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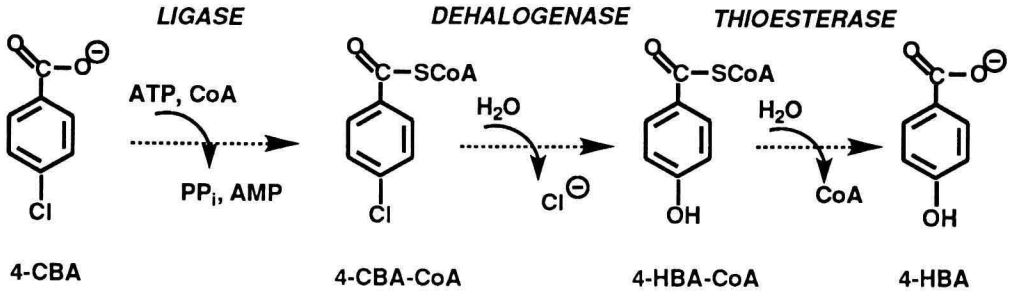
Studies on the Structure, Catalytic Mechanism and Ancestry of the 4-Chlorobenzoate-Coenzyme A Dehalogenase

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).

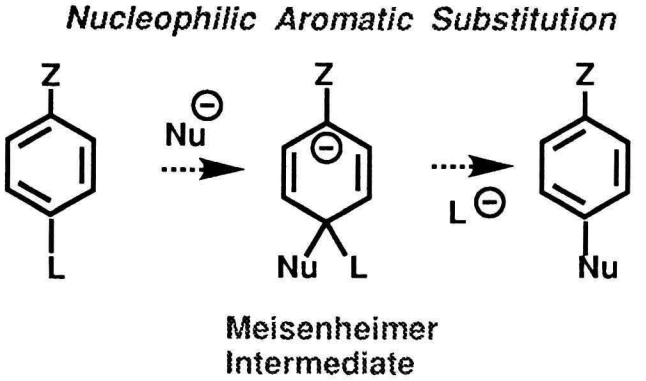
Inhibitor	K_i (μM)
4-HBA-CoA	2.5
BA-CoA	70
CoA	140
4-HBA-Pantothenate	>2000
ADP, 5′-AMP, Ade, PP_i	>5000
3′-AMP	1200

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.

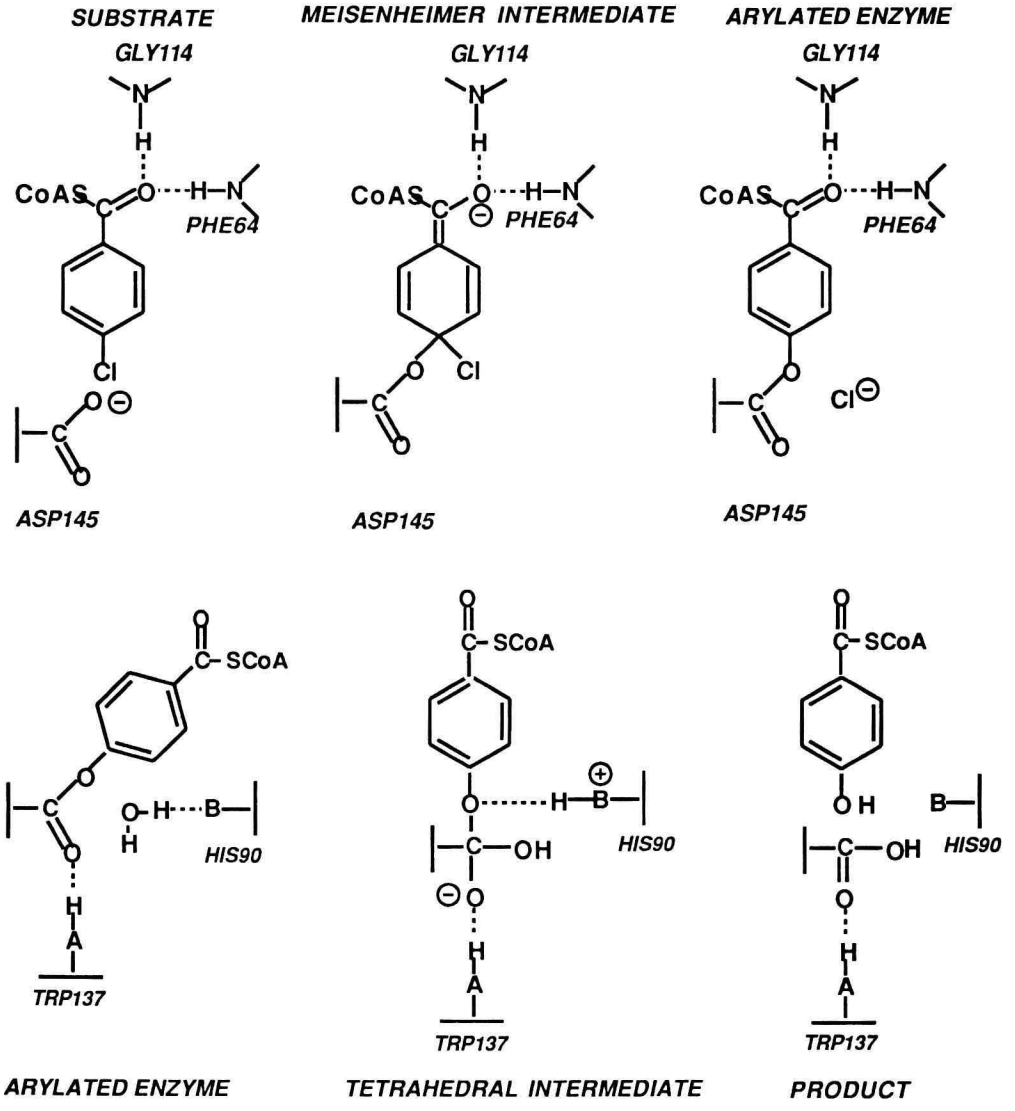
Presteady state kinetic studies of the dehalogenase reaction carried out with equivalent amounts of [^{14}C]4-CBA-CoA and enzyme in a rapid quench apparatus allowed us to detect the formation of a covalent adduct formed



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

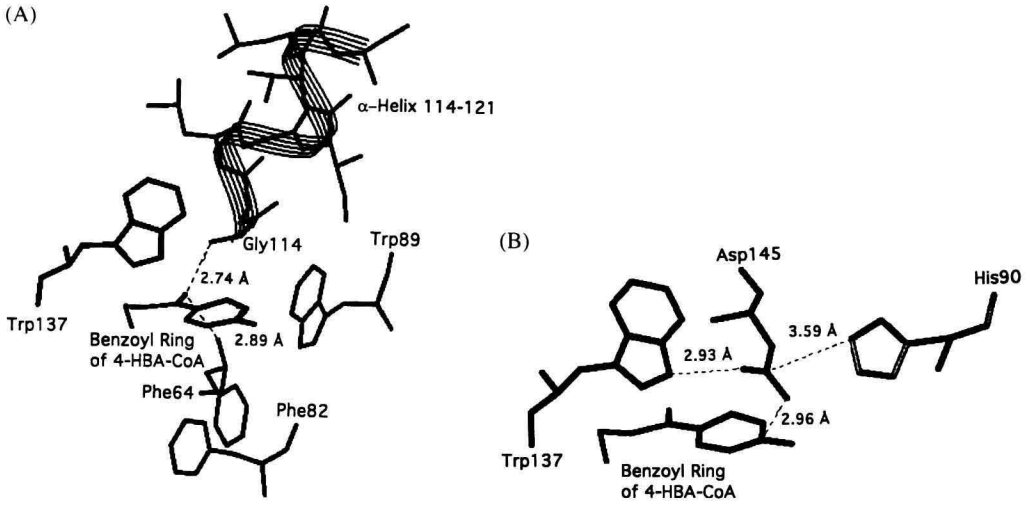


Fig. 1. A picture of the 4-CBA-CoA dehalogenase active site derived from the X-ray crystal stucture of the 4-HBA-CoA-dehalogenase complex (Benning, 1996) showing (A) the catalytic residues involved in the formation/stabilization of the Meisenheimer intermediate and (B) the three catalytic residues involved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

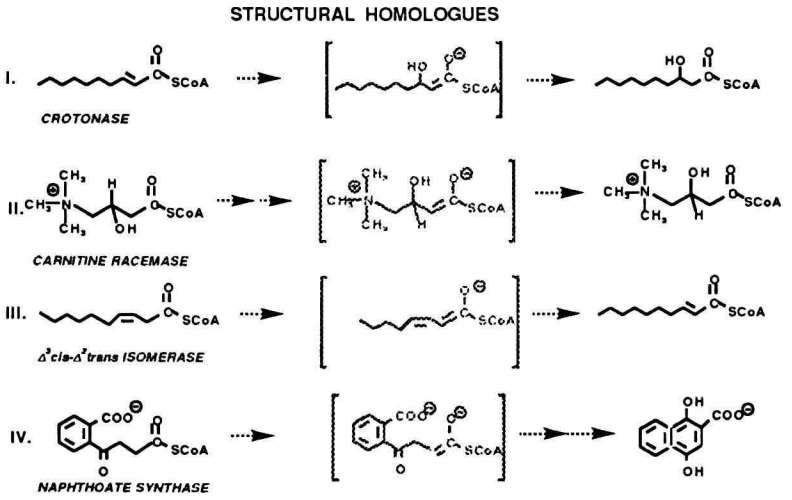
The second half of the dehalogenase reaction involves hydrolysis of the arylated enzyme. The ^{18}O labeling studies described above showed that the water adds to the acyl C = O (Yang, 1994). In Scheme 3 we represent this process as a classical two-step ester hydrolysis reaction. The solvent deuterium 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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