the oxygen atom of the 4-HBA-CoA product C(4)-OH is derived from the enzyme and not from the solvent water. Transfer of an oxygen atom from the enzyme during catalytic turnover can be rationalized in mechanistic terms by attack of an active site carboxylate at C(4) to form a Meisenheimer complex followed by expulsion of the chloride to form the arylated enzyme which, in a second partial reaction, is hydrolyzed at the acyl carbon (Scheme 3). The identity of the active site carboxylate as Asp145 is suggested by the X-ray structure of the 4-HBA-CoA-dehalogenase complex which shows the Asp145 C-O- directed at the benzoyl C(4) less than 3 Å away (Benning, 1996) and site directed mutagenesis studies which show that the D145A mutant binds the substrate analog 4-methyl benzoyl-CoA (4-MeBA-CoA) with a $K_d = 1\ \mu M$ (Vs $3\ \mu M$ for wild-type enzyme) (Hong, unpublished) but does not under go catalytic turnover with bound substrate (Yang, 1996). The catalytic advantage of using the active site carboxylate instead of an activated water molecule to displace the ring chloride is retained by having the hydrolysis step occur at an acyl linkage, and not at the aromatic ring. This is indeed a clever enzyme.

Electrophilic catalysis was first evidenced by spectral studies of the enzyme bound substrate analog ligand, 4-MeBA-CoA and product ligand, 4-HBA-CoA (Taylor, 1995). Upon binding to the enzyme the benzoyl rings of these ligands under go electrostatic interaction with the active site resulting in the redistribution of ring π-electron density. The polarization of the benzoyl ring is evidenced by (1) the red shift observed in the benzoyl ring λ_{max} (from 260 nm to 302 nm for 4-MeBA-CoA; from 292 nm to 330 and 373 nm for 4-HBA-CoA) as seen in the UV-visible difference spectra, (2) the 2.8 ppm down field shift observed for the ^{13}C-NMR peak from the benzoyl C = O in 4-HBA-CoA and (3) the dramatic changes observed in the Raman bands corresponding to the benzoyl C = O stretch, in plane phenyl C-H stretches and benzene ring modes 8a and 8b observed for 4-MeBA-CoA and 4-HBA-CoA all of which take place when the ligand binds to the enzyme. Control experiments which measured spectral changes deriving from the benzoyl chromophore upon switching the ligand from buffer to organic solvent ruled out simple desolvation as the cause of the spectral perturbations observed upon ligand-enzyme complexation (Taylor, 1995). Insight into the identity of the enzyme active site groups responsible for polarization of the benzoyl ring is provided by the X-ray structure of the



Scheme 3.

4-HBA-CoA dehalogenase complex (see Figure 1). First, the benzoyl C = O is positioned for hydrogen bond formation with the backbone amide hydrogens from Gly114 and Phe64. These interactions should serve to withdraw electron density from the ring (hence from C(4)). Second, the Asp145 directs its charged carboxylate oxygen at the benzoyl C(4) thereby enhancing the polarization of π-electron density by a possible "push-pull" mechanism. Third, the benzoyl ring is enveloped by the aromatic side chains of Trp137, Trp89, Phe64 and Phe82. The

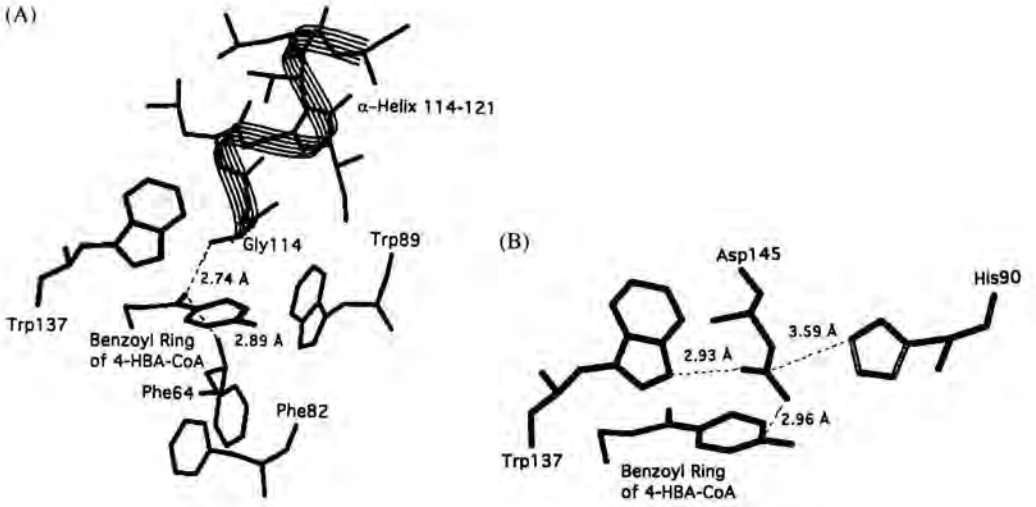


Fig. 1. A picture of the 4-CBA-CoA dehalogenase active site derived from the X-ray crystal stucture of the 4-HBA-CoA-dehalogenase complex (Benning, 1996) showing (A) the catalytic residues involved in the formation/stabilization of the Meisenheimer intermediate and (B) the three catalytic residues involved in the hydrolysis partial reaction.

hydrophobic environment thus provided may serve to facilitate the electrostatic interactions between ligand and enzyme and thus, the polarization of the benzoyl ring π-electrons.

We are in the process of testing the model of electrophilic catalysis described above by replacing the active site amino acids and measuring the impact on benzoyl ring polarization. The red shift of the 4-MeBA-CoA or 4-HBA-CoA benzoyl λ_{max} is used to detect benzoyl ring polarization in the dehalogenase mutant complex. The UV-visible absorption difference spectra of the 4-MeBA-CoA-D145A dehalogenase complex $\lambda_{max} = 304$ nm and 4-HBA-CoA-D145A dehalogenase complex ($\lambda_{max} = 330$, 373, 400 nm) reveal the red shift indicative of benzoyl ring polarization. We conclude that the Asp145 carboxylate does not contribute at a detectable level to the polarization of the benzoyl ring and thus, have eliminated the "push-pull" mechanism. To test the role of the Gly114 and Phe64 amide hydrogen bonds to the benzoyl C = O in ring polarization the local backbone conformation must be altered. Gly114 is the N-terminal residue of an α-helix that extends from G114 to A121 while Phe64 is positioned in a nearby loop that contributes to the active site wall. The F64L mutant was designed to alter side chain packing and hopefully, the orientation of the backbone amide hydrogen. However, the UV-visible absorption difference spectra of the 4-MeBA-CoA and 4-HBA-CoA complexes of the F64L dehalogenase mutant ($\lambda_{max} = 304$ nm and $\lambda_{max} = 330$, 369 nm, respectively) reveal the red shift indicative of benzoyl ring polarization. Intact substrate activation is supported by the steady-state kinetic constants ($k_{cat} = 0.2\ s^{-1}$, $K_m = 5\ \mu M$) determined for the mutant which are similar in value to those measured for the wild-type

dehalogenase ($k_{cat} = 0.6\ s^{-1}$, $K_m = 4\ \mu M$) (Liu, 1996). Presently, other substitutions are being made at this position to perturb the hydrogen bonding interaction with the substrate ligand. The G114P dehalogenase mutant which was designed to probe hydrogen bonding between the Gly114 backbone amide hydrogen and the benzoyl C = O proved to be too unstable for isolation. A more conservative replacement Gly114 with Ala was made next. The G114A mutant is stable but relatively inactive ($k_{cat} = 0.00001\ s^{-1}$) (Hong, unpublished). Benzoyl ring polarization by this mutant has not yet been tested by UV-visible difference spectroscopy.

The contribution of the hydrophobic environment of the active site to the polarization of the substrate benzoyl ring was examined by first substituting the target residue with a different hydrophobic residue (to serve as a steric control) and then with a more polar residue. The UV-visible absorption difference spectra of the 4-MeBA-CoA and 4-HBA-CoA complexes of the W137F ($\lambda_{max} = 306$ nm and $\lambda_{max} = 330$, 378 nm, respectively), W89F ($\lambda_{max} = 302$ nm and $\lambda_{max} = 338$ nm, respectively), F64L ($\lambda_{max} = 304$ nm and $\lambda_{max} = 330$, 369 nm, respectively) and F82L ($\lambda_{max} = 302$ nm and $\lambda_{max} = 330$, 373 nm, respectively) dehalogenase mutants indicate that the red shift and hence, benzoyl ring polarization is still intact (Liu, 1996). With the exception of the W137F mutant, in which a catalytic residue has been replaced, these mutants remain reasonably active ($k_{cat} = 0.002\ s^{-1}$, $K_m = 8\mu M$ for W137F; $k_{cat} = 0.01\ s^{-1}$, $K_m = 5\ \mu M$ for W89F; $k_{cat} = 0.2\ s^{-1}$, $K_m = 5\ \mu M$ for F64L; $k_{cat} = 0.1\ s^{-1}$, $K_m = 6\ \mu M$ for F82L), despite the change in packing in the vicinity of the benzoyl ring that must occur as a result of the difference in size of the side chain (Liu, 1996). Next, we set out to examine "polar" mutants. The W89Y dehalogenase mutant was thus prepared for comparison to the W89F mutant. This mutant displays the stability and chromatographic behavior of the wild-type enzyme but is catalytically inactive. The binding affinity of 4-MeBA-CoA and 4-HBA-CoA to the W89Y mutant is considerably reduced, $K_d \sim 100\ \mu M$. Nevertheless, the UV-visible absorption difference spectra of the 4-MeBA-CoA and 4-HBA-CoA complexes of the W89Y dehalogenase mutant reveal absorption maxima at 308 nm and 330 nm, respectively, suggesting that while the hydroxyl group on the tyrosine ring weakens ligand binding it does not preclude the polarization of the benzoyl ring once the ligand is bound. Other polar mutants are currently being constructed for examination.

The nucleophilic catalysis and electrophilic catalysis facilitates the formation of the Meisenheimer intermediate (see Scheme 3). The driving force for expulsion of the chloride leaving group from this intermediate is the reinstatement of charge balance and aromaticity in the benzoyl ring. There is no indication from the X-ray structure of the 4-HBA-CoA-dehalogenase complex of a "chloride binding" pocket (Benning, 1996). While the rate of formation of the Meisenheimer intermediate is $75\ s^{-1}$ (as determined from stopped-flow absorption single turnover experiments) the rate at which chloride ion departs to form the arylated enzyme is considerably slower, $2\ s^{-1}$ (Liu, 1995). For the 4-fluorobenzoyl-CoA substrate (4-FBA-CoA) the rate at which the Meisenheimer com-

plex is formed remains unchanged while the rate at which it loses the halide ion falls to 0.00001 s^{-1}, reflecting the difference in the C-Cl and C-F bond dissociation energies (Liu, 1995).

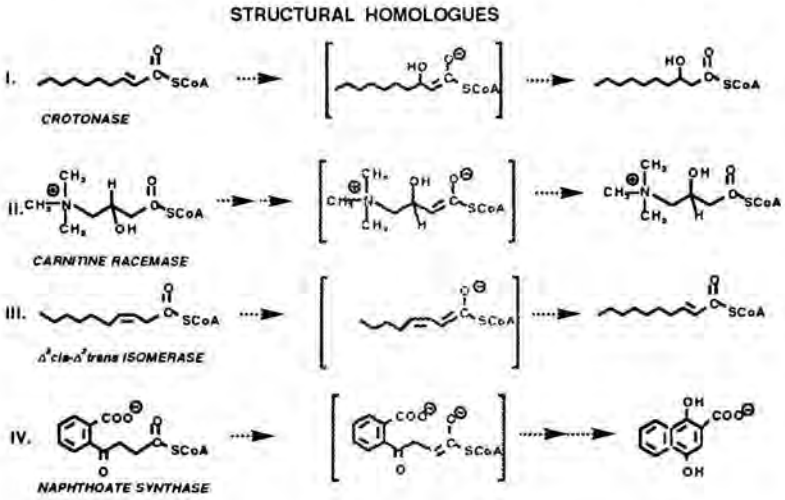
The second half of the dehalogenase reaction involves hydrolysis of the arylated enzyme. The ^{18}O labeling studies described above showed that the water adds to the acyl C = O (Yang, 1994). In Scheme 3 we represent this process as a classical two-step ester hydrolysis reaction. The solvent deuterium kinetic isotope effect measured for E · P formation is 1.7 (Liu, unpublished). Enzyme mediated ester hydrolysis is subject to general acid-general base catalysis by active site residues. Examination of the 4-HBA-CoA-dehalogenase crystal structure reveals that the Trp137 indole NH is within hydrogen bonding distance of the Asp145 C = O and thus may serve to stabilize the tetrahedral intermediate and the two flanking transition states. Indeed, the steady-state kinetic constants of the W137F dehalogenase mutant are $k_{cat} = 0.002\ s^{-1}$ and $K_m = 8\ \mu M$ (Yang, 1996). The Asp145 C = O is located towards the back of the active site, formed by two helices (residues 75–97 of one subunit and residues 225–244 of the adjacent subunit) which run anti-parallel. The region at the helix interface has numerous water molecules bound, however, none appear to be within striking distance of the benzoyl ring C(4). This is not surprising though because we are looking at the product complex and not the substrate complex. It is apparent from the structure that His90 is properly positioned to bind and activate the attacking water molecule. The H90Q dehalogenase mutant is not active. The conjugate acid of His90 may in turn facilitate the departure of the phenol from the tetrahedral intermediate through proton transfer to the C(4)-oxygen atom. In this regard we found an interesting comparison in the reaction rates of 4-CBA-CoA and its 2,3-dihydro derivative. The rate limiting step of the catalysis of 4-CBA-CoA is hydrolysis of the arylated enzyme which occurs at 3.5 s^{-1} (Liu, 1995). The arylated enzyme intermediate is formed from turnover of the 2,3-dihydro adduct at a rate of A s^{-1} (Vs 2 s^{-1} for 4-CBA-CoA) while the hydrolysis occurs at the much reduced rate of 0.08 s^{-1} (Taylor, unpublished). The reduction in rate may reflect the difference in the pK_a values of the phenol Vs allylic alcohol leaving groups, and hence charge accumulation on the departing oxygen in the C-O bond charge step.

Relatives of 4-CBA-CoA Dehalogenase

4-CBA-CoA dehalogenase could be either an old enzyme or a new enzyme on the evolution time scale. The existence of a variety of halogenated aromatic natural products (Gribble, 1994) can be used to argue that the dehalogenase is part of a degradative pathway which may have emerged along with the halogenated aromatic biosynthetic pathways, ages ago. If this is the case, then the 4-CBA-CoA dehalogenase has had ample time to develop and perfect its dehalogenation catalytic machinery through classical mechanisms of protein evolution. An alternate view has the emergence of the 4-CBA-CoA dehalogenase

coinciding with prolonged exposure of a population of bacteria to 4-CBA, as might occur at a 4-CBA or PCB dump site. Such an enzyme might be considered the product of recent, adaptive mutation and as such may not have had ample evolution time to fine tune its structure/catalysis. Thus, the dehalogenase and its progenitor may be structurally very similar. Ongoing gene scanning in *Pseudomonas* sp. strain CBS3 is being carried out to attempt to identify such a closely related protein in the very strain which harbors the dehalogenase.

In the mean time, protein sequence homology searches have allowed us to identify enzymes in a variety of other organisms which appear to share a common peptide backbone fold with the 4-CBA-CoA dehalogenase. The closest relative to the *Pseudomonas* sp. strain CBS3 4-CBA-CoA dehalogenase is the 4-CBA-CoA dehalogenase isolated from *Arthrobacter* sp. strain SU (Schmitz, 1992) (shares 46% identity with the *Pseudomonas* sp. strain (Babbitt, 1992) and the *Alcaligenes* sp. strain (Lai, unpublished) (86% identity with the *Pseudomonas* sp. strain and 47% identity with the *Arthrobacter* strains). We wonder whether the differences existing among the dehalogenase sequences reflect divergence of the dehalogenase gene or divergence of the dehalogenase progenitor gene. A family of enzymes, which we will refer to as the enoyl-CoA enzyme family, share between 25–32% sequence identity with the 4-CBA-CoA dehalogenase (Dunaway-Mariano, 1994). Four of the reactions catalyzed by the members of this family are represented in Scheme 4. The reactions catalyzed by four other members of this family are not represented in Scheme 4 because they have not been identified but their sequences are sufficiently distinct from those of the enzymes represented to suggest that these reactions are distinct as well. The catalytic functions that are represented in Scheme 4 include 2-enoyl-CoA hydratase, carnitine racemase, Δ^3-cis, Δ^2-trans enoyl-CoA isomerase and nahpthoate synthetase. Like the dehalogenase, these enzymes catalyze reactions



Scheme 4.

of enoyl-CoA substrates which are likely to proceed through enolate-thioester intermediates and/or transition states.

To date the only structure determined for a member of this family of enzymes is that of the *Pseudomonas* sp. strain 4-CBA-CoA dehalogenase. If the protein fold and hence, active site structure is assumed to be roughly the same among this family of enzymes, then sequence alignments can be used to identify the active site residues in each. For instance, from a the sequence alignment constructed by P. Babbitt at UCSF (unpublished extension of the sequence alignment reported in Dunaway-Mariano, 1994) which contains all of the currently known sequences representing this family of enzymes, the loop corresponding to that containing the dehalogenase Phe64, the α-helix corresponding to the dehalogenase α-helix terminating in Gly114, and the stretch of sequence corresponding to the stretch of sequence in the dehalogenase which contains the Trp137 and Asp145 residues can be identified. We find that the residues of the Phe64 loop and Gly114 α-helix are highly conserved suggesting that the enoyl-CoA thioester C = O is polarized through hydrogen bonding interaction with the backbone amide hydrogens contributed by the loop Phe and α-helix Gly. On the other hand, the single active site residue which resides in striking distance of the enoyl-CoA C = C, Trp137 in the dehalogenases, is Glu in the 2-enoyl hydratases, carnitine racemases and isomerases where it functions in acid/base catalysis while in the naphthoate synthetases, where it plays no obvious catalytic role, it is a Gly. We wonder whether or not the catalytic functions of say the dehalogenase and crotonase can be interchanged by exchange of the catalytic Trp and Glu residues? If this were true then retooling a crotonase to function as a dehalogenase might be a small task indeed.

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2-Haloacid Dehalogenases: Structure and Catalytic Mechanism

Abstract

We have analyzed the structure and function of L-2-haloacid dehalogenase and DL-2-haloacid dehalogenase. The carboxylate group of Asp^{10} of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL was shown to act as a nucleophile on the α-carbon of L-2-haloalkanoic acid to form an ester intermediate, which is hydrolyzed to produce the corresponding 2-hydroxyalkanoic acid. In contrast, in the reaction of DL-2-haloacid dehalogenase from *Pseudomonas* sp. 113, a water molecule activated by the enzyme directly attacks the α-carbon of the substrate. We have determined the crystal structure of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL, and found that it is an α/β-type hydrolase, but does not belong to the α/β hydrolase fold family to which haloalkane dehalogenase belongs. Asp^{10} is located on a loop following the amino-terminal β-strand, and other functional group detected by site-directed mutagenesis experiment are arranged around Asp^{10}. We have cloned and sequenced the gene encoding DL-2-haloacid dehalogenase, and found that its amino acid sequence is similar to that of D-2-haloacid dehalogenase from *Pseudomonas putida* AJ1.

Introduction

2-Haloacid dehalogenases (EC class: 3.8.1.2) catalyze the hydrolysis of 2-haloalkanoic acids to produce the corresponding 2-hydroxyalkanoic acids (Fetzner and Lingens, 1994; Janssen *et al.*, 1994). They are classified into four groups as follows. L-2-Haloacid dehalogenase catalyzes the dehalogenation of L-2-haloalkanoic acids to produce the corresponding D-2-hydroxyalkanoic acids. D-2-Haloacid dehalogenase catalyzes the hydrolysis of D-2-haloalkanoic acids to produce the corresponding L-2-hydroxyalkanoic acids. DL-2-Haloacid dehalogenase (inversion type) dehalogenates both L- and D-2-haloalkanoic acids to the corresponding D- and L-2-hydroxyalkanoic acids, respectively. The reactions catalyzed by these three kinds of dehalogenases proceed with inversion of the C_2-configuration of the substrates. DL-2-Haloacid dehalogenase (retention type) acts on both L- and D-2-haloalkanoic acids to produce the corresponding L- and D-2-hydroxyalkanoic acids, respectively.

Namely, the reaction of this dehalogenase proceeds with retention of the C_2-configuration. We describe here the structure and catalytic mechanism of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL (L-DEX YL) (Hasan *et al.*, 1994; Liu *et al.*, 1994) and DL-2-haloacid dehalogenase (inversion type) from *Pseudomonas* sp. 113 (DL-DEX 113) (Motosugi *et al.*, 1982a; Motosugi *et al.*, 1982b).

Results and Discussion

Analysis of reaction mechanism of L-DEX YL by means of ^{18}O incorporation experiments

Two reaction mechanisms have been proposed for L-2-haloacid dehalogenases and other dehalogenases whose reactions proceed with inversion of the C_2-configuration of the substrate (Fig. 1). According to the Fig. 1A mechanism, a carboxylate group of Asp or Glu acts as a nucleophile to attack the α-carbon of 2-haloalkanoic acid, leading to the formation of an ester intermediate. This is hydrolyzed by an attack of water molecule activated by a basic amino acid residue of the enzyme. Alternatively, water is activated by a catalytic base of the enzyme, and directly attacks the α-carbon of 2-haloalkanoic acid to displace the halogen atom (Fig. 1B).

We carried out single- and multiple-turnover enzyme reactions with L-DEX YL in $H_2{}^{18}O$. The single-turnover reaction was carried out in the solution containing the enzyme in excess of substrate, whereas an excess amount of substrate was used in the multiple-turnover reaction. If the reaction proceeds through the Fig. 1B mechanism, ^{18}O is incorporated into the product both in single- and multiple-turnover reactions. If the reaction proceeds as shown in

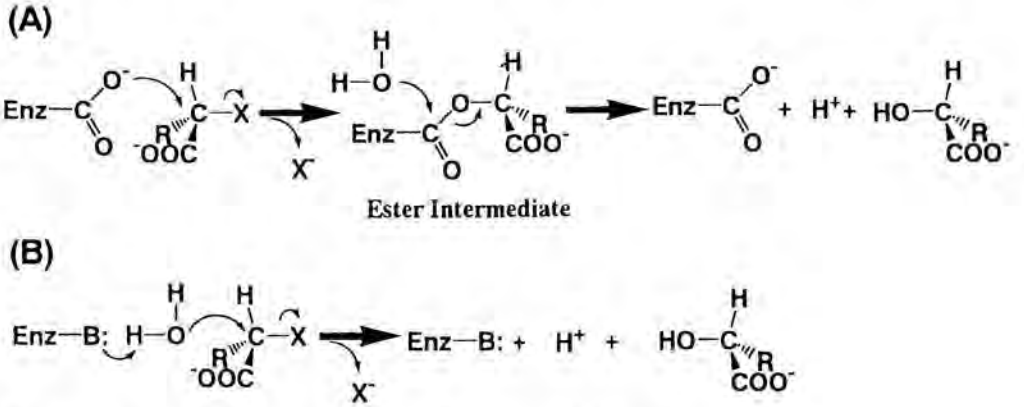

Fig. 1. Proposed reaction mechanisms of 2-haloacid dehalogenase.

Fig. 1A, the single-turnover reaction causes ^{18}O-incorporation into the carboxylate group of the enzyme, but does not into the product. In the multiple-turnover reaction, both the product and the carboxylate group of the catalytic residue are labeled with ^{18}O.

When we conducted the single-turnover enzyme reaction with L-2-chloropropionate as a substrate, less than 10% D-lactate produced in $H_2{}^{18}O$ contained ^{18}O, whereas under the multiple-turnover conditions, more than 95% D-lactate contained ^{18}O (Liu *et al.*, 1995). These suggest that an oxygen atom of water molecule is first incorporated into the enzyme, and then into the product. This supports the Fig. 1A mechanism, but does not the Fig. 1B mechanism.

If the reaction proceeds through the Fig. 1A mechanism, the catalytic residue is expected to be labeled with ^{18}O after multiple-turnover enzyme reaction in $H_2{}^{18}O$. In order to identify the position of the ^{18}O incorporated into the enzyme, a mutant enzyme L-DEX T15 was constructed by introducing three lysyl residues at the positions of 11, 176 and 185 of L-DEX YL by site-directed mutagenesis. The mass spectrometrical analysis can be carried out more precisely with L-DEX T15 than with wild-type L-DEX YL because shorter peptide fragments can be obtained from L-DEX T15 by lysyl endopeptidase treatment. Using L-DEX T15, a multiple-turnover reaction was carried out in $H_2{}^{18}O$ with L-2-chloropropionate as a substrate. After completion of the reaction, the enzyme was digested with lysyl endopeptidase, and the resulting peptide fragments were separated on a capillary column interfaced with an ionspray mass spectrometer as a detector. The molecular mass of peptide 6–11 (Gly-Ile-Ala-Phe-Asp-Lys) was 654.5 Da, which is approximately 4 Da higher than the predicted molecular mass (650.75 Da) (Liu *et al.*, 1995). Molecular masses of all other peptides were indistinguishable from the predicted ones. These results indicate that two ^{18}O atoms were incorporated solely into the peptide 6–11, which contains Asp10.

Fragmentation of the ^{18}O-labeled and unlabeled peptide 6–11 was performed using mass spectrometer in the daughter ion scan mode in order to determine the incorporation position of ^{18}O (Liu *et al.*, 1995). The parent ions of m/z 654.5 and m/z 650.2, corresponding to ^{18}O-labeled and unlabeled hexapeptides, respectively, were selected in the first quadrupole, and subjected to collision-induced fragmentation in a collision cell in the second quadrupole. The Y″ series ions were found at m/z 484.0, 413.1, and 266.0 of ^{18}O-labeled peptide, which corresponded to the fragments of Ala-Phe-Asp-Lys, Phe-Asp-Lys, and Asp-Lys, respectively. They are about 4 Da higher than those of ions at m/z 480.3, 409.1, and 262.0 of the unlabeled peptide. However, after the deletion of Asp, molecular masses of the remaining portions (Lys) of these two peptides were identical with each other (146.8). These results suggest that two atoms of ^{18}O of solvent water are incorporated into Asp^{10} of the enzyme during the dehalogenation reaction.

All these observations are not consistent with the general base mechanism shown in Fig. 1B, but with the Fig. 1A mechanism involving ester intermediate. Mass spectrometrical analysis showed that an oxygen atom of solvent water was

incorporated into the carboxylate group of Asp^{10}. Accordingly, the dehalogenation reaction of L-DEX YL probably proceeds through the ester intermediate mechanism in which Asp^{10} acts as a nucleophile (Fig. 1A). Since two ^{18}O atoms were incorporated into Asp^{10}, both two oxygen atoms of the carboxylate group of Asp^{10} are equivalent and either can attack the substrate.

The same reaction mechanism in which a nucleophilic carboxylate group functions has been proposed for three types of hydrolases: rat liver microsomal epoxide hydrolase (Lacourciere and Armstrong, 1993), haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (Pries *et al.*, 1994), and (4-chlorobenzoyl)coenzyme A dehalogenase from *Pseudomonas* sp. strain CBS3 (Yang *et al.*, 1994). Primary structures of epoxide hydrolase and haloalkane dehalogenase are similar to each other, but (4-chlorobenzoyl)coenzyme A dehalogenase is not similar to these two enzymes. L-DEX YL does not show a significant sequence similarity to any of these three enzymes. Therefore, L-DEX YL resembles these hydrolases solely by the presence of an active site nucleophilic carboxylate.

Paracatalytic Inactivation of L-DEX YL by Hydroxylamine

As described above, Asp^{10} of L-DEX YL acts as a nucleophile to attack the α-carbon atom of the substrate, producing an ester intermediate and a halide ion. Since such an ester intermediate is very reactive with water, nucleophiles other than water are expected to react with this intermediate to modify it. We used hydroxylamine as a nucleophile, and examined whether L-DEX YL is paracatalytically inactivated by hydroxylamine, and the active site Asp^{10} is specifically labeled.

We found that treatment of L-DEX YL with hydroxylamine in the presence of monochloroacetate or L-2-chloropropionate led to an irreversible inactivation of the enzyme, whereas the treatment in the absence of the substrate or in the presence of monofluoroacetate, a substrate analogue, caused no significant alterations in the enzyme activity. The enzyme was inactivated as the chloride ions were released, and the inactivation followed pseudo-first-order kinetics. The initial rates of the inactivation followed saturation kinetics with respect to the substrate concentrations.

We determined molecular masses of L-DEX YL treated with hydroxylamine in the presence or absence of the substrate by ionspray mass spectrometry. The molecular mass of the native L-DEX YL was 25,863 Da, which indicates that the C-terminal of this enzyme preparation lacks the last three amino acid residues. The predicted molecular mass of this truncated L-DEX YL is 25,862.4 Da, which is in good agreement with the above value. The molecular mass of the enzyme treated with hydroxylamine in the absence of the substrate was 25,863 Da, which is closely similar to the above value. In contrast, the molecular masses of the enzymes incubated with hydroxylamine in the presence of L-2-chloropropionate and monochloroacetate were 25,952 and 25,937 Da, respectively, which are higher by 89 and 74 Da than that of the native protein. These

indicate that L-DEX YL was modified by a molecule (or molecules) derived from the substrate and/or hydroxylamine to give molecular masses increased by 89 and 74 Da, respectively. We carried out amino acid sequencing of the enzyme incubated with hydroxylamine in the presence of the substrate, and found that Asp^{10} was modified.

The enzyme modified with hydroxylamine in the presence of L-2-chloropropionate was digested with TPCK-treated trypsin, and the molecular masses of the resulting peptide fragments were measured by LC/MS. Three peaks appearing on the chromatogram were shown to be derived from the peptide 6–24 containing Asp^{10} (Gly-Ile-Ala-Phe-Asp-Leu-Tyr-Gly-Thr-Leu-Phe-Asp-Val-His-Ser-Val-Val-Gly-Arg). The molecular mass of one of the peptides (2,066 Da) was closely similar to that of the unmodified peptide. However, the molecular masses of other two peptides were 2,081 and 2,153 Da, which are 15 and 87 Da higher, respectively.

Next, we incubated L-DEX T15 with hydroxylamine in the presence or absence of monochloroacetate, and digested with lysyl endopeptidase. The resulting peptide fragments were separated by HPLC. Amino acid sequencing of the isolated peptides showed that a modified amino acid occurs at the position corresponding to the active site Asp^{10} in the hexapeptide, Gly-Ile-Ala-Phe-Asp-Lys. The modified hexapeptide was subsequently introduced into ionspray mass spectrometer for detailed analysis. Two major peaks, derived from peptides with molecular masses of 708 and 723 Da, were found on the chromatogram. These values are 58 and 73 Da higher, respectively, than that of the native hexapeptide (650 Da). These peptide fragments were in more detail analyzed by tandem MS/MS spectrometry, and the active site Asp^{10} was shown to be specifically modified.

All these data obtained here indicate that hydroxylamine inactivates L-DEX YL paracatalytically. The molecular mass of L-DEX YL inactivated by hydroxylamine in the presence of monochloroacetate was about 74 Da higher than that of the native L-DEX YL. The mass spectrometrical analysis of the Asp^{10} containing peptides showed that there are two different species whose molecular masses are 58 and 73 Da higher than that of the unmodified peptide. The peptide species with 58-Da higher mass number are probably formed artificially by a degradation of the other peptide species with 73-Da higher mass number during the preparation of the peptide fragments, because we could not detect the modified L-DEX YL whose molecular mass is about 58 Da higher than that of the native L-DEX YL. The increment of the molecular mass of 73 Da, which is more reliable than that of 74 Da observed for the whole modified protein, can be ascribed to the modification of Asp^{10} leading to the formation of aspartate β-hydroximate carboxymethyl ester (Fig. 2). The species with 58 Da higher molecular mass is probably produced by removal of the hydroxylamine moiety from the modified aspartate residue to form carboxymethylated Asp^{10}.

The treatment of L-DEX YL with hydroxylamine in the presence of L-2-chloropropionate caused 87 Da increment in the molecular mass, and the species

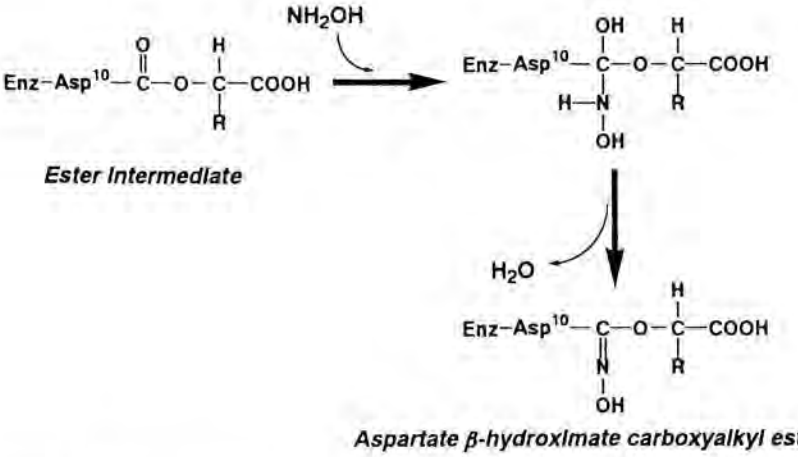

Fig. 2. Paracatalytic inactivation of L-DEX YL by hydroxylamine.

whose molecular mass is 15 Da higher than that of the native one was also produced during the preparation of the peptide fragments. The increment of 87 Da can be explained by the formation of aspartate β-hydroximate carboxyethyl ester residue substituting for Asp^{10} (Fig. 2). The species with 15 Da higher molecular mass is probably produced by the removal of L-2-chloropropionate-derived moiety to form aspartate β-hydroxamate residue.

All the above data show that the active-site Asp^{10} of L-DEX YL is specifically modified by hydroxylamine, and the modified residue is aspartate β-hydroximate carboxyalkyl ester, which contains the moiety derived from the substrate, 2-haloalkanoic acid, as well as hydroxylamine. This indicates that the ester intermediate, whose acyl moiety is derived from Asp^{10}, is produced in the reaction, which strongly supports the mechanism shown in Fig. 1A.

Probing Essential Residues of L-DEX YL by Site-Directed Mutagenesis

Seven L-2-haloacid dehalogenases from various bacterial strains are significantly similar to one another in their amino acid sequences (36–70% identity) (Nardi-Dei, *et al.*, 1994; Kawasaki, *et al.*, 1994; Jones, *et al.*, 1992; Schneider, *et al.*, 1991; van der Ploeg, *et al.*, 1991; Murdiyatmo, *et al.*, 1992). They are supposed to catalyze the reaction by the same mechanism, and catalytically important residues are expected to be conserved among them. We mutated all the 36 highly conserved charged and polar amino acid residues of L-DEX YL, which consists of 232 amino acid residues, by replacement of Asp by Asn, Glu by Gln, Arg by Lys, and vice versa, Ser and Thr by Ala, Tyr and Trp by Phe, Met by Leu and His by Asn. We found that the replacement of Asp^{10}, Lys^{151}, Ser^{175}, Asp^{180},

Arg^{41}, Thr^{14}, Tyr^{157}, and Asn^{177} led to a significant loss in the enzyme activity, and the replacement of Ser^{118} caused an increase in *K*m value for the substrate (Kurihara, *et al.*, 1995).

D10N mutant enzyme, which lacks Asp^{10} required for the wild-type enzyme reaction, showed about 0.4% activity of the wild-type enzyme. We analyzed the reaction mechanism of D10N mutant enzyme. We carried out single-turnover enzyme reactions in $H_2{}^{18}O$ containing the enzyme in excess of the substrate. In contrast to the wild-type enzyme reaction, D-lactate produced from L-2-chloropropionate was labeled with ^{18}O. This shows that water is activated by a catalytic base of D10N mutant enzyme and directly attacks the α-carbon of the substrate to displace the halogen atom (Fig.1B). Thus, the reaction mechanisms of wild-type and D10N enzymes are different from each other.

Asp^{180} and Asn^{177} are thought to play essential roles in the catalysis because D180N and N177D mutant enzymes showed no activity. We incubated these mutant enzymes with L-2-chloropropionate, and measured their molecular masses by ionspray mass spectrometry. The molecular masses of these mutant enzymes were about 72 Da higher than those of the enzymes that were not incubated with the substrate. These increments are ascribable to the accumulation of the ester intermediate. Accordingly, Asp^{180} and Asn^{177} are probably involved in the hydrolysis of the ester intermediate.

Crystal Structure of DL-DEX YL

We have obtained rhombohedral crystals by the vapor diffusion of a 15mg/ml enzyme solution against a 50mM potassium dihydrogenphosphate solution containing 15% (w/v) polyethylene glycol 8000 and 1% (v/v) n-propanol at 4° C (Hisano, *et al.*, 1996a). Two kinds of isomorphous heavy atom derivative crystals were prepared by soaking native crystals in 1mM $UO_2(NO_3)_2$ and 3mM $K[Au(CN)_2]$. We have determined the crystal structure at 2.5 Å resolution, and found that it consists of two domains: the core domain and the subdomain (Hisano, *et al.*, 1996b). The core domain has an α/β-type structure consisting of a central six-stranded parallel β-sheet (β1 and β4-β8) flanked on both sides by five α-helices (α5-α9). The subdomain has a distorted four-helix-bundle structure (α1-α4). This crystallographical analysis revealed that L-DEX YL does not belong to the α/β hydrolase fold family to which haloalkane dehalogenase belongs. Asp^{10} is located on a loop following the amino-terminal β-strand (β1) in the core domain, and other functional residues, Thr^{14}, Arg^{41}, Ser^{118}, Lys^{151}, Tyr^{157}, Ser^{175}, Asn^{177}, and Asp^{180}, described above are arranged around Asp^{10}.

Primary structure of DL-DEX 113

DL-2-Haloacid dehalogenase from *Pseudomonas* sp. 113 (DL-DEX 113) catalyzes the hydrolytic dehalogenation of both D- and L-2-haloalkanoic acids

to produce the corresponding L- and D-2-hydroxyalkanoic acids, respectively. We have isolated and sequenced the gene encoding DL-DEX 113. The open reading frame consists of 921 bp corresponding to 307 amino acid residues. The amino acid sequence of DL-DEX 113 was similar to that of D-2-haloacid dehalogenase from *Pseudomonas putida* AJ1 that specifically acts on D-2-haloalkanoic acids: 23.5% residues were completely conserved between these two enzymes. In contrast, no sequence similarity was found with L-2-haloacid dehalogenases.

Reaction mechanism of DL-DEX 113

We conducted the single-turnover reaction of DL-DEX 113 in $H_2{}^{18}O$ using L- and D-2-chloropropionate as substrates, and found that more than 80% D-lactate or L-lactate produced contained ^{18}O. This suggests that an oxygen atom of water molecule is directly transferred from water to the product. This supports the Fig.1B mechanism. An amino acid of the enzyme probably acts as a base to activate water, and water directly attacks the α-carbon of the substrate. Thus, the reaction mechanism of DL-DEX 113 is different from that of DL-DEX YL.

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Characteristics of DL-2-haloacid dehalogenase

Abstract

We have analyzed the structure and function of DL-2-haloacid dehalogenase from *Pseudomonas* sp. 113 (DL-DEX 113), which acts on both L- and D-2-haloalkanoic acids to produce the corresponding D- and L-2-hydroxyalkanoic acids. The gene encoding this enzyme was isolated, and its nucleotide sequence was determined. The open reading frame consists of 921 bp corresponding to 307 amino acid residues. The amino acid sequence of DL-DEX 113 was similar to that of D-2-haloacid dehalogenase from *Pseudomonas putida* AJ1 that specifically acts on D-2-haloalkanoic acids (23.5% residues were completely conserved between these two enzymes), but was not similar to that of L-2-haloacid dehalogenases. We mutated 26 charged and polar amino acid residues of DL-DEX 113 conserved between DL-DEX 113 and D-2-haloacid dehalogenase, and found that Thr^{65}, Glu^{69} and Asp^{194} are essential for the dehalogenation of both D- and L-2-haloalkanoic acids. The activities of each of all the 26 mutant enzymes toward D- and L-2-chloropropionate were almost equal to each other. We also found that D-2-chloropropionate competitively inhibits the enzymatic dehalogenation of L-2-chloropropionate, and vice versa. These suggest that DL-DEX 113 has a common active site for both enantiomers. ^{18}O incorporation experiment showed that a water molecule activated by DL-DEX 113 directly attacks the α-carbon of the substrate to displace the halogen atom. This reaction mechanism is different from that of L-2-haloacid dehalogenase, whose active-site Asp^{10} acts as a nucleophile to attack the α-carbon of the substrate to form an ester intermediate.

Introduction

DL-2-Haloacid dehalogenase from *Pseudomonas* sp. 113 (DL-DEX 113) catalyzes the hydrolytic dehalogenation of both D- and L-2-haloalkanoic acids to produce the corresponding L- and D-2-hydroxyalkanoic acids, respectively (Motosugi, *et al.*, 1982a; Motosugi, *et al.*, 1982b). The reaction proceeds with inversion of the C_2-configuration of the substrates. This enzyme is unique in

respect that it acts on the chiral carbon of both enantiomers indiscriminately. However, it remains unclear whether there is a single active site for both enantiomers, or two distinct sites for each enantiomer.

Other kinds of 2-haloacid dehalogenases which are different from DL-DEX 113 in their substrate and stereochemical specificities have been isolated from several bacteria (Fetzner and Lingens, 1994; Janssen, *et al.*, 1994). L-2-Haloacid dehalogenases from *Pseudomonas* sp. YL (Liu, *et al.*, 1994), *Pseudomonas putida* no. 109 (Motosugi, *et al.*, 1982c), *Xanthobacter autotrophicus* GJ10 (van der Ploeg, *et al.*, 1991), etc. specifically act on L-2-haloalkanoic acids to produce the corresponding D-2-hydroxyalkanoic acids. D-2-Haloacid dehalogenase from *Pseudomonas putida* AJ1 dehalogenates D-2-haloalkanoic acids to L-2-hydroxyalkanoic acids (Smith, *et al.*, 1990). These dehalogenases are similar to DL-DEX 113 in respect that they catalyze the hydrolysis of 2-haloalkanoic acids with inversion of the C_2-configuration of the substrates. However, structural comparison of DL-DEX 113 with L- and D-2-haloacid dehalogenases has not been carried out. Also, the reaction mechanism of DL-DEX 113 has not been studied thus far, and it remains unclear whether the mechanism is similar to those of other 2-haloacid dehalogenases.

In the present study, we isolated and sequenced the gene encoding DL-DEX 113, and compared its primary structure with those of L- and D-2-haloacid dehalogenases. We found that DL-DEX 113 has a sequence similarity with D-2-haloacid dehalogenase (Barth, *et al.*, 1992), but not with L-2-haloacid dehalogenase (Nardi-Dei, *et al.*, 1994). We characterized the active site of DL-DEX 113 by means of site-directed mutagenesis and inhibition experiment, and the results of these experiments suggested that the enzyme has a common active site for both enantiomers. We also found that the reaction mechanism of DL-DEX 113 is different from that of L-2-haloacid dehalogenase (Liu, *et al.*, 1995).

Results and Discussion

Cloning of the gene encoding DL-DEX 113

DNA was isolated from *Pseudomonas* sp. 113, and partially digested with *Sau*3AI. The fragments whose molecular sizes are between 1 and 12 kbp were isolated by electroelution after agarose gel electrophoresis, and ligated to the *Bam*HI site of pUC118. The resultant plasmids were introduced into *Escherichia coli* XL1-Blue.

The library was screened either by (i) bromoacetate method or (ii) immunoscreening method as follows. (i) According to the bromoacetate method, the transformed *E. coli* cells were cultivated in LB broth containing 100 μg/ml of ampicillin, 1mM of IPTG and 0.3 mg/ml of bromoacetate. After another transfer to the fresh medium of the same composition, the cells were cultivated on agar plates of the same composition, and the dehalogenase activity of the colonies

appearing on the plates was measured. This selection method is based on the toxicity of bromoacetate to the host cells that do not express the dehalogenase gene. (ii) According to the immunoscreening method, the colonies appearing on the plates were replicated on nitrocellulose filter, and screened with rabbit antiserum raised against DL-DEX 113 and a goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate as described by Sambrook *et al.* (1989). The colonies found positive were then plated on LB agar plates containing 100 μg/ml of ampicillin, 1mM of IPTG and 0.3 mg/ml of bromoacetate. The dehalogenase activity of the colonies appearing on these plates were measured.

We obtained 2 positive clones from 2,000 transformants by bromoacetate method. However, both clones were found to produce L-2-haloacid dehalogenase. The gene for L-2-haloacid dehalogenase might be present as a cryptic gene, because only DL-2-haloacid dehalogenase was produced by *Pseudomonas* sp. 113 when the cells were cultivated in the medium containing DL-2-chloropropionate.

By immunoscreening method, we obtained two positive clones named 4b(1) and 5b(1) from 5,000 transformants. Both showed DL-2-haloacid dehalogenase activity. The plasmids isolated from 4b(1) and 5b(1), named p4b(1) and p5b(1), respectively, contained inserted DNAs of 1.2 and 11kbp, respectively. Since the clone 4b(1) showed a higher activity than 5b(1), p4b(1) was used in the following experiment.

Primary structure of DL-DEX 113

Nucleotide sequence analysis revealed that the open reading frame of the DL-DEX 113 gene consists of 921 bp corresponding to 307 amino acid residues. The predicted molecular weight of the protein is 34,242, which agrees well with that of the enzyme purified from *Pseudomonas* sp. 113 (35,000) (Motosugi, *et al.*, 1982b). Deduced amino acid composition is also in good agreement with the value previously reported for the purified enzyme. The deduced N-terminal amino acid sequence is identical to that determined for the enzyme purified from *Pseudomonas* sp. 113 except that the initial methionine was not found in the purified enzyme. Putative Shine-Dalgarno sequence (GGAGG) was found 9 to 13 nucleotides upstream of the initiation codon, ATG. Possible -35 sequence (TTGAAA) and -10 sequence (TACGAT) were found at positions from -79 to -74 and from -57 to -52, respectively. Five out of six nucleotides of *Escherichia coli* -35 consensus sequence (TTGACA) and four out of six nucleotides of *E. coli* -10 consensus sequence (TATAAT) are present.

We found that there is a sequence similarity between DL-DEX 113 and D-2-haloacid dehalogenase from *Pseudomonas putida* AJ1, which specifically acts on D-2-haloalkanoic acids (Barth, *et al.*, 1992): 72 amino acid residues are conserved between these enzymes (23.5% identity). Although the overall sequence similarity is not so high, there should be an evolutional relationship between these enzymes because successive amino acid residues are found to be conserved.

In contrast, DL-DEX 113 has no sequence similarity with L-2-haloacid dehalogenases, which have been isolated from many bacteria such as *Pseudomonas* sp. YL (Nardi-Dei, *et al.*, 1994), *Pseudomonas putida* no. 109 (Kawasaki, *et al.*, 1994), and *Xanthobacter autotrophicus* GJ10 (van der Ploeg, *et al.*, 1991). These suggest that DL-DEX 113 is not composed of two domains one of which is similar to D-2-haloacid dehalogenase and the other of which is similar to L-2-haloacid dehalogenase. Sequence similarity with D-2-haloacid dehalogenase suggests that DL-DEX 113 has an active site for D-2-haloalkanoic acids similar to that of D-2-haloacid dehalogenase.

We also found that the primary structure of DL-DEX 113 is similar to that of DL-2-haloacid dehalogenase from *Alcaligenes xylosoxidans* ssp. *xylosoxidans* ABIV registered in the protein sequence data base. Among 307 amino acid residues of DL-DEX 113, 123 residues were conserved in the sequence of the *Alcaligenes* enzyme (40.1% identity).

Characterization of DL-DEX 113 from the recombinant E. coli cells

DL-DEX 113 was purified from the recombinant *E. coli* JM109 harboring pb4(1) 4.9-fold with 66% yield by ammonium sulfate fractionation and Butyl-Toyopearl column chromatography. The enzyme had a molecular weight of about 68,000, and appeared to be composed of two subunits identical in molecular weight. The K_m values for L-2-chloropropionate, D-2-chloropropionate and monochloroacetate were 0.9, 4.2 and 4.8 mM, respectively. These values are similar to those of DL-DEX 113 purified from *Pseudomonas* sp. 113: the values for L-2-chloropropionate, D-2-chloropropionate and monochloroacetate are 1.1, 4.8 and 5.0 mM, respectively (Motosugi, *et al.*, 1982b). We carried out the following experiment using DL-DEX 113 purified from the recombinant *E. coli* cells.

Probing catalytically important residues of DL-DEX 113

Twenty charged and polar amino acid residues are completely conserved among DL-DEX 113, DL-2-haloacid dehalogenase from *Alcaligenes xylosoxidans* and D-2-haloacid dehalogenase from *Pseudomonas putida*: three aspartate (Asp^{28}, ^{73} and ^{194}), one asparagine (Asn^{117}), three glutamate (Glu^{69}, ^{102} and ^{129}), one lysine (Lys^{119}), four arginine (Arg^{34}, ^{43}, ^{76} and ^{134}), one serine (Ser^{193}), two threonine (Thr^{65} and ^{219}), two tryptophan (Trp^{37} and ^{201}), and three tyrosine residues (Tyr^{26}, ^{115} and ^{120}). Ser^{125}, Glu^{161}, Asp^{167}, Asp^{250}, Thr^{256} and Asn^{271} are also highly conserved. To probe the important residues for the hydrolysis of D-2-haloalkanoic acids, we replaced each of all these 26 residues by another residue as follows: Asp by Asn, Asn by Asp, Glu by Gln, Lys by Arg, Arg, Ser and Thr by Ala, and Trp and Tyr by Phe. The enzyme activity toward D-2-haloalkanoic acids became less than 10% of the wild-type enzyme activity by replacing Asp^{28}, Thr^{65}, Glu^{69}, Asn^{117}, Tyr^{120}, Asp^{194}, Thr^{219} and Asp^{250}. The activities toward

L-2-haloalkanoic acids were also decreased by these mutations. In particular, T65A, E69Q and D194N mutant enzymes showed no activity toward both enantiomers. E69D, E69N, D194E, D194G, D194A and D194S mutant enzymes also did not act on D- nor L-2-chloropropionate. Accordingly, Thr^{65}, Glu^{69}, and Asp^{194} probably play an essential role in the catalysis. The activities of each of all the 26 mutant enzymes toward D- and L-2-chloropropionate were almost equal to each other. These suggest that the active site for L-2-haloalkanoic acids is located in the close vicinity to that for D-2-haloalkanoic acids.

Active site of DL-DEX 113 for D- and L-2-haloalkanoic acids

If DL-DEX 113 has a common active site for D- and L-2-haloalkanoic acids, competition between D- and L-2-haloalkanoic acids is expected to be observed. To examine the inhibitory effect of D-2-chloropropionate on the enzymatic hydrolysis of L-2-chloropropionate, the enzyme reaction was carried out in the mixture containing L-2-chloropropionate (0.5, 1.0 or 2.0 mM), D-2-chloropropionate (0, 10 or 20 mM), 100mM Tris-sulfate (pH9.0), 1mM NAD, 16 units of D-lactate dehydrogenase and DL-DEX 113. An increase of the absorbance at 340nm was measured at 30° C. Because D-lactate dehydrogenase specifically acts on D-lactate produced from L-2-chloropropionate, not on L-lactate produced from D-2-chloropropionate, it is possible to determine the enzymatic activity toward L-2-chloropropionate selectively. To examine the effect of L-2-chloropropionate on the dehalogenation of D-2-chloropropionate, the reaction was carried out in the mixture containing D-2-chloropropionate (2.0, 4.0 or 8.0 mM), L-2-chloropropionate (0, 1.0, 4.0 or 10 mM), 100mM Tris-sulfate (pH9.0), 1mM NAD, 16 units of L-lactate dehydrogenase and DL-DEX 113. An increase in absorbance at 340nm was monitored at 30° C.

The dehalogenations of D- and L-2-chloropropionate catalyzed by DL-DEX 113 were inhibited by L- and D-2-chloropropionate, respectively. Plots of reciprocals of initial velocities versus reciprocals of L-2-chloropropionate concentrations at several fixed concentrations of D-2-chloropropionate gave a group of straight lines intersecting at the point of $1/V_{max}$. Thus, D-2-chloropropionate is a competitive inhibitor for the dehalogenation of L-2-chloropropionate: the K_i value was 5.0 mM. Similarly, L-2-chloropropionate was shown to be a competitive inhibitor for the dehalogenation of D-2-chloropropionate with an apparent K_i value of 1.4 mM. The K_i values for D- and L-2-chloropropionate are similar to the K_m values of DL-DEX 113 for D- and L-2-chloropropionate, respectively (D-2-chloropropionate, 4.2 mM; L-2-chloropropionate, 0.9 mM).

These suggest that DL-DEX 113 does not have two active sites apart from each other, but has only one active site for both enantiomers. If the alkyl group and hydrogen atom bound to the α-carbon of 2-haloalkanoic acids are not distinguished by the enzyme, it is possible for the single active site to accommodate both enantiomers.

Analysis of the reaction mechanism of DL-DEX 113 by ^{8}O incorporation experiment

Two mechanisms have been proposed for the 2-haloacid dehalogenase reactions that involve the inversion of the C_2-configuration of the substrate. According to the mechanism shown in Fig.1(A), a carboxylate group of an acidic amino acid residue attacks the α-carbon of the substrate, producing an ester intermediate and a halide ion. Subsequently, the ester intermediate is hydrolyzed. Alternatively, a water molecule activated by a basic amino acid residue of the enzyme directly attacks the α-carbon of the substrate to displace the halogen atom (Fig.2 (B)). We conducted single-turnover enzyme reactions in $H_2{}^{18}O$ using L- and D-2-chloropropionate as substrates in order to elucidate through which mechanism the enzyme reaction proceeds. If the reaction proceeds through the Fig.1(B) mechanism, ^{18}O should be incorporated into the product. In contrast, ^{18}O should not be incorporated into the product in the case of Fig.1(A) mechanism. We found that more than 80% D-lactate or L-lactate produced contained ^{18}O. This indicates that an oxygen atom of water is directly transferred to the product. This supports the Fig.1(B) mechanism, but is not consistent with the Fig.1(A) mechanism.

In the case of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL, the reaction proceeds through the Fig.1(A) mechanism, where Asp^{10} acts as a nucleophile (Liu, *et al.*, 1995). Nucleophilic acidic amino acid residues also play an essential role in the reactions of haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (Pries, *et al.*, 1994) and (4-chlorobenzoyl)coenzyme A dehalogenase from *Pseudomonas* sp. strain CBS3 (Yang, *et al.*, 1994), both of which catalyze the hydrolytic dehalogenation of the substrates. In these enzyme reactions, a carboxylate group of the catalytic residue, not water molecule, attacks the substrate in the first step. Accordingly, DL-DEX 113 is different from these dehalogenases in its reaction mechanism. DL-DEX 113 is the first enzyme whose reaction is shown to proceed through the Fig.1(B) mechanism.

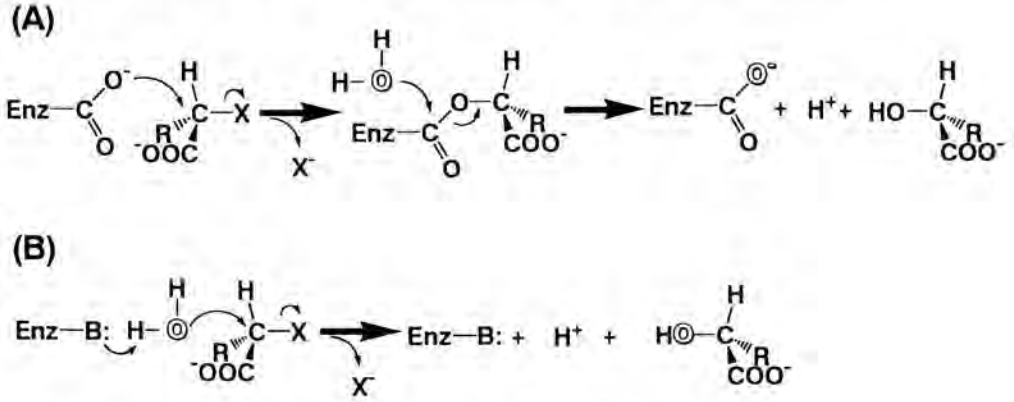

Fig. 1. Proposed reaction mechanism of 2-haloacid dehalogenase.

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The Haloacetate Dehalogenase Gene *dehH2* Carried on a Transposon Residing in a Plasmid of *Moraxella* sp. B

Abstract

The plasmid pUO1 of *Moraxella* sp. B carries two haloacetate dehalogenase genes, *dehH1* and *dehH2*. The spontaneous deletion of an about 5-kb plasmid DNA containing *dehH2* was frequently observed. The nucleotide sequence analysis of the deletion region revealed the presence of 3.2-kb repeated sequences on both sides of *dehH2*. This sequence, which was flanked by 110-bp inverted repeats and contained an open reading frame that seemed to be a transposase gene, was almost identical to IS1071, which was found in chlorobenzoate catabolic transposon Tn*5271* that resides in the plasmid of *Alcaligenes* sp. As to the structure, the *dehH2* gene flanked by two IS1071 was considered to be a class I composit transposon.

Introduction

The enzymes that cleave halogen-carbon bonds are generally called dehalogenases. Many kinds of dehalogenases are known that differ in their reaction types and substrate ranges. Among them, the enzymes that catalyze hydrolytic dehalogenation of haloaliphatic compounds are more widespreading among microorganisms and have been well studied.

The haloaliphatic dehalogenases are classified into two groups; haloalkane dehalogenase and haloalkanoic acid dehalogenase. The haloalkanoic acid dehalogenases are divided into two sub-groups on the basis of their substrate ranges. One group, termed haloacetate dehalogenase (EC 3.8.1.3), acts specifically on haloacetates to yield glycolate. The other group, termed 2-haloacid dehalogenase (EC 3.8.1.2), acts on short-chain haloalkanoic acids to produce 2-hydroxy acids. The 2-haloacid dehalogenases, which are all inactive towards fluorinated compounds, are further classified into 4 groups according to their stereo-specificity as shown in Fig. 1. Most of them act specifically on L- or D-enantiomers converting to D- or L-products, respectively. A few enzymes being active towards both stereoisomers are divided into two groups by the reaction mechanism inverting or retaining the substrate configuration.

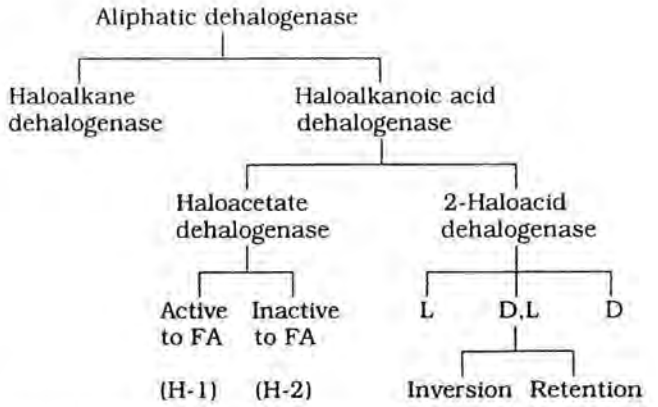

Fig. 1. Classification of hydrolytic dehalogenases acting on haloaliphatic compounds. FA indicates fluoroacetate, and L, D, and DL mean stereo-specific enzyme groups.

The group of haloacetate dehalogenase includes two types of enzymes differing in their halogen specificity. One is able to cleave the very stable fluorine carbon bond of fluoroacetate, but the other is unable. Haloacetate dehalogenases played pioneer roles in dehalogenase studies. Davies & Evans (1962) reported an enzymatic study on a haloacetate dehalogenase from *Pseudomonas dehalogenans*, being inactive towards fluoroacetate, and after that, Goldman (1965) reported a fluoroacetate-defluorinating enzyme. We also found two haloacetate dehalogenases, H-1 and H-2, which were similar with former two types of enzymes, respectively (Kawasaki *et al.*, 1981a).

In this paper, I will describe that the two types of haloacetate dehalogenases have no genetic relationship to each other. The enzyme H-1 is related to the haloalkane dehalogenase group, whereas H-2 is related to the L-stereospecific 2-haloacid dehalogenase group. It will be also reported that the H-2 gene residing in a plasmid assumes the form of a transposon.

Relationships between haloacetate dehalogenases and other dehalogenases

1. Two haloacetate dehalogenases, H-1 and H-2, are genetically unrelated.

A species of *Moraxella*, isolated from industrial waste-water, can utilize fluoroacetate and chloroacetate as sole carbon and energy souces, and has two kinds of haloacetate dehalogenases, designated H-1 and H-2, which differ in halogen specificity (Table 1). H-1 acts preferentially on fluoroacetate but has little activity against iodoacetate. H-2 acts on chloro-, bromo-, and iodoacetate, but not on fluoroacetate. Since both enzymes have only slight activity toward 2-chloropropionate, it is rational that they come under the group of haloacetate dehalogenase. The enzymes have been purified and characterized. The molecular weight estimated by SDS-PAGE is 33,000 for H-1 and 26,000 for H-2. Both

Table 1. Substrate specificity of H-1 and H-2

Substrate	Relative activity* and Km (mM) H-1	H-2
Monofluoracetate	510 (2.0)	0.0
Monochloroacetate	100 (4.8)	100 (2.5)
Monobromoacetate	70 (6.5)	160 (0.5)
Monoiodoacetate	0.2	150 (1.1)
Dichloroacetate	1.4	3.3
Trichloroacetate	0.0	0.0
2-Chloropropionate	2.6	9.5
3-Chloropropionate	<0.1	0.0
2,2-Dichloropropionate	0.0	0.1
2-Chlorobutyrate	<0.1	0.0
3-Chlorobutyrate	0.8	0.0
4-Chlorobutyrate	0.0	0.0
Chloroacetamide	<0.1	0.0

* The relative activity was expressed as percentage to the activity against monochloroacetate. Substrate concentration was 2.5 mM.

enzymes are sensitive to thiol-blocking reagents, and show similar properties as to pH and temperature (Kawasaki *et al.*, 1981b).

It was evidenced that both enzymes are specified by a plasmid named pUO1, which is about 65-kb long and transmissible into *Pseudomonas* and *E. coli*. A restriction map of this plasmid was constructed (Fig. 2) and the loci of the H-1 and H-2 genes were estimated by the cloning of the restriction fragments onto pBR322 (Kawasaki *et al.*, 1984).

When Southern hybridization was done using the *Sal*I-G fragment (Fig. 2) containing the H-2 gene as a probe, it hybridized with the *Eco*RI-F fragment containing the H-1 gene, implying that the two fragments contain common sequences. This finding and the fact that the genes for two similar enzymes were located closely on one plasmid suggested an evolutionary relationship between H-1 and H-2. However, the comparison of the H-1 and H-2 genes, termed *dehH1* and *dehH2*, respectively, in nucleotide and amino acid sequences (Fig. 3) showed no homology between two genes, implying that H-1 and H-2 must have evolved from different ancestors (Kawasaki *et al.*, 1992).

2. The H-1 enzyme is related to haloalkane dehalogenases.

Proteins and genes homologous to H-1 and H-2 and their genes were searched for in the data bases. The H-1 enzyme showed slight similarity in the amino acid sequence to a haloalkane dehalogenase (*dhlA*) of *Xanthobacter autotrophicus* GJ10 (Janssen *et al.*, 1989) and three hydrolases of *Pseudomonas putida*, i.e. 2-hydroxymuconic semialdehyde hydrolase (*dmpD*, Nordlund & Shingler, 1990), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (*bphD*, Kimbara *et al.*, 1989), and tropin esterase (Hessing, 1983). The alignment of their amino acid sequences in relatively similar parts of N-terminal regions is shown in Fig. 4.

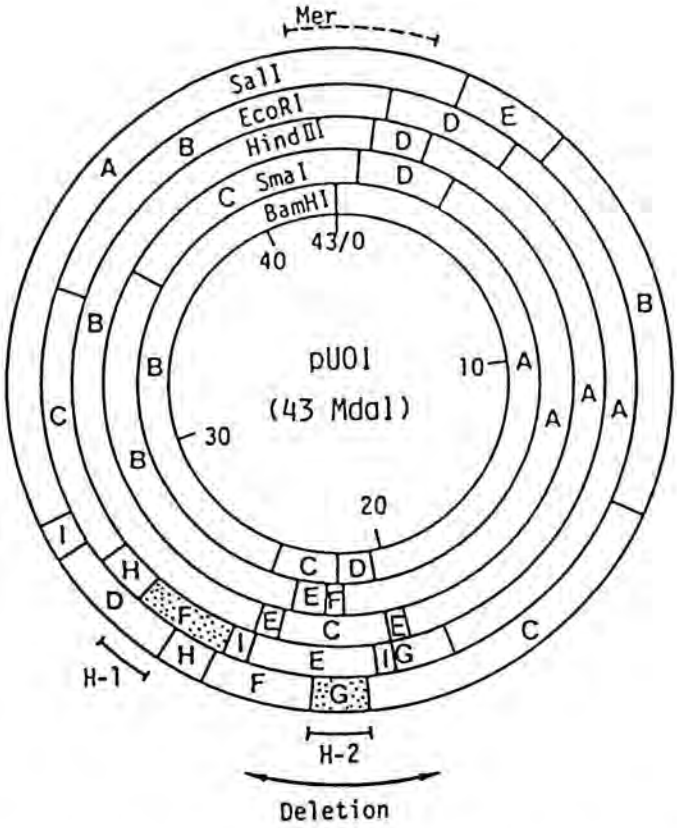

Fig. 2. A restriction map of plasmid pUO1 and the H-1 and H-2 coding regions. Dotted fragments hybridized each other.

Recently, Janssen *et al.* (1994) have reported that H-1 and two haloalkane dehalogenases encoded by the *dhlA* gene from *Xanthobacter* and the *linB* gene from *Pseudomonas paucimobilis* have a similar overall structure and catalytic mechanism.

3. The H-2 enzyme comes within L-2-haloacid dehalogenase group.

The H-2 gene showed high degrees of sequence similarity against the genes of the L-specific 2-haloacid dehalogenase group. The alignment of the amino acid sequences of seven L-2-haloacid dehalogenases and H-2 is shown in Fig. 5. H-2 shows 53% identity to DehYL from *Pseudomonas* sp. YL (Nardi-Dei *et al.*, 1994), 51% to HadL from *P. putida* AJ1 (Jones *et al.*, 1992), 51% to H-109 from *P. putida* No.109 (Kawasaki *et al.*, 1994), 50% to C-II from *Pseudomonas* sp. CBS3 (Schneider *et al.*, 1991), 44% to DhlB from *X. autotrophicus* GJ10 (Ploeg *et al.*, 1991), 38% to C-I from *Pseudomonas* sp. CBS3 (Schneider *et al.*, 1991), and 37% to Hdl IVa from *P. cepacia* MBA4 (Murdiyatmo *et al.*, 1992).

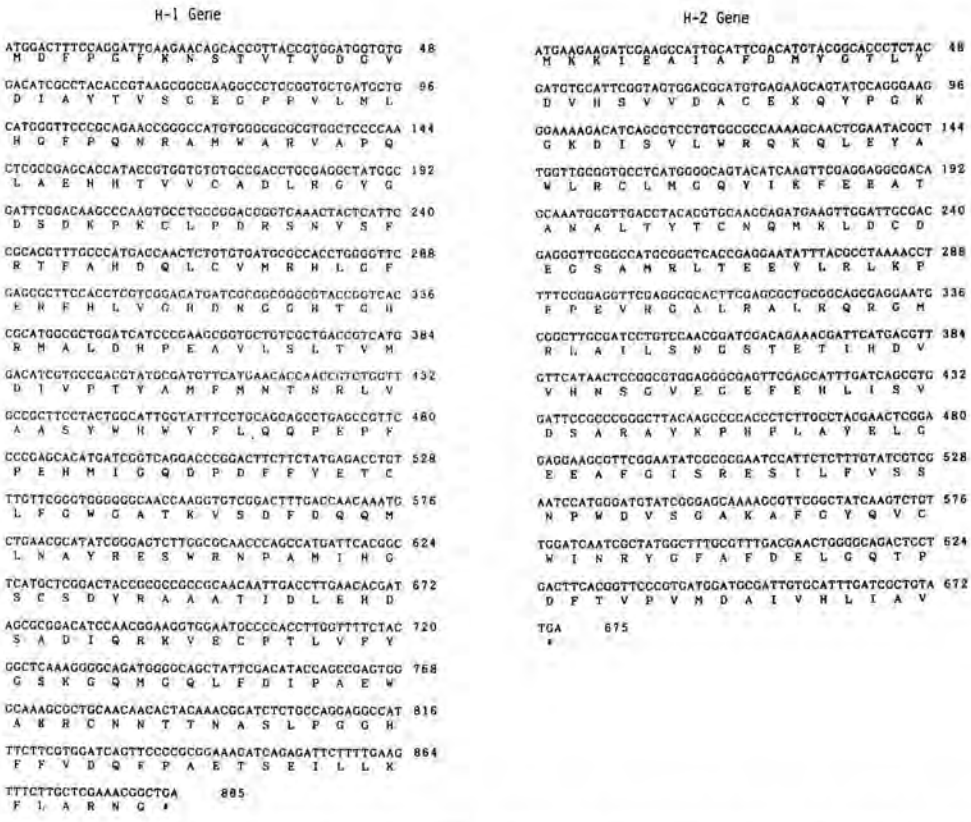

Fig. 3. The nucleotide sequences and deduced amino acid sequences of the H-1 and H-2 genes.

A tentative common sequence that was constructed by choosing the amino acid residues commonest to all 8 enzymes might be considered to be close to an original sequence, and when the 8 enzymes were compared with this sequence, H-2 showed a similarity as high as 60%, whereas other 7 dehalogenases showed 71 ~ 52% similarities. Therefore, the H-2 type haloacetate dehalogenase that has no fluorine-eliminating activity may be regarded as a variant of L-2-haloacid dehalogenase having an extremely narrow substrate range.

DehH1	23	SGEGPPVLMLHG.....	94	LGFERFHLVGHDRGG	(294 a.a.)
DmpD	26	SGAGFPLMMIHG.....	96	LEIEQADLVGNSFGG	(283 a.a.)
BphD	30	AGQGERVIMLHG.....	101	LGIEKAHLVGNSMGG	(277 a.a.)
Tpes	33	NPSGDPVLLLHG.....	99	MGLHNTTVIGHSMGS	(272 a.a.)
DhlA	44	SDAEDVFLCLHG.....	113	LDLRNITLVWQDWGG	(310 a.a.)

Fig. 4. Alignment of the predicted amino acid sequences of H-1, three hydrolases, and a haloalkane dehalogenase.

The numbers of amino acid residues in parentheses show enzyme sizes. Well conserved amino acids are emboldened. DehH1, H-1; DmpD, 2-hydroxymuconic semialdehyde hydrolase of *P. putida*; BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienate hydrolase of *Pseudomonas* sp. KKS102; Tpes, tropin esterase of *P. putida*; DhlA, haloalkane dehalogenase of *X. autotrophicus* GJ10.

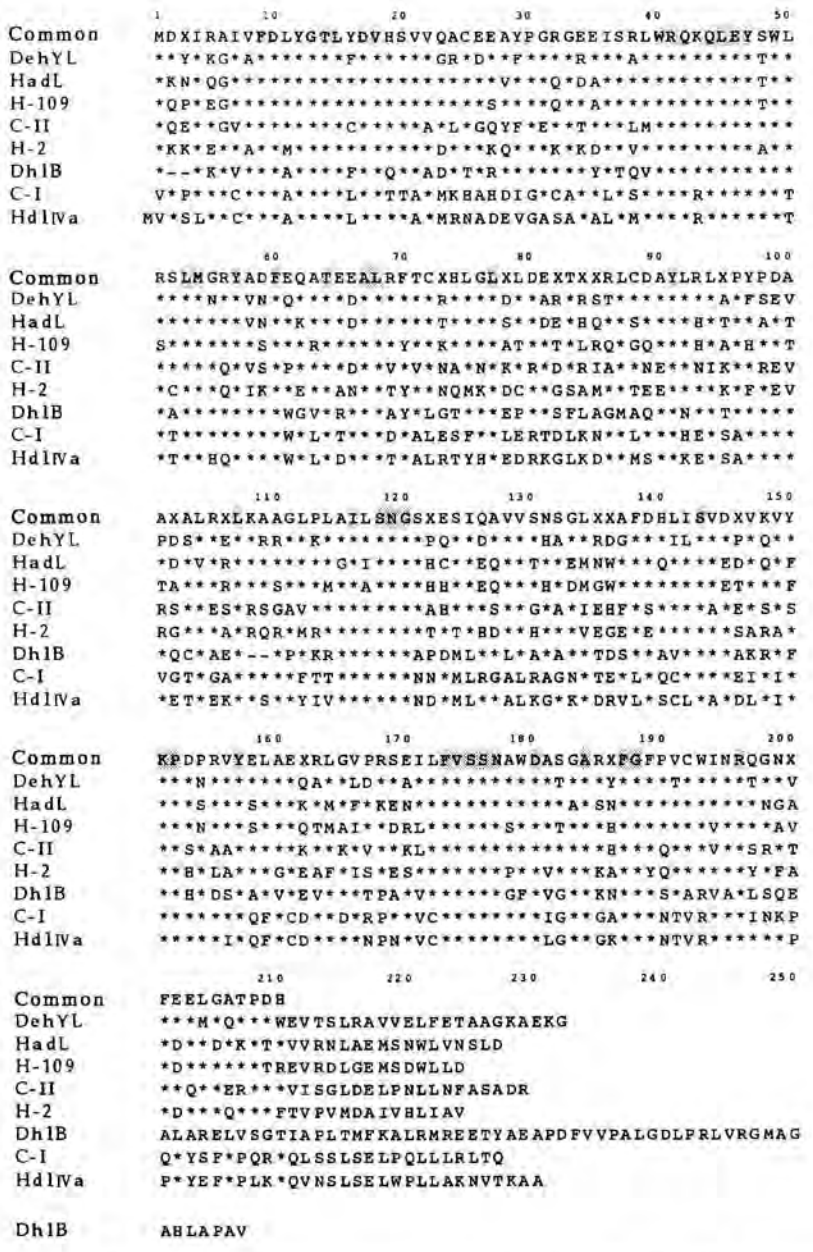

Fig. 5. Alignment of the predicted amino acid sequences of seven L-2-haloacid dehalogenases and H-2.

Asterisks indicate identical amino acids with a tentative common sequence that consists of amino acids the most common to 8 enzymes. When no common amino acid is found, X is placed. DehYL is a enzyme from *Pseudomonas* sp. YL, HadL from *P. putida* AJ1, H109 from *P. putida* No.109, C-I and C-II from *Pseudomonas* sp. CBS3, H-2 from *Moraxella* sp. B, DhlB from *X. autotrophicus* GJ10, and HdlIVa from *P. cepacia* MBA4.

A new transposon carrying the *dehH2* gene

1. The dehH2 gene is a class I composit transposon flanked by IS1071.

We knew that the phenotype H-2 of *Moraxella* was so labile that an overnight cultivation caused a few percent of the cells to lose the H-2 activity. These H-2 deficient cells harbored a uniform deletion plasmid being 5 kb smaller than pUO1. The deleted region, which was estimated by restriction analysis, included the H-2 gene and its flanking regions (Fig. 2).

The two restriction fragments, *Eco*RI-F and *Sal*I-G, of pUO1 hybridized tightly with each other as described, and besides similar restriction patterns were noticed on both sides of the deletion region. These facts suggested the presence of similar nucleotide sequences on both sides of *dehH2*. So, a plasmid DNA of about 12-kb long area including the *dehH2*-coding region and the flanking region was sequenced and analyzed.

As expected, 3.2-kb directly repeated sequences were found that holding the *dehH2* gene between (Fig. 6). Each sequence was flanked by 110-bp inverted repeats and contained single open reading frame (2913-bp) that seemed to be a transposase gene. The sequence comparison revealed that this sequence was identical, excepting three bases, to an insertion sequence element IS1071, which was first found on both ends of chlorobenzoate catabolic transposon Tn *5271* that resides in a plasmid of *Alcaligenes* sp. BR60 (Nakatsu *et al.*, 1991).

It is of interest that the same IS element was found at two distant places beyond the ocean; *Alcaligenes* strain BR60 carrying IS1071 was isolated from an industrial landfill runoff in the Niagara River watershed, USA, while *Moraxella* strain B carrying the H-2 gene was isolated in Japan.

Several transposons of class I and class II are shown in Fig. 7. The class II transposons carry short terminal inverted repeats, while class I transposons have

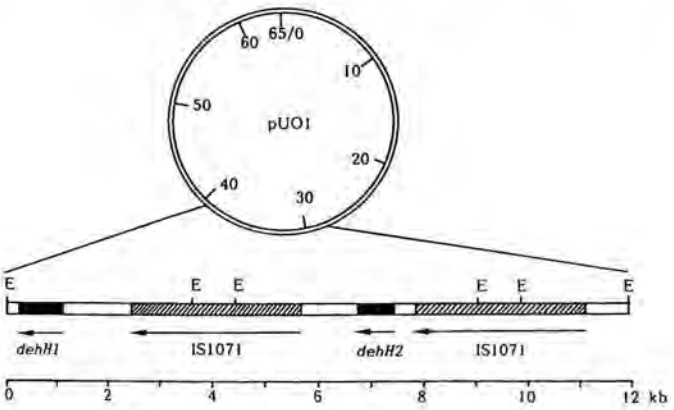

Fig. 6. Loci of *dehH1*, *dehH2* and directly repeated insertion sequences, IS1071, on plasmid pUO1.

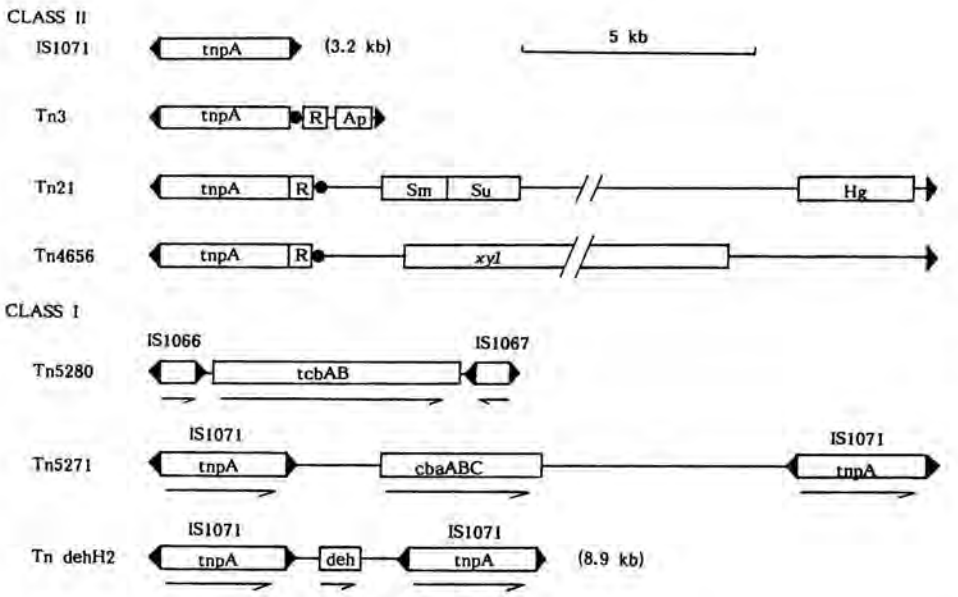

Fig. 7. Structures of class I and II transposons.

IS elements on both ends. The *dehH2* gene is sandwiched between two IS1071. Such structure supports an idea that *dehH2* is a class I composite transposon. This transposon-like element, tentatively designated Tn*dehH2*, is 8.9-kb long and consists of two 3.2-kb IS DNAs and a 2.5-kb intervening DNA containing the *dehH2* gene.

Thomas *et al.* (1992) have reported that the 2-haloacid dehalogenase gene of *Pseudomonas putida* PP3 is a transposon, designated *DEH*.

2. Functions of Tn dehH2; Deletion and transposition.

As described above, the H-2 gene is spontaneously deleted from the plasmid pUO1. The size of the deletion is corresponding to the sum of the intervening DNA (2.5 kb) and one IS1071 (3.2 kb). This deletion is seemed to be mediated by recombination between the two IS.

The transposition function of this element has been examined using the plasmid pUO1 carrying Tn*dehH2* as a donor and a drag resistant plasmid RP4 as a recipient. Two plasmids were successively introduced into the cells of *Pseudomonas* sp. E, in which pUO1 and RP4 seemed to be incompatible so that both plasmids would not coexist in the cell after several generations. Since most of the cells should have either of the two plasmids, the cells carrying recombinant plasmids could be selected by a pair of phenotypes of both plasmids, e.g. the ability to assimilate chloroacetate and kanamycin resistance. Many kinds of recombinants were obtained, and one of them, pDR4, showed suitable phenotypes, namely, the H-2 activity and all resistances (Ap, Tc, Km) and the

transmissibility owing to RP4 (Kawasaki *et al.*, 1985). Although the detailed structural analysis of recombinant DNA is in progress, I am sure that this recombinant has resulted from the transposition of this element.

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Bacterial Dehalogenation of Chlorinated Methanes

Abstract

Chlorinated methanes are metabolized by various aerobic and strictly anaerobic bacteria. The highly chlorinated compounds trichloromethane and tetrachloromethane undergo fortuitous reactions with enzymes or biocatalysts and do not provide carbon or energy to the cell. Chloromethane and dichloromethane are also subject to fortuitous transformation reactions, but in addition serve as growth substrates for facultatively methylotrophic bacteria and for specialized acetogenic bacteria. These organisms use specific dehalogenases for converting chloromethane or dichloromethane to intermediates of their primary metabolism. The chloromethane dehalogenase of aerobic methylotrophs and the dichloromethane dehalogenase of an anaerobic bacterium fermenting this compound to formate and acetate are still uncharacterized. In contrast, the chloromethane dehalogenase of a homoacetogenic bacterium has been characterized in crude extracts, and extensive genetic and biochemical studies, including protein engineering, have been performed with dichloromethane dehalogenase/glutathione *S*-transferases of aerobic methylotrophs.

Introduction

All four chlorinated methanes are industrially produced in considerable quantities, primarily for use as solvents in large scale chemical reactions and as degreasing agents. Chloromethane, and less significantly trichloromethane and tetrachloromethane are also of biogenic origin (Gribble, 1994). Because of their high volatility, the chlorinated methanes are distributed to various oxic and anoxic ecosystems where they are met by microorganisms craving for carbon and energy sources or for terminal electron acceptors respectively. In this contribution we discuss how bacteria interact with chlorinated methanes, particularly how they make use of chloromethane and dichloromethane as carbon and energy sources for growth. The subject has been addressed in an earlier communication (Leisinger *et al.*, 1993), and the mechanistic features of bacterial metabolism of chlorinated methanes have been discussed in a review by Wackett *et al.* (1992).

Microbial metabolism of the various chlorinated methanes is initiated by dehalogenation reactions. The biochemistry of these reactions, their evolutionary origin and their integration into cellular metabolism are topics of intrinsic interest. Microbial dehalogenation of chlorinated methanes is also of environmental relevance on two accounts. First, bacteria that dehalogenate these compounds are used as catalysts in the bioremediation of contaminated soil and groundwater (McCarty, 1993). Second, bacterial metabolism of chlorinated methanes in the environment may represent a sink for these compounds. For chloromethane and bromomethane it is particularly important to identify these sinks and to estimate their capacity, since both compounds are thought to make very significant contributions to the destruction of the stratospheric ozone layer (Manö & Andreae, 1994).

Table 1 gives an overview of how chlorinated methanes are metabolized by bacteria. Chloromethane and dichloromethane serve as growth and energy substrates for both aerobic and strictly anaerobic bacteria. With a view to bioremediation processes, this is clearly the preferred type of metabolism. It confers a selective advantage to the dehalogenating bacteria and thus leads to an increase of the biological catalyst when the chlorinated pollutant is present. In addition, all chlorinated methanes are subject to cometabolic transformations (Table 1). Such fortuitous reactions remain without direct benefit to the dehalogenative organisms. In biodegradation processes they are more difficult to control than dehalogenations based on growth. Dehalogenation by co-metabolic transformation thus is of interest for trichloromethane and tetrachloromethane, the chlorinated methanes not used as growth substrates.

Table 1. Metabolism of chlorinated methanes by pure cultures of aerobic and anaerobic bacteria

Compound	aerobic		anaerobic	
	Growth	Co-metabolism	Growth	Co-metabolism
CH_3Cl	yes	yes	yes	-
CH_2Cl_2	yes	yes	yes	yes
$CHCl_3$	-	yes	-	yes
CCl_4	-	-	-	yes

Co-metabolic transformation of trichloromethane and tetrachloromethane

Trichloromethane is aerobically transformed to phosgene by bacterial oxygenases with relaxed substrate specificity. Systems using methanotrophic organisms, the ammonia-oxidizing *Nitrosomonas europaea* (Wackett *et al.*, 1992), a recombinant pseudomonad expressing soluble methane monoooxygenase (Jahng & Wood, 1994), and toluene-oxidizing bacteria (McClay *et al.*, 1996) have been described. However, the formation of a reactive transformation product and the requirement for dissolved oxygen to dehalogenate the volatile chlorinated substrate impose practical limitations on these processes.

Co-metabolic anaerobic dehalogenation of trichloromethane to CO_2 and dichloromethane has also been explored. This transformation is carried out by methanogenic archaea and by anaerobic bacteria. Transition metal coenzymes found in these organisms, such as coenzyme F_{430}, cobalamins and hematin dehalogenate trichloromethane in vitro under reduced conditions to products and with kinetics similar to the *in vivo* systems. These catalysts thus are thought to be responsible for the non-specific metabolism of the compound in anaerobic microbes (Wackett *et al.*, 1992). The potential of degrading trichloromethane in pure or mixed cultures of methanogens was found to be limited by the strong inhibitory action of the compound on methanogenesis (Bagley & Gossett, 1995).

Tetrachloromethane is not known to be transformed by microorganisms under aerobic conditions. The compound, however, is efficiently dehalogenated to trichloromethane, dichloromethane and chloromethane by the same anaerobic archaea and bacteria that react with trichloromethane. Since tetrachloromethane is metabolized exclusively by anaerobic transformation reactions, the potential of such processes for the dehalogenation of this toxic pollutant is of particular interest. Recent studies have uncovered bacteria capable of transforming tetrachloromethane to CO_2 without the accumulation of trichloromethane as an end product. This is achieved by *Pseudomonas* sp. strain KC, an organism that rapidly transforms tetrachloromethane under denitrifying conditions (Dybas *et al.*, 1995), and by an as yet uncharacterized anaerobic bacterium which is not a methanogen or a sulfate reducer (deBest *et al.*, 1996). In both cases the reactions involved in tetrachloromethane transformation appear to be different from the reactions catalyzed by transition metal coenzymes, and it will be interesting to observe the elucidation of the underlying biochemistry.

Chloromethane and dichloromethane as growth substrates for acetogenic bacteria

Chloromethane

Diekert and collaborators (e.g. Messmer *et al.*, 1996) have isolated a strictly anaerobic bacterium capable of growth at the expense of chloromethane as the sole energy source. The organism, strain MC, was enriched from an anaerobic sludge digester, and in its morphology it resembled the homoacetogenic bacterium *Peptostreptococcus productus*. Strain MC fermented chloromethane to acetate as the sole organic product according to the fermentation balance shown in Fig. 1A.

Analysis of the biochemistry of chloromethane utilization revealed that the compound is metabolized via the acetyl-CoA pathway operative in acetogenic bacteria. Subsequently an elaborate enzyme assay was developed which allowed the characterization of chloromethane dehalogenase activity in crude extracts of strain MC. This key enzyme introduces chloromethane into the acetyl-CoA pathway. It catalyzes methyl transfer from chloromethane to tetrahydrofolate (Fig. 1A) and, for as yet unexplained reasons, requires substoichiometric

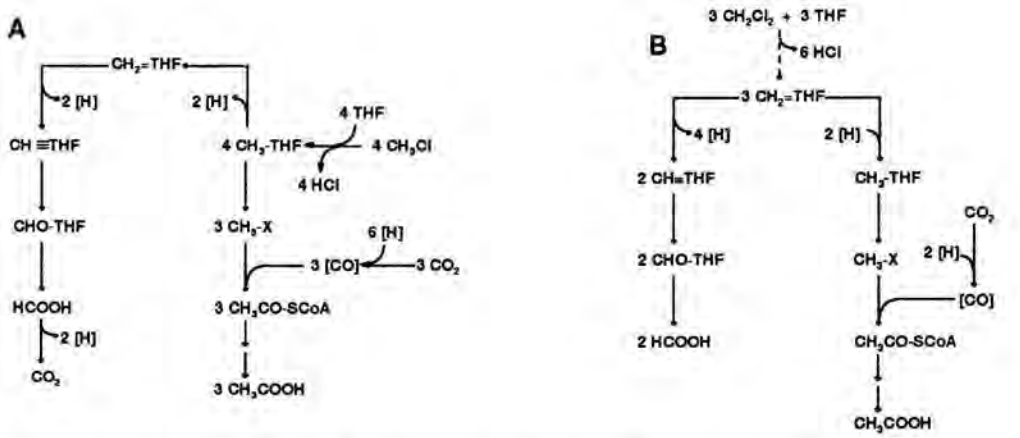


Fig. 1. Metabolism of chloromethane and dichloromethane by strictly anaerobic acetogenic bacteria.
A: Chloromethane utilization by the homoacetogenic bacterium strain MC (modif. from Messmer *et al.*, 1996). Fermentation balance: $4CH_3Cl + 3CO_2 + 2H_2O \rightarrow 3CH_3COOH + CO_2 + 4HCl$.
B: Hypothetical pathway for dichloromethane utilization by *Dehalobacterium formicoaceticum* strain DMC (Mägli *et al.*, 1996) in accordance with the observed fermentation balance: $3CH_2Cl_2 + CO_2 + 4H_2O \rightarrow 2HCOOH + 3CH_3COOH + 6HCl$.
THF, tetrahydrofolic acid; CH_3-X, methylated corrinoid-iron/sulfur protein.

amounts of ATP. It is induced by its substrate and is not identical with O-demethylase, an enzyme transferring the methyl group from methoxylated aromatics onto tetrahydrofolate and thereby enabling acetogenic growth of the organism on these compounds. Purification and characterization of chloromethane dehalogenase is under way (Messmer *et al.*, 1996). It is expected to provide information on the number and the properties of the protein components involved in the reaction and to clarify whether a reduced corrinoid or a thiol is involved as a nucleophile in this novel dehalogenation reaction.

Dichloromethane

Two anaerobic methanogenic enrichment cultures capable of growth with dichloromethane as the sole carbon and energy source have been described (Leisinger *et al.*, 1996). From one of these we have recently isolated a strictly anaerobic bacterium which converts dichloromethane to formate and acetate in a molar ratio of 2:1 and to biomass and traces of pyruvate. The fermentation balance observed for this organism is shown in Fig. 1B. Phylogenetic analysis of the 16S ribosomal DNA sequence placed the dichloromethane utilizing anaerobe in the *Clostridium-Bacillus* subphylum of the gram positive bacteria. The lack of sequence identity above 90% with any previously described taxon indicated that it was a representative of a new genus, and the organism was named *Dehalobacterium formicoaceticum* (Mägli *et al.*, 1996). More than fifty potential substrates

and combinations of substrates, including methoxylated aromatics and various C1 compounds were tested, but only dichloromethane supported growth.

It thus appears that *D.formicoaceticum* is specialized for the utilization of an anthropogenic compound. A metabolic explanation for this trait is at present not available. NMR-analysis of the products formed by resting cells from ^{13}C-dichloromethane showed that formate and the methyl group, but not the carboxyl group of acetate are derived from the labelled substrate (A. Mägli, unpublished). This observation and the presence of key enzymes of the acetyl-CoA pathway in cell extracts have led us to propose the pathway shown in Fig. 1B for the degradation of dichloromethane by *D.formicoaceticum*. The first step in this scheme, the conversion of dichloromethane plus tetrahydrofolate to methylene tetrahydrofolate plus chloride, is purely hypothetical. The development of a sensitive assay for this reaction therefore is a prerequisite for analysis of the dehalogenation enzyme in this system.

Chloromethane and dichloromethane as growth substrates for aerobic facultative methylotrophic bacteria

Chloromethane

Aerobic mineralization of chloromethane has previously been observed with a *Hyphomicrobium* sp. that utilizes the compound as a carbon and energy source (Hartmans *et al.*, 1986). More recently, further facultative methylotrophs capable of growth with chloromethane were isolated from contaminated environments (Doronina *et al.* 1996). With one of these, *Methylobacterium* sp. CM4, we have initiated studies to elucidate the metabolism of chloromethane (T.Vannelli and A.Sokolov, unpublished). Efforts to measure chloromethane dehalogenation in cell-free extracts have so far been unsuccessful, but a preliminary view of chloromethane metabolism by the organism has been obtained from studies with whole cells.

Oxygen uptake studies with resting cells indicated that chloromethane dehalogenation is inducible (Doronina *et al.*, 1996). As shown in Fig. 2, there are at least three possible pathways for the metabolism of chlormethane to carbon dioxide and hydrochloric acid by *Methylobacterium* CM4. They all generate formaldehyde but differ in the enzymatic step(s) that lead to this intermediate. In the hydrolytic mechanism, methanol and hydrochloric acid are produced without oxygen being required. The methanol produced would then be oxidized to formaldehyde, generating two electrons in an oxygen-dependent manner by methanol dehydrogenase. In the dehydrogenase mechanism, formaldehyde is produced without methanol as an intermediate, again generating two electrons in an oxygen-dependent manner. In the monooxygenase mechanism, formaldehyde is produced by an oxygen-dependent reaction, but two electrons are consumed.

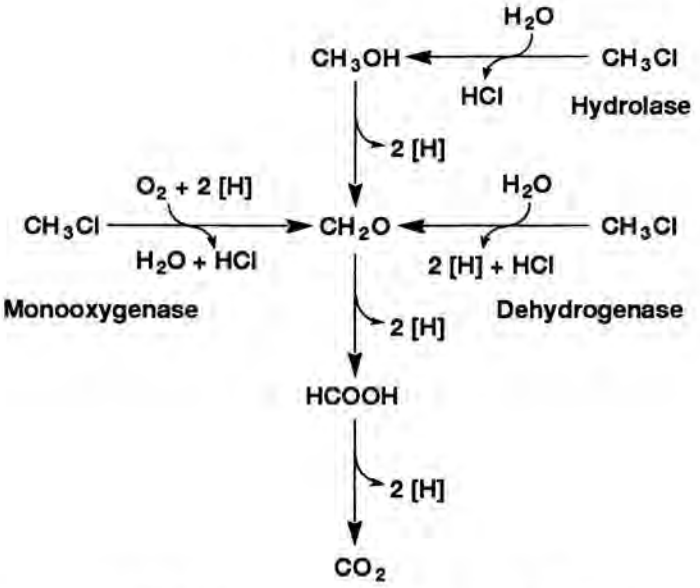


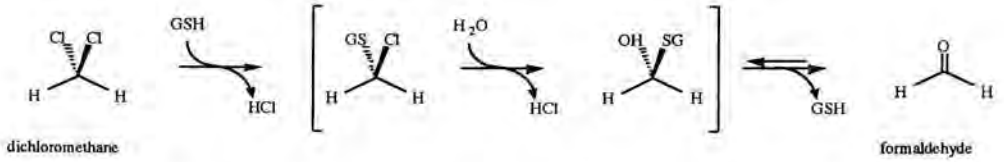
Fig. 2. Possible reactions for the dehalogenation of chloromethane by *Methylobacterium* sp. strain CM4

Based on the following observations we consider that a dehydrogenase mechanism is likely to be involved in chloromethane dehalogenation: i) The growth yields of *Methylobacterium* CM4 on chloromethane or methanol were both 3 times higher than when cells were grown on formate. Since two electrons are generated from the oxidation of formate and six from methanol, these growth yields suggest that six electrons were also generated from chloromethane and thereby argue against the monooxygenase mechanism (Fig. 2). ii) When cells were incubated anaerobically with chloromethane, no chloride was produced, but when oxygen was subsequently introduced, release of chloride was initiated. Since chloride production by a hydrolase is not dependent on oxygen, such a mechanism appears unlikely. Further, when the cells were incubated aerobically with EDTA, methanol oxidation was inhibited, but chloromethane oxidation was not, indicating that methanol was not an intermediate in the metabolism of chloromethane.

Dichloromethane

Dichloromethane degradation by methylotrophic proteobacteria that use this compound as the sole source of carbon and energy for growth has been investigated both at the biochemical and the genetic level in our laboratory (Leisinger *et al.*,1996). These bacteria contain an inducible enzyme, dichloromethane dehalogenase/glutathione *S*-transferase, which requires glutathione as a cofactor to hydrolyse dichloromethane to formaldehyde and hydrochloric acid

(Table 2). Dichloromethane dehalogenases are atypical enzymes among glutathione S-transferases (GST). In contrast to most enzymes of the GST superfamily, their substrate range is very narrow and restricted to dihalomethanes. Further, the tripeptide glutathione is not consumed in an initial conjugation with the electrophilic substrate, but regenerated in the course of the dehalogenation reaction:



Although the function of dichloromethane dehalogenase enzymes in metabolism is reminiscent of glutathione-dependent dehydrogenases, no significant sequence similarity can be detected between such proteins and dichloromethane dehalogenases. At the protein sequence level, dichloromethane dehalogenases are distantly but unequivocally related to GST enzymes of the Theta-class. Database sequences most closely related to dichloromethane dehalogenases are GSTT1 and GSTT2 enzymes from human, rat and mouse (25–28% identity) rather than bacterial GST enzymes (13–23% identity). Interestingly, it appears from the recent literature that GSTT1 enzymes are active with dichloromethane whereas GSTT2 enzymes are not, although the level of sequence identity of dichloromethane dehalogenase is about the same with GSTT1 and GSTT2 enzymes. However, no sequence regions or residues unique to both dichloromethane dehalogenase and GSTT1 sequences are readily apparent from sequence alignments. Thus, only low sequence identity is required to generate enzymatic activity with dihalomethanes within the glutathione *S*-transferase scaffold. This suggests that organisms as far apart as mammals and bacteria may have independently evolved GST enzymes with dichloromethane dehalogenase activity, well before the onset of anthropogenic contamination with dihalomethanes. Indeed, the sequence divergence of 44% between dichloromethane dehalogenase

Table 2. Kinetic properties of dichloromethane dehalogenases / glutathione *S*-transferases

Dichloromethane dehalogenase from	K_m^{DCM} (μM)	K_s^{DCM} (μM)	K_m^{GSH} (μM)	k_{cat} (s^{-1})
Hyphomicrobium sp. strain DM2[a]	11	ND	741	0.6
Methylobacterium sp. strain DM4[a]	9	0.5	2075	0.6
Methylophilus sp. strain DM11[b]	59	5.6	66[c]	3.3
rat liver Theta GSTT1 (GST5-5)[d]	300	ND	4200-5900	10

[a] Helga Sorribas, unpublished results.
[b] Data from Vuilleumier & Leisinger (1996)
[c] $S_{0.5}^{GSH}$ value (sigmoid saturation curve)
[d] Data from Blocki *et al.* (1994), and Meyer (1993)

from *Methylophilus* sp. DM11 and its closest known relative from *Methylobacterium* sp. DM4 indicates that the latest common ancestor of these enzymes may have existed as long as hundreds of millions years ago (Vuilleumier & Leisinger, 1996).

In the absence of a three-dimensional structure for dichloromethane dehalogenase or closely related GST enzymes, we are following several experimental approaches to elucidate the molecular determinants of catalytic efficiency and specificity of dichloromethane dehalogenase enzymes. First, protein engineering studies on the enzyme from strain DM11 have pinpointed several key residues required for activity and specificity (Vuilleumier & Leisinger, 1996) or glutathione affinity (Vuilleumier *et al.*, unpublished). Further, we have recently used PCR methods to amplify and sequence dichloromethane dehalogenase genes from previously isolated strains. Most variations in dichloromethane dehalogenase sequences observed so far are base substitutions leading to changes in the corresponding protein sequence. Whether these amino acid exchanges represent random drift or functional adaptation of the enzymes to the various host environments is currently under investigation.

Finally, we performed chemostat studies to determine the effect of dehalogenase properties on the growth behaviour of the model organism *Methylobacterium* sp. strain DM4. Transconjugants of this strain that carry a plasmid-encoded, constitutively expressed copy of a given dichloromethane dehalogenase or GST gene under the control of the promoter from the strain DM4 dehalogenase gene are being characterized with respect to μ_{max} and K_s, the Monod kinetic constants of the organism. For example, at low steady state concentrations of dichloromethane, a transconjugant expressing the catalytically superior enzyme from strain DM11 was outcompeted by an otherwise identical strain carrying the dichloromethane dehalogenase gene from strain DM4, due to the much higher affinity of the strain DM4 enzyme for dichloromethane (Table 2). These results show that dehalogenase characteristics are directly reflected in the growth properties of the host. They may contribute to explain our observations that methylotrophs expressing enzymes closely related to the strain DM4 dichloromethane dehalogenase are often more abundant in natural samples. Our recent results, however, also indicate that under conditions where dichloromethane is plentiful, growth of the strain with the better dehalogenase from strain DM11 occurs at a higher rate. Moreover, both strain DM4 and strain DM11 types of dichloromethane dehalogenase genes can be detected in sludge from some wastewater treatment plants. This suggests that, depending on the rate of dichloromethane supply, organisms with either strain DM4 or strain DM11 type genes may prevail in these environments.

Acknowledgments

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Tetrachloroethene Respiration

Abstract

Dehalospirillum multivorans is a strictly anaerobic bacterium, which is able to utilize H_2 as electron donor and tetrachloroethene (PCE) as electron acceptor in its energy metabolism. Energy is derived from PCE reduction *via* electron transport phosphorylation (tetrachloroethene respiration). PCE is reductively dechlorinated via trichloroethene (TCE) to *cis*-1,2-dichloroethene (DCE). This process was characterized in detail with respect to its physiology and biochemistry.

Introduction

Tetrachloroethene (PCE) is a volatile chlorinated hydrocarbon, which is a frequent pollutant of ground water or soil due to ist extensive use during the last 50 years in e. g. fat extraction, dry cleaning of textiles, and machine scouring. Because of its toxic effects on biological systems on the one hand and its effect on the ozone layer of the atmosphere on the other hand, the application of PCE is no longer desirable. Efforts have been undertaken to substitute for this compound and to decontaminate polluted environments. During the last few years, the application of bioremediation has been discussed (see e. g. Vogel and McCarty, 1985; Freedman and Gossett, 1989). A prerequisite for bioremediation of PCE polluted environments is the availability of bacteria capable of PCE dechlorination.

Due to the four chlorine substituents, the carbon backbone of PCE is highly oxidized. Therefore, it cannot be easily attacked by oxygen, resulting in persistence of PCE under aerobic conditions. Biotransformation of PCE obviously occurs exclusively under anaerobic conditions involving reductive dechlorination of the compound.

So far, complete reductive dechlorination of PCE to ethene (or ethane) has only been observed in mixed cultures (Freedman and Gossett, 1989; DiStefano *et al.*, 1991; DeBruin *et al.*, 1992), which converted PCE *via* trichloroethene (TCE), dichloroethene (DCE), and vinyl chloride (VC) to ethene (or ethane).

Recently, efforts were undertaken to isolate pure cultures of anaerobic bacteria able to dechlorinate PCE. Pure cultures of '*Dehalobacter restrictus*'

(Holliger and Schumacher 1994) and of *Dehalospirillum multivorans* (Neumann *et al.*, 1994; Scholz-Muramatsu *et al.*, 1995) have been described to reductively dechlorinate PCE to *cis*-1,2-dichloroethene (DCE) in their energy metabolism and to couple this reaction to energy conservation (tetrachloroethene respiration) (Scholz-Muramatsu *et al.*, 1995; Schumacher and Holliger, 1996). Both organisms are able to grow at the expense of H_2 and PCE as sole energy sources. The process of tetrachloroethene respiration upon growth on these substrates is schematically summarized in Fig. 1.

A coupling of ATP synthesis to reductive dechlorination is only feasible if thermodynamically favourable. The $\Delta G^{\circ\prime}$ value for PCE reduction with H_2 according to the equation

$$PCE + 2H_2 \rightarrow \textit{cis}\text{-1,2-DCE} + 2H^+ + 2Cl^-$$

is about -376 kJ/mol. Hence, PCE reduction with H_2 is a highly exergonic reaction, which is due to the positive standard redox potential at pH 7, $E^{\circ\prime}$, of the couples PCE/TCE ($+580$ mV) and TCE/DCE ($+540$ mV). For microorganisms, PCE is — besides several transition metal ions — the most positive-potential electron acceptor under anoxic conditions. PCE reduction was studied in detail with *D. multivorans*. In this communication, the physiology, energetics, and biochemistry of PCE reduction in this organism will be described.

Dehalospirillum multivorans

D. multivorans is a gram-negative strict anaerobe, which clusters with the ε-subgroup of the *Proteobacteria*. In contrast to '*D. restrictus*', which is restricted to

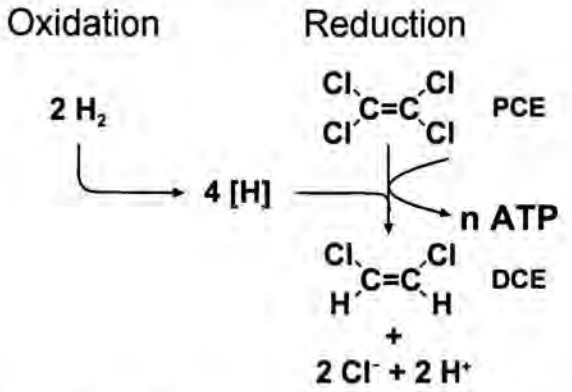

Fig. 1. Simplified scheme of tetrachloroethene respiration. ATP is formed exclusively in the reductive part of the energy metabolism via electron transport phosphorylation.

H_2 and PCE as energy source, *D. multivorans* is an organism with a relatively wide substrate spectrum. Besides H_2, the organism utilizes formate, pyruvate, glycerol, lactate, and ethanol as electron donors for catabolic reduction reactions. Alternative electron acceptors in energy metabolism are fumarate, nitrate, and possibly also S° (Scholz-Muramatsu *et al.*, 1995). The only substrate utilized in the absence of an electron acceptor is pyruvate.

The ability to dechlorinate PCE appears to be constitutive, at least in the strain investigated. This facilitated the investigations on tetrachloroethene reduction considerably, since with pyruvate plus fumarate and in the presence of yeast extract growth is very fast ($t_d \approx 1$–2 h) and results in high cell yields. When the bacterium was grown on a defined medium with H_2 and PCE as sole energy sources and acetate as carbon source (the organism is not capable of autotrophic CO_2 fixation), the organism grew much slower ($t_d \approx 20$ h) at a specific growth yield of at most 2.8 g cells (dry weight) per mol Cl^- released. The low growth yields with PCE are surprising with respect to the favourable thermodynamics of PCE reductive dechlorination (see above). A possible explanation for this observation will be discussed below.

Purification and properties of tetrachloroethene reductive dehalogenase

The tetrachloroethene reductive dehalogenase (PCE dehalogenase) catalyzes in vitro the reduction of PCE to TCE and of TCE to DCE with reduced methyl viologen as artificial electron donor (Neumann *et al.*, 1994, 1995). Methyl viologen is blue in its reduced state and colourless in its oxidized state. PCE reduction can easily be measured spectrophotometrically by the decrease in absorbance at 578 nm. Although in crude extracts the enzyme is not oxygen sensitive, the assay has to be performed under anoxic conditions. The reduction of PCE *via* TCE to DCE is shown in Fig. 2.

The enzyme exhibits catabolic activities in crude extracts. Incubation of extracts in the presence of low concentrations of propyl iodide led to a rapid inactivation of the enzyme; this was only observed under reducing conditions, i.e.

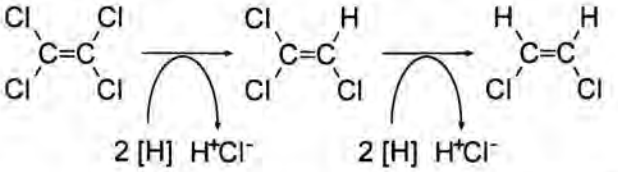

Fig. 2. Tetrachloroethene reductive dechlorination to dichloroethene catalyzed by tetrachloroethene reductive dehalogenase.

in the presence of titanium(III) citrate. In the absence of an electron donor, the enzyme remained unaffected by the alkyl halide. The inactivation could be reversed by illumination. These findings pointed to the involvement of a corrinoid in reductive PCE dechlorination (Neumann *et al.*, 1995). From the data, the reaction mechanism depicted in Fig. 3 may be derived.

It is known for 'free' and protein-bound corrinoids that the standard redox potential at pH 7, depending on the corrinoid type, of the couple cob(II)/cob(I)alamin is lower than −0.5 V, whereas that of cob(III)/cob(II)alamin is far more positive. According to the scheme depicted in Fig. 3, one low-potential electron and one electron at a more positive potential would be required for the reductive dechlorination of PCE or TCE.

The involvement of a corrinoid in reductive dechlorination is not surprising, since it was known from earlier studies, that corrinoids mediate the abiotic dehalogenation of halogenated methanes in the presence of an electron donor (Krone *et al.*, 1989, 1991). However, no significant abiotic dechlorination of PCE occurred in the presence of corrinoids and Ti(III) citrate (unpublished results). Therefore, PCE dehalogenation has to be considered as a biotic rather than an abiotic process.

The PCE dehalogenase was purified approximately 100-fold to apparent homogeneity from pyruvate/fumarate-grown cells (Neumann *et al.*, 1996). The purified enzyme mediated PCE dechlorination at a rate of near 2600 nkat.mg^{-1}. It consisted of one single subunit of an apparent molecular mass of 57 kDa; gel filtration revealed an apparent molecular mass of 58 kDa. Per 1 mol monomeric enzyme 1 mol corrinoid (or 1 mol cobalt), about 8 mol Fe, and 8 mol acid-labile sulfur were detected.

In the meantime, the gene encoding PCE dehalogenase has been cloned in *Escherichia coli*. Sequencing of the gene is currently performed in our laboratory.

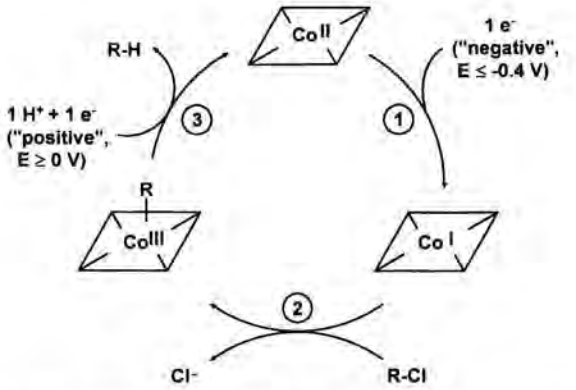

Fig. 3. Tentative scheme of the reaction mechanism of PCE dehalogenase. [Co] indicates the corrinoid in the respective oxidation state. The cobalt has to be in the +1 state to be alkylated by the alkyl halide (PCE or TCE = R-Cl). For the reduction of cob(II)alamin to cob(I)alamin (reaction 1) a low-potential electron is required.

Studies on energy conservation *via* tetrachloroethene respiration

It has already been mentioned that PCE reduction with H_2 is a thermodynamically favourable process due to the positive standard redox potentials $E^{\circ\prime}$ of the couples PCE/TCE (+580 mV) and TCE/DCE (+540 mV). The potential difference between H_2 ($E^{\circ\prime}$ $H^+/H_2 = -414$ mV) and PCE or TCE, respectively, is more than 0.9 V (under standard conditions at pH 7), which would theoretically account for about 9 mol electrogenic protons translocated across the cytoplasmic membrane per mol Cl^- released. Assuming that 3 protons are required for the synthesis of 1 ATP in the ATP synthase reaction (like in other organisms), the synthesis of approximately 3 mol ATP per mol Cl^- would be feasible. In this case, a specific growth yield Y_S of about 15–20 g cells (dry weight) per mol Cl^- could be obtained.

This theoretical value is far away from what has been measured with *D. multivorans* (2.8 g/mol; see above), even if the value given for standard conditions is corrected for the actual experimental conditions. This finding indicated that a proton pump is not involved in energy conservation *via* PCE respiration.

The reason for this discrepancy is probably the reaction mechanism of the enzyme, especially the involvement of a corrinoid as prosthetic group. As outlined in Fig. 3, The redox potential of the cob(II)/cob(I)alamin couple is in theory lower than or equal to −0.5 V. Therefore, it was assumed that a low-potential electron donor is required for PCE reduction. We studied the influence of the redox potential on PCE reduction as well as the enzyme activity with electron donors of similar structure (mostly viologens) and different redox potentials (Miller *et al.*, unpublished results). The enzyme activity was dependent on the redox potential with an estimated half-maximal velocity at about −0.4 V. With different artificial electron donors, a significant enzyme activity was only observed when the redox potential of the electron donor was lower than −0.36 V. These findings support the assumption that a low-potential electron donor is required for reductive dechlorination of PCE and that the redox potential of the prosthetic group is lower than or close to −0.4 V.

To elucidate the mechanism of energy conservation in tetrachloroethene respiration, it is important to know the cellular localization of the enzymes involved in the process. The hydrogenase was membrane-associated and faced the periplasm, whereas the PCE dehalogenase appeared to be a soluble, cytoplasmic enzyme. The electrons derived from H_2 oxidation have to cross the membrane from the outside to the inside of the cell. A simple model for energy conservation coupled to PCE reduction is shown in Fig. 4. This model involves an energy conservation mechanism similar to that of fumarate respiration in *Wolinella succinogenes* (Kröger *et al.*, 1992), with the exception that fumarate reductase is membrane associated.

The problem with the model depicted in Fig. 4 is the transfer of an electron ($E^{\circ\prime} = -414$ mV) derived from H_2 oxidation outside the cell to the corrinoid ($E^{\circ\prime}$ cob(II)/cob(I)alamin $\leqslant -400$ mV) of the PCE dehalogenase inside the cell (see Fig. 3, reaction 1) against an electrochemical proton potential Δp of about

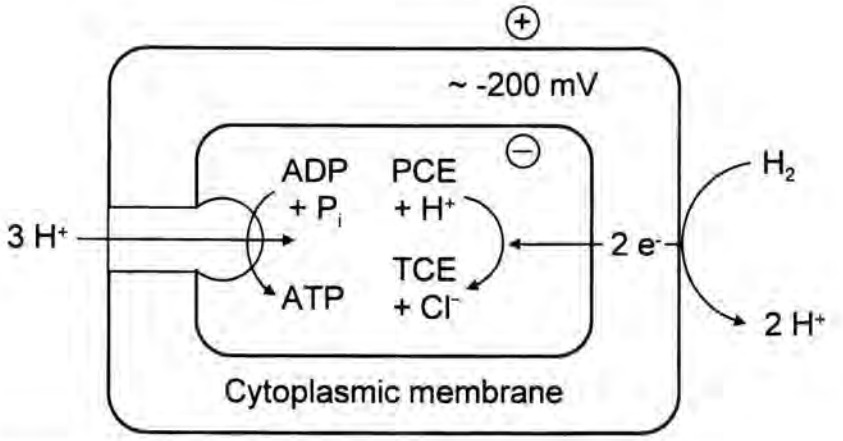

Fig. 4. Simple model of tetrachloroethene respiration in *D. multivorans*.

−200 mV (which is the average Δp found so far for all organisms). This problem can be solved assuming that the low-potential electron required for cob(II)alamin reduction has to be translocated across the cytoplasmic membrane involving a reversed electron flow according to the model depicted in Fig. 5.

The other, positive-potential electron (required for reaction 3 in Fig. 3) is driven across the membrane by the potential difference between H^+/H_2 and cob(III)/cob(II)alamin. Assuming a H^+/ATP stoichiometry of 3:1, the resulting ATP yield per mol chloride ion released would be about 1/3. This would be in accordance with the low growth yields observed for the organism (see above).

To confirm or disprove the involvement of a reversed electron flow, preliminary studies on the influence of ionophores on PCE reduction in whole

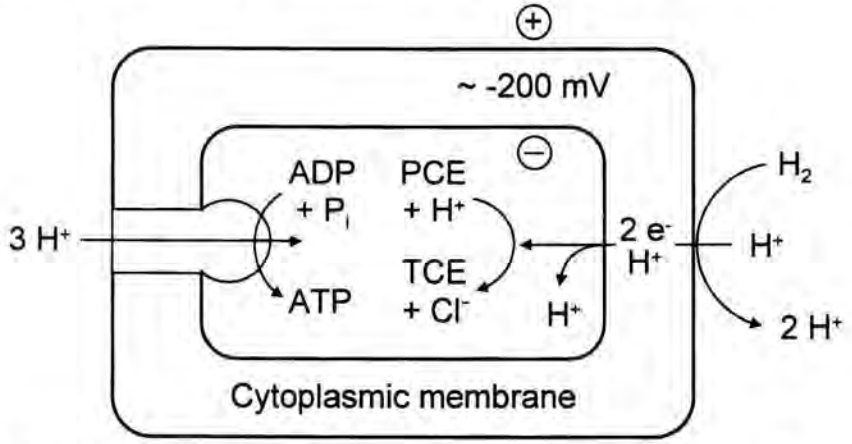

Fig. 5. Model of tetrachloroethene respiration involving a reversed electron flow. One electron is translocated along with a proton in a reversed electron flow.

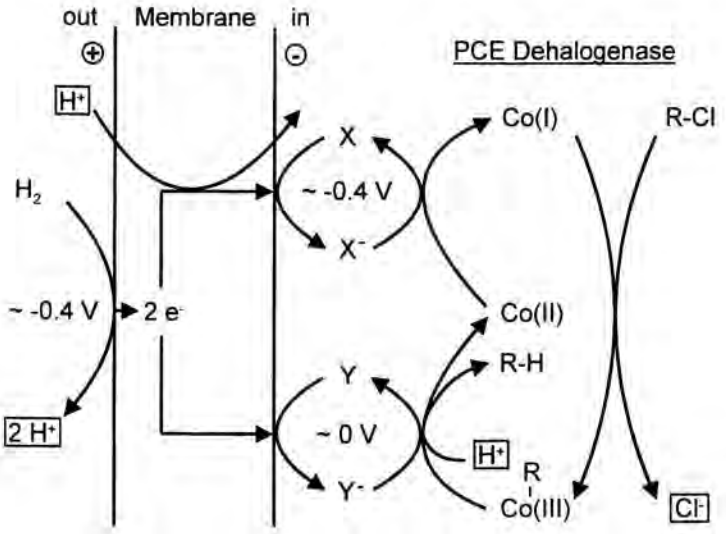

Fig. 6. Detailed tentative scheme of tetrachloroethene respiration. The low-potential electron required for the reduction of cob(II)alamin to cob(I)alamin (represented by Co(II) and Co(I)) is driven across the membrane by a reversed electron flow. X and Y are unknown secondary electron carriers.

cells were performed. Assuming that an eletrochemical proton potential is required to drive the low-potential electron across the membrane, ionophores should prevent reductive dechlorination of PCE. This was indeed observed for the ionophores tetrachlorosalicylanilide, FCCP, nigericin and/or valinomycin (plus K^+) (data not shown). These results were interpreted to support the hypothesis that a reversed electron flow is involved in PCE reductive dechlorination in *D. multivorans*. Whether this is a general feature of dehalorespiration (i.e. reductive dehalogenation coupled to energy conservation via a chemiosmotic mechanism), will have to await further studies on other bacteria mediating reductive dehalogenation in their energy metabolism. These studies are currently underway in our laboratory. Fig. 6 summarizes a more detailed model of PCE reductive dechlorination in *D. multivorans* involving a reversed electron flow.

Acknowledgements

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Production of Chiral Chloropropanols using Stereospecifically Assimilating Bacteria

Abstract

Bacteria stereospecifically assimilating (S)- and (R)-2,3-dichloro-1-propanol or (S)- and (R)-3-chloro-1,2- propanediol were isolated from soils. These bacteria have dehalogenase activity for various halohydrins and epoxyhydrolase activity for aliphatic epoxides. Microbial resolution of (RS)-2,3-dichloro-1-propanol or (RS)-3-chloro-1,2-propanediol was performed and using these bacteria an industrial process was developed for the production of the respective highly optically active enantiomers.

Introduction

Large amounts of halogenated compounds are manufactured on an industrial scale and they have been utilized as solvents, agrochemicals, pharmaceuticals, and polymers. Recently, there have been many reports concerning the microbial degradation of low-molecular halogenated aliphatics (Vogel *et al.*, 1987). The enzymatic dehalogenation of haloacids (Hardman and Slater 1981; Kawasaki *et al.*, 1981a,b; Motosugi and Soda, 1983), haloalkanes (Janssen *et al.*, 1987; Keuning *et al.*, 1987; Scholtz *et al.*, 1987) and dichloromethane (Brunner *et al.*, 1980) have been reported. However, the microbial degradation of halogenated aldehydes, alcohols and esters has rarely been reported.

Stucki and Leisinger (1983) isolated a bacterium capable of degrading chloroethanol via chloroacetic acid, and with respect to the dehalogenation of a halogenated C3 alcohol by microorganisms, Castro and Bartnicki (1968) reported the degradation of 2,3-dibromo-1-propanol by *Flavobacterium*. Recently, Janssen and van den Wijngaard (1989) isolated three bacterial cultures capable of growing on 1,3-dichloro-2-propanol, epichlorohydrin (EP) and 3-chloro-1,2-propanediol (CPD) and purified haloalcohol dehalogenase from the strain *Arthrobacter* sp. AD2 (van den Wijngaard *et al.*, 1991). This enzyme catalyzed the conversion of halogenated alcohols such as 1,3-dichloro-2-propanol, 1,3-

dibromo-2-propanol, CPD, 1-chloro-2-propanol and 1-bromo-2-propanol to the corresponding epoxides.

We have long studied the development of the microbial conversion of C3 halogenated aliphatic compounds to useful chemicals. Recently, we isolated novel bacteria capable of stereospecifically assimilating 2,3-dichloro-1-propanol (DCP) with the liberation of chloride ion, and developed the microbial resolution of optically active DCP and the synthesis of optically active epichlorohydrin (EP) (Kasai *et al.*, 1992a, 1992b). Our microbial resolution was very effective and practical, so that the optically active epichlorohydrin obtained with this method was utilized for the syntheses of many useful compounds such as pharmaceuticals (Kawamura *et al.*, 1990), pheromones (Imai and Nishida, 1990), and ferro-electric liquid crystals (Koden *et al.*, 1990). More recently, we isolated novel bacteria stereospecifically assimilating (R)- and (S)-CPD from soil, and succeeded in the preparation of (S)- and (R)-CPD with highly optical purity using the bacterium by virtue of its stereospecific selectivity (Figure 1). These bacteria can produce optically active EP and CPD as useful chiral building blocks; they are not only useful for the degradation of halogenated compounds but also excellent biocatalysts for new chemistry. This report describes the characterization of the strains, the properties of the dehalogenating activity in the cell-free extracts of the bacteria used in the production of optically active EP, and CPD.

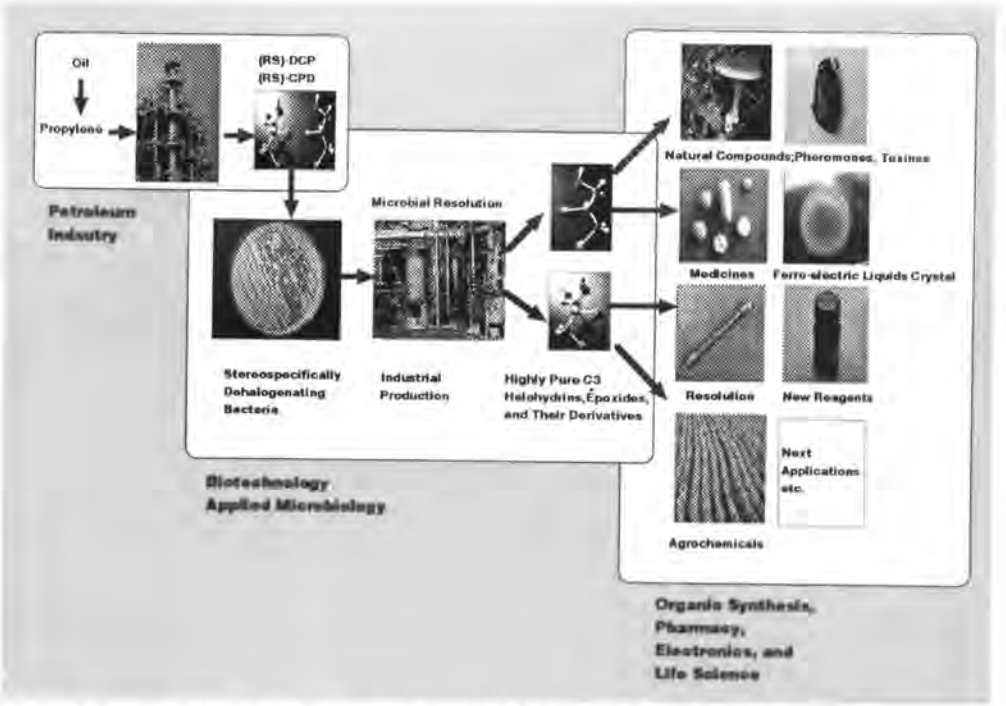

Fig. 1. Microbial production of optically active C_3 building blocks and their use.

(R)- and (S)-DCP assimilating bacteria

Isolation of an (R)-DCP assimilating strain

From 300 soil samples, a bacterial strain, OS-K-29, which assimilated DCP as a sole carbon source, was isolated. Morphological and physiological characteristics of OS-K-29 showed that it belonged to the genus *Pseudomonas*.

The strain OS-K-29 could grow on DCP, which was gradually degraded, and CPD was formed with liberation of chloride ion. CPD accumulated in the logarithmic phase and disappeared completely in the stationary phase. The growth of the cells ceased and the final degree of degradation was estimated to be 50% regardless of the concentration of 2,3-dichloro-1-propanol (DCP) used. Therefore, the conversion of DCP was considered to be stereospecific. When the strain was cultured on DCP or 2,3-dibromo-1-propanol(DBP), the specific rotations of residual DCP, formed CPD, and residual DBP gave $[\alpha]_D^{25} - 10.50$ ($c = 1.3$, in CH_2CI_2), $[\alpha]_D^{25} + 6.90$ ($c = 1.2$, in H_2O), and $[\alpha]_D^{25} - 12.00$ ($c = 1.3$, in CH_2C1_2), respectively.

A possible degradation route by OS-K-29 of DCP was from CPD to glycidol to glycerol with EP as a tentative intermediate. The dehalogenation or epoxidation of halohydrins was slower than hydrolysis of epoxides, so accumulation of epoxide was hardly observed. However, alcohols formed from halohydrins were easily detected. The degradation activity of OS-K-29 seems to be similar to that of the previously reported *Flavobacterium* sp. which degrades 2,3-dibromo-1-propanol (Castro and Bartnick, 1968). The latter strain assimilated 2,3-dibromo-1-propanol via epibromohydrin, 3-bromo-1,2-propanediol, glycidol, and glycerol, and accumulated more epoxides than dehalogenated alcohols because of strong bromohydrin epoxidase and weak epoxyhydrolase activities. However, some distinct features between two strains were noted by us; first, in the conversion of DCP, EP was not detected in OS-K-29, and CPD accumulated instead. This might be due to a fast epoxy-opening reaction by strong epoxyhydrolase activity. Second, the conversion of DCP was stereospecific in OS-K-29, whereas *Flavobacterium* sp. did not show stereospecificity. The enzyme of OS-K-29 that causes stereospecific conversion of DCP seemed to be novel. This could be used for preparation of chiral compounds. Synthesis of optically active EP has been tried by various methods (Golding, 1988).

Isolation of an (S)-DCP assimilating strain

From about 1000 samples of soils collected at petrochemical plants, six (RS)-DCP assimilating bacteria and six (R)-DCP assimilating bacteria were found, while only one bacterial strain, DS-K-S38, which preferentially assimilated (S)-DCP as a sole source of carbon was isolated. This strain was found to belong to the genus *Alcaligenes* according to its morphological and physiological characteristics.

When strain DS-K-S38 was cultivated on (RS)-DCP, (S)-DCP was degraded with liberation of chloride ions. The cell growth ceased when the final degradation was estimated to be 52%. A trace amount of CPD (Kasai *et al.*, 1990) and glycerol was detected in the logarithmic phase. These results were similar to those obtained with the (R)-DCP-assimilating strain OS-K-29 (Kasai *et al.*, 1992 a). The specific rotation of residual (R)-DCP gave $[\alpha] = +10.7$ ($c = 1.25$, in CH_2Cl_2) and the value was in good accord with that of (S)-DCP ($[\alpha] = -10.5$, $c = 1.36$, in CH_2Cl_2) (Kasai *et al.*, 1992 a).

Bacteria preferentially assimilating (R)-DCP ((R)-type) or (S)-DCP((S)-type) were isolated from soils. During the cultivation, in which (RS)-DCP was the single source of carbon, each bacterium degraded and assimilated half of the DCP; (R)-DCP or (S)-DCP.

Degradation of halogenated compounds

Table 1 shows the degradation activity of the (R)-DCP-assimilating strain OS-K-29 and the (S)-DCP assimilating strain DS-K-S38. Various low-molecular mass chlorinated aliphatic hydrocarbons and related epoxides (mainly C3 chlorinated compounds) were tested as to whether or not they were assimilated. Both strains could degrade chlorohydrins such as CPD, DCP, and glycidol, but

Table 1. Degradation of various halogenated compounds by strain OS-K-29 and DS-K-S-38

	OS-K-29 Growth (at 660 nm)	Degradation (%)	DS-K-S38 Growth (at 660 nm)	Degradation (%)
Halohydrins				
3-Chloro-1,2-propanediol	0.52	63.4	0.85	56.8
1,3-Dichloro-2-propanol	0.14	73	0.04	0
2,3-Dichloro-1-propanol	0.2	42.6	0.17	36.7
2,3-Dibromo-1-propanol	0	47.6	0	0
Ethylenechlorohydrin	0.02	16.8	0.08	3.1
Propylenechlorohydrin	0.11	58.2	0.07	20.7
Butylenechlorohydrin	0.04	44.3	0.07	24.2
Epoxides				
Epichlorohydrin	0.48	>99	0	0
Epibromohydrin	0.01	75.1	0	0
Glycidol	0.75	>99	0.02	16.2
Propylene oxide	0.01	17.2	0	0
Haloacids and others				
Chloroacetone	0	4.5	0	0
n-Propylchloride	0	1.2	0	0
2-Chloropropionic acid	0	0	0	0
3-Chloropropionic acid	0	0	0	0

Five ml of synthetic medium containing various haloginated compounds (0.2%, v/v) in screw capped test tubes was inoculated with 0.1 ml of a preculture, and incubated at 30 °C for 24 h and shaken.

not chloroacetone and chloropropionic acid. CPD was a good carbon source for growth, but the other compounds were poor carbon sources. Comparing the degradation activity between strain DS-K-S38 ((S)-type) and strain OS-K-29 ((R)-type), it was found that the range of assimilable and degradable compounds was smaller for the (S)-type organism than for the (R)-type. 1,3-Dichloro-2-propanol and EP were good carbon sources for growth of the (R)-type, however, the (S)-type could not degrade and assimilate them, regardless of the concentration.

Enzymatic conversion

Figure 2 shows the activity of the cell-free extracts of the strains OS-K-29 and DS-K-S38. C3 halohydrins were dehalogenated, yielding dehalogenated alcohols. Epoxides, except for propylene oxide, were hydrolyzed and converted to the diols. Comparing the relative activity of cell-free extracts between the (S)-type and the (R)-type, some notable differences were found. In the case of the (R)-type, the conversion activity was high and broad; especially, high epoxyhydrolase activity was found. On the other hand, in the case of the (S)-type, the pattern of the relative activity for halohydrins was similar to that of the (R)-type, however, the activity was weak. The epoxyhydrolase activity of the (S)-type was very weak except for EP. Although EP was not assimilated, the epoxyhydrolase activity for EP was the highest.

The (S)-type and (R)-type were isolated as stereospecific DCP assimilating bacteria from soil samples collected from the same petrochemical plant site. CPD and glycerol were detected during degradation of DCP, and the pattern of relative activity

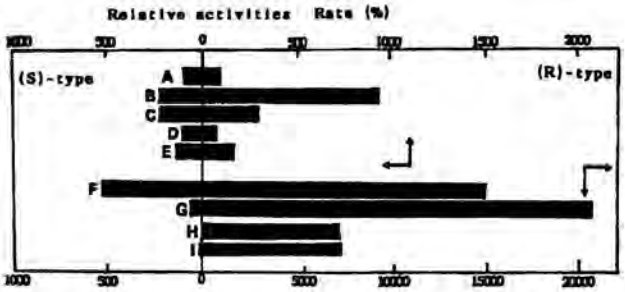

Fig. 2. Comparison of relative activities of cell-free extracts for halohydrins and epoxides between (S)-type and (R)-type. The conversion activities are expressed as percentages of the rate found with DCP(S)-type, 1.5 mU/mg protein; (R)-type, 1.1 mU/mg protein). Substrates A, DCP; B, 1,3-dichloro-2-propanol; C, CPD; D, propylene chlorohydrin; E, DBP, F, EP; G, epibromohydrin; H, propylene oxides; I, glycidol.

for halohydrins in the cell-free extracts resembled each other. Therefore, we considered that the degradation patterns were similar except the stereospecificity for DCP, which was dehalogenated and degraded to glycerol via CPD. Bacterial degradation of (RS)-DBP, in which the Cl of DCP is substituted for Br, was reported by Castro and Bartnicki (1968). DBP was converted by a halohydrin epoxidase to epibromohydrin, resulting in its accumulation. It was not determined whether or not dechlorination of (R)- and (S)-DCP occurred via EP, because epoxyhydrolase activity for EP was high compared to the dechlorinating activity for DCP which caused that EP did not accumulate. The enzyme which caused the dehalogenation of (R)- and (S)-DCP in the bacteria seemed to be novel because of its high stereospecificity. Stereospecific dehalogenation for DCP had not yet been described.

Production of (R)- and (S)-DCP and their conversion to (S)- and (R)-EP

Microbial resolution of DCP was carried out in simple synthetic medium using the stereospecific assimilating bacteria. The strain OS-K-29 or DS-K-S38 was precultured and inoculated into a synthetic medium containing (RS)-DCP as the single source of carbon. After the cultivation, the residual (R)- or (S)-DCP was purified using a charcoal column treatment, elution with acetone, and distillation *in vacuo*. The chiral purity of the each optically active DCP was 100% ee. (R)- and (S)-epichlorohydrin was obtained from DCP via treatment with aqueous NaOH with a yield of 74%. The chiral purity was determined as 99.3-99.4% ee through an examination of complexation gas chromatography (Kasai *et al.*, 1992a, 1992b).

Production of D-lactate from L-2-chloropropionate by a stereospecific dehalogenase of L-2-chloro-propionate-assimilating *Pseudomonas putida* has been reported. This system is similar to ours in the sense that the substrates are C3 compounds having the same chlorinated chiral center at the C2 position and the enzymes are stereospecific dehalogenases. Our substrate, however, has a hydroxyl group instead of a carboxyl group in the Cl position. Further characterization of the enzyme of these bacteria will be needed.

Generally, the resolution of liquid substances is very difficult since crystallization can not be carried out. Asymmetric syntheses of chiral EP has been developed, but these methods are not simple. For the resolution of DCP, some biochemical methods also have been reported. Iriuchijima and Kojima (1982) reported asymmetric hydrolysis of 1-acetoxy-2,3-dichloropropane with pancreatic or *Mucor* sp. lipases but the desired optical purity was not obtained.

This microbial resolution reported here was effective and useful for preparing (R)-and (S)-DCP and (S)- and (R)-EP. Residual (R)- and (S)-DCP were found to be optically pure, and highly pure (R)-and (S)-EP were prepared from these products. (RS)-DCP is produced economically by the petroleum industry and therefore, this method is considered applicable in practice. Habets-Crutzen *et al.*(1985) reported the formation of (S)-EP from allylchloride by microbial stereospecific epoxidation yielding product with an optical purity of 80-98% ee.

However, we consider that our method has advantages such as the higher solubility in water, its lower toxicity, and its higher boiling point than allylchloride. Since 1994, the microbial resolution is used on an industrial scale in Matsuyama, Japan. Currently, the (R)-and (S)-EP are being used in the synthesis of chiral drugs or natural products. It should also be possible to use these optically active compounds for easily obtaining C3 chiral building blocks.

(R)- and (S)-CPD assimilating bacteria

Isolation and characterization of strains assimilating (R)- and (S)-CPD

From 1000 soil samples, two bacterial strains, named DS-K-2D1 and DS-S-7G, were isolated that preferentially assimilated (S)-CPD and (R)-CPD as sole source of carbon, respectively. Strain DS-K-2D1 was identified as a member of the genus *Pseudomonas*, and DS-S-7G was identified as *Alcaligenes*.

Utilization of halogenated compounds

Table 2 shows the utilization and degradation of various halogenated and related compounds by strains DS-K-2D1 and DS-S-7G. The strains grew on racemic CPD, (R)-CPD or (S)-CPD and 3-bromo-1,2-propanediol but not on the other halohydrins, epoxides, haloacids and chloroacetone. Although (R)-CPD and 1,3-dichloro-2-propanol or (S)-CPD and DCP were slightly degraded by DS-K-2D1 and DS-S-7G, respectively, they did not support growth of the strains. Growth of the strain on racemic CPD medium was inhibited by the presence of 1,3-dichloro-2-propanol or 2,3-dibromo-1-propanol.

Degradation of halogenated and related compounds by cell-free extracts

The activities toward various halohydrins and related epoxides by cell-free extracts of strains DS-K-2D1 and DS-S-7G are summarized in Table 2.
In case of DS-K-2D1, halohydrins such as CPD, 3-bromo-1,2-propanediol, 1,3-dichloro-2-propanol, DCP, and propylene chlorohydrin were converted to the corresponding epoxides. The cell-free extracts had higher activity for bromohydrins than for chlorohydrins. The respective conversions were accompanied by the release of halide ions. The cell-free extracts also converted epichlorohydrin, epibromohydrin, GLD, and propylene oxide to the corresponding diols. The epoxide-opening activity for halogenated epoxides by cell-free extracts was high compared to that for non-halogenated epoxides. The degradation activity for (R)-CPD was 2.3 times higher than that for (S)-CPD.
In case of DS-S-7G, the cell-free extracts had activities towards several halohydrins and epoxides which were converted to the corresponding epoxides and alcohols, respectively. Various halohydrins such as racemic CPD, 3-bromo-

Table 2. Comparison of degradation activities of cells and relative activities of the cell free extracts between strain DS-K-2D1 and DS-S-7G

	DS-K-2D1			DS-S-7G		
	Growth (at 660 nm)	Degradation (%)	Relative activity by cell free extracts	Growth (at 660 nm)	Degradation (%)	Relative activity by cell free extracts
Halohydrins						
3-Chloro-1,2-propanediol	1.07	56.5	100	0.75	52.7	100
(R)-CPD	0.06	10	145	0.88	66.1	116
(S)-CPD	0.93	65.1	62.7	0.05	<5	47.5
3-Bromo-1,2-propanediol	0.35	67.6	894	0.44	49.5	72.7
1,3-Dichloro-2-propanol	0	13	136	0	<5	0
2,3-Dichloro-1-propanol	0	<5	23.3	0	11.5	36.4
Propylenechlorohydrin	0	<5	23.3	0	8.2	12.1
Epoxides						
Epichlorohydrin	0	<5	283	0	<5	30.3
Epibromohydrin	0	<5	300	0	<5	0
Glycidol	0	<5	34.2	0	<5	39.4
Propylene oxide	0	<5	39.1	0	13	0
Haloacids and others						
Chloroacetone	0	8	0	0	<5	0
n-Propylchloride	0	<5	0	0	<5	0
2-Chloropropionic acid	0	<5	0	0	<5	0

Method for degradation activities were the same as Table 1.
Relative activities were expressed as percentages of the rate found with CPD.

1,2-propanediol, DCP, and propylenechlorohydrin were mainly converted to the corresponding epoxides. These conversions were accompanied by the release of halide ions. Epichlorohydrin and glycidol were converted to the corresponding diols. Further enzymatic study of stereospecificity for (R)-CPD was done. We also found that (R)-CPD was converted to hydroxyacetone, and that the enzyme responsible was new, namely, a halohydrin dehydro-dehalogenase (HDDase) (Suzuki and Kasai, 1994).

Preparation of (R)- and (S)-CPD

A bacterial culture was inoculated into a synthetic medium containing (RS)-CPD as a single carbon source. (S)- or (R)-CPD was assimilated preferentially with the release of chloride ions, and (R)- or (S)-CPD was not converted. After cultivation, each CPD was purified by condensation and distillation from the culture filtrate. The optical purity of the purified (R)- and (S)-CPD was estimated to be greater than 99.5% ee by HPLC analysis of the tosylated derivatives (3-tosyloxy-1-chloro-2-propanol), respectively. Conversion to GLD was carried out according to the standard procedures. The optical purities of the formed (R)- and (S)-GLD were estimated to be greater than 99.3%ee and 99.4%ee, respectively, by gas chromatography (Suzuki and Kasai, 1991).

Chemical syntheses of (S)-CPD from methyl-6-chloro-6-deoxy-α-D-glucopyranoside and D-mannitol (Porter and Jones, 1982) are known, and also enzymatic and microbial preparations of (R)-CPD from racemic 1,2-diacetoxy-3-chloro-propane (Iriuchijima and Kojima, 1982b) and 1,3-dichloro-2-propanol, have been reported. Klunder *et al.* have reported a method for the preparation of optically active glycidol on the basis of the asymmetric oxidation of allyl alcohol (Klunder *et al.*, 1986., Gao *et al.*, 1987). However, these methods for generation of (R)-CPD and (S)-glycidol result in low optical purity or are not practical. Our microbial preparation of (R)-and (S)-CPD and the synthesis of (R)-and (S)-glycidol seems to be simpler, more effective and more practical than the other preparation processes described above. It is likely that these compounds can be produced on an industrial scale and it is possible using our procedure to produce highly optically active (R)- and (S)-CPD as well as optically active DCP and EP on a multiton scale.

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