Peroxisomal Protein Import

Introduction

Several important facts were elucidated during the last few years that contribute considerably to our present understanding of the mechanisms of peroxisomal protein import. There are still many unknown details, however, a rough outline of the process has become clear. All experimental evidence indicates that import into peroxisomes occurs posttranslationally [24] and with that respect resembles the import into mitochondria [12, 8], chloroplasts [4] and the cell nuclei [17] rather than the endoplasmic reticulum where most proteins are imported cotranslationally [31]. The newly synthesized peroxisomal polypeptides, as a rule, have the same size as the mature proteins and thus contain uncleavable targeting signals within their polypeptide chain. So far two exceptions are known, 3-ketoacyl-CoA thiolase and the sterol carrier protein 2 which both are made as larger precursors [15, 28]. In the case of 3-ketoacyl-CoA thiolase the peroxisomal targeting signal 2 (PTS2) has been confined to 11 amino acids of the 26 amino acid long N-terminal presequence [40] which is split off after import by the activity of a thiolase processing peptidase. Most other peroxisomal matrix proteins are targeted to peroxisomes by a consensus tripeptide signal consisting of the amino acids SKL or an SKL-related motif [39] called peroxisomal targeting signal 1 (PTS 1). These targeting signals are sufficient to direct the corresponding proteins to peroxisomes.

According to a posttranslational import mechanism, peroxisomal proteins are released into the cytosol before they are taken up by the target organelle. There are several experimental indications that cytosolic factors are involved in the import [39–36], although at present the nature of the cytosolic components is not clear. Insertion of membrane proteins into the membrane and translocation of matrix proteins into the lumen of peroxisomes most likely are initiated by binding of the targeting signal of the precursor proteins to specific receptors present on the surface of the organelles. McCollum et al. [26] recently reported on the existence of such a receptor. The authors characterized the P. pastoris PAS8 gene product as an SKL-binding protein that is located to the peroxisomal membrane.

Several peroxisomal membrane components were recently identified to play an important role in peroxisome assembly. These are the gene products of PAF1 [41] and XALD [29] from rat liver and human skin fibroblasts, respectively,

and PAS3 from S. cerevisiae [16]. The latter has been identified as a 48kD integral peroxisomal membrane protein with yet unknown function. PAF1p is of rather low abundance and contains a zink-finger-like motif that is also found in the gene products of PAS4 and PAS5 from S. cerevisae [5]. PAF1 mutant cells closely resemble fibroblasts from patients with the autosomal recessive, peroxisome-defective disorder, Zellweger syndrome [33]. Peroxisome biogenesis in the mutant cells as well as in a fibroblast cell line from a Zellweger patient [34] was restored by PAF1 complementation.

The analysis of the specific function in protein import of all these components involved in peroxisome assembly is best carried out by in vitro import systems. Lazarow and coworkers [18] were able to demonstrate that in vitro import of peroxisomal acyl-CoA oxidase into isolated rat liver peroxisomes requires energy in the form of ATP but no membrane potential, and Fujiki et al. [27] used this system to study the in vitro uptake of expression products of various acyl-CoA oxidase constructs in order to characterize the targeting signal of this protein. Using a rather similar approach Behari and Baker [2] recently reported on the in vitro import of isocitrate lyase into glyoxysomes isolated from sunflower cotyledons.

Starting with experiments in which peroxisomal reporter proteins were introduced into the cell by microinjection [38] we searched for alternative systems which could be used to study the mechanisms of peroxisomal protein import [32]. In this chapter we describe our first experiences with the different systems worked out. Independent from our efforts, Subramani and coworkers have explored rather similar experimental systems [39, 43, 44].

1. Peroxisomal protein import by microinjection

Introduction of the marker by microinjection offers the possibility to analyze the import process under rather physiological conditions, comparable to those in vivo. A disadvantage of the system might be that its external manipulation is limited by the presence of an intact plasma membrane which allows access to the peroxisomal membrane of only a few types of molecules. With this system we investigated the import of firefly luciferase (FL) into peroxisomes of CHO cells and analyzed the time, temperature, energy and membrane potential dependence of this import [38]. We used FL as an import marker since its import into peroxisomes of cells transfected with the luciferase gene has been demonstrated [22], and since it is normally not present in peroxisomes of CHO cells and therefore its import can be documented simply by the immunofluorescent colocalization with the endogenous peroxisomal marker catalase (Fig. 1).

The true intraperoxisomal localization of the microinjected FL is demonstrated by a differential permeabilization of the cellular membranes by Triton X-100 and digitonin. The use of Triton X-100 in the immunofluorescence protocol permeabilizes all cellular membranes, including the peroxisomal mem-

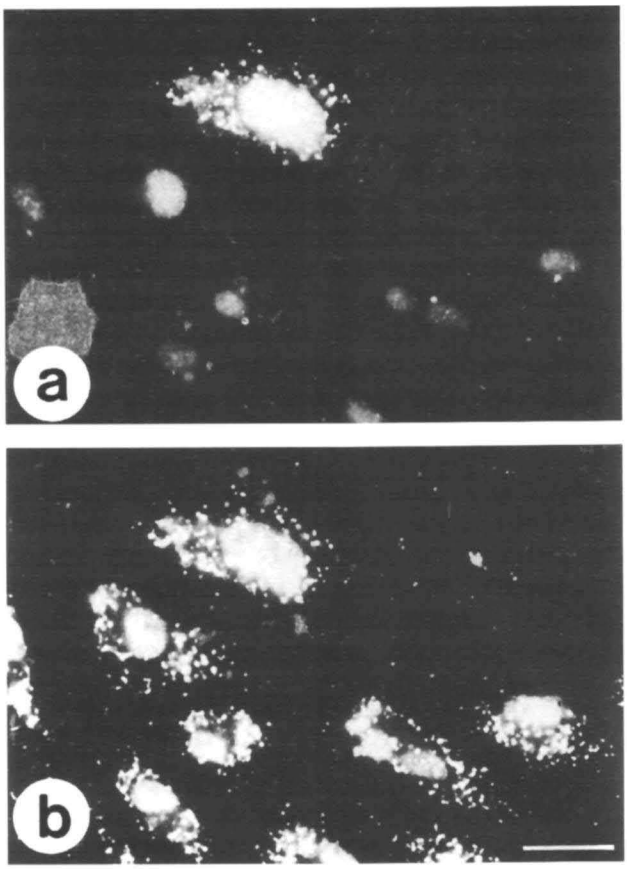


Fig. 1. Colocalization of microinjected FL (a) with endogenous catalase (b) in CHO wild-type cells by double immunofluorescence. FL was visualized by a monospecific polyclonal rabbit anti-FL antibody which was reacted with a TRITC-labeled goat anti-rabbit IgG antibody. Catalase was stained by a monospecific polyclonal mouse anti-catalase antibody which was recognized by a FITC-labeled goat anti-mouse IgG antibody. One microinjected cell bearing FL is shown in (a) whereas all cells are positively stained for endogenous catalase in (b). Bar, 20 μm. From ref. 38 with permission.

brane, and makes the intraorganellar antigens available for the antibodies. Under these conditions both catalase and the imported FL are recognized by their punctate immunofluorescence (Fig. 2). Low concentrations of digitonin, on the other hand, permeabilize the plasma membrane but leave the peroxisomal membrane impermeable for the antibody. Under these conditions peroxisomes are stained by neither the catalase nor the FL antibodies, however, a punctate immunofluorescence pattern of peroxisomes is obtained using antibodies directed against a peroxisomal surface antigen such as PMP69 [10, 21]. The differential permeabilization produced by digitonin thus easily allows to discriminate by immunofluorescence between proteins solely attached to membranes and those that were imported [38].

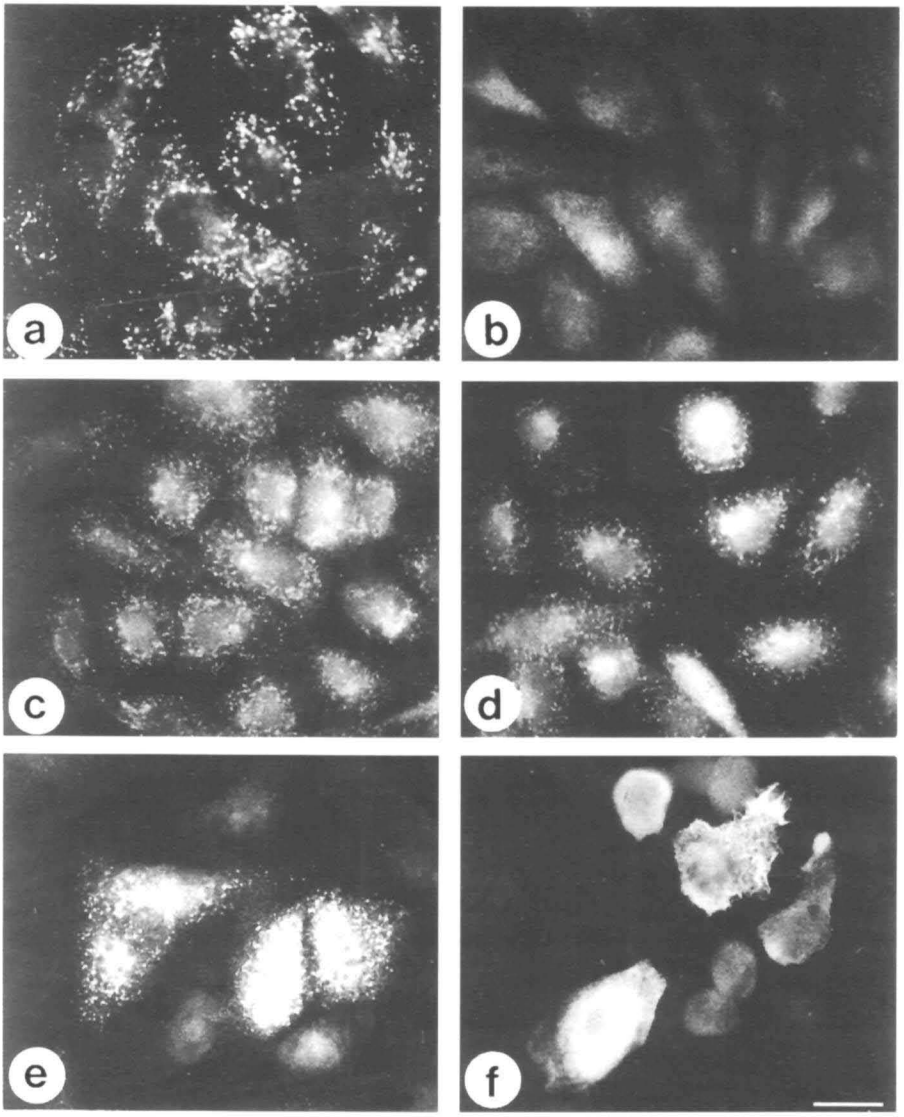


Fig. 2. Demonstration of intraperoxisomal localization of microinjected and imported FL in CHO wild-type cells. Endogenous catalase, which serves as a marker for the peroxisomal matrix, is only detected when all cellular membranes are permeabilized with Triton X-100 (a), but is not visualized by permeabilizing the plasma membrane with digitonin (b). PMP 69, a peroxisomal membrane marker, a portion of which extends into the cytosol, is recognized by the antibody under conditions of both Triton X-100 (c) and digitonin (d) permeabilization. FL microinjected into the cytosol, like catalase, gives a punctate immunofluorescence staining only after Triton X-100 (e), but not after digitonin (f) permeabilization, suggesting that a punctate immunofluorescence reflects its intraperoxisomal localization. In all cases the FITC-labeled goat anti-rabbit IgG antibody was used to detect the first antibody. Bar, 20 μm. From ref. 38 with permission.

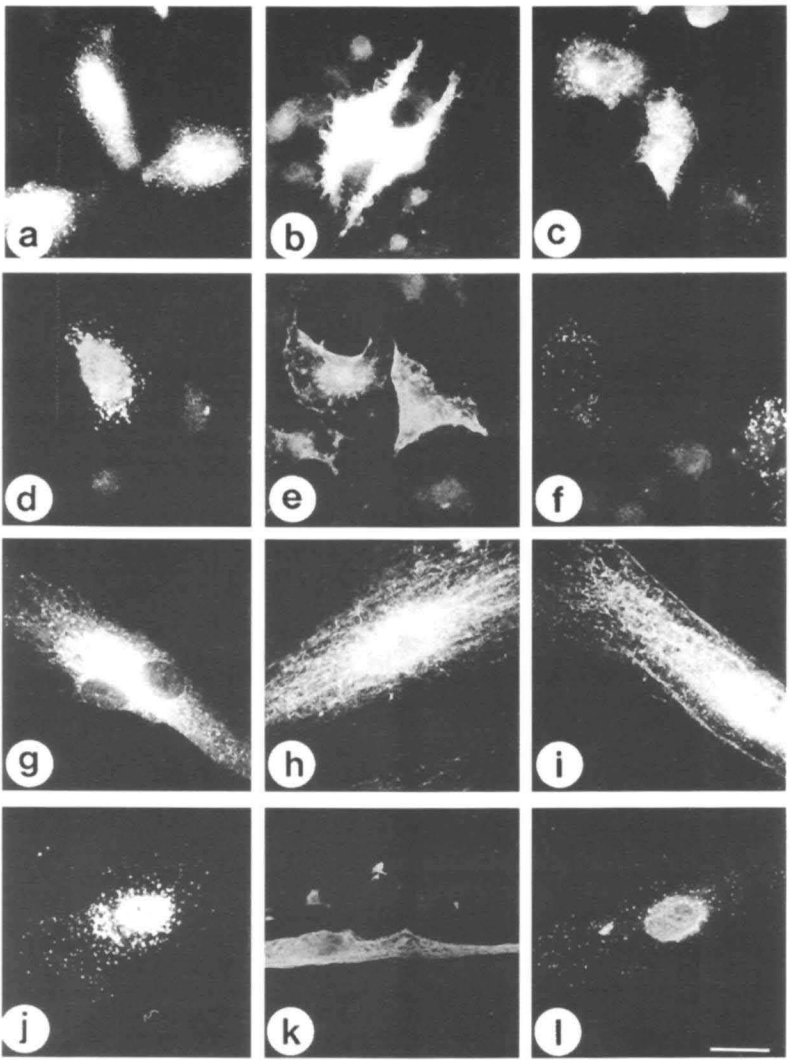


Fig. 3. NTP dependence of FL import into CHO wild-type cells (a–f) and human skin fibroblasts (g–l). Control cells (a, d, g, j) were kept in normal medium, NTP-depleted cells (b, e, h, k) in glucose-free medium containing the inhibitors 2-deoxyglucose (6 mM) and NaN3 (10 mM), and reenergized cells (c, f, i, l) in normal medium after washing out the inhibitors. The experiments were conducted by the following schedule. Cells were depleted of NTPs by a 15-min preincubation at 37° C in glucose-free medium containing the inhibitors prior to microinjection. Control cells and NTP-depleted cells were incubated for 60–90 min at 37° C after microinjection to allow FL import. To study FL import after reenergetisation, cells were depleted on NTPs for 15 min, followed by microinjection and continued incubation under conditions of NTP depletion for 60 min. After this time, the cells were transferred to normal medium and incubated for additional 2 h to allow FL import. The concentration of microinjected FL for these experiments was 0.6 mg/ml. Cells were processed for immunofluorescence either by the standard protocol, including Triton X-100 permeabilization (a–c, g–i), or by preferential permeabilization of the plasma membrane with digitonin (d–f, j–l) prior to fixation, Triton X-100 permeabilization, and immunofluorescence staining. Bar, 20 μm. From ref. 38 with permission.

The import of FL was dependent on time and temperature and could be demonstrated as early as 15–30 min after microinjection. The imported enzyme was still visible after 5–6 days. Import was observed within a broad temperature range between 16–37° C, was optimal at 26° C and did not occur at temperatures of 14° C and lower [38].

The energy requirements for FL import were studied in both CHO cells and human skin fibroblasts by lowering the level of intracellular NTPs by more than 90% using the metabolic inhibitors NaN3 and 2-deoxyglucose [38]. FL import was largely abolished under these conditions but was restored upon removal of the inhibitors suggesting the need of NTPs, most likely that of ATP and/or GTP (Fig. 3). However, no decision could be made by this type of experiment which nucleoside triphosphate is actually involved or in which step of the import process the energy is consumed.

Nevertheless important findings were made by these microinjection experiments which demonstrated that a mature, completely folded and enzymatically active protein can be imported in an energy dependent manner. No indication for the need of a functional membrane potential were observed. The possibility to import mature FL means that peroxisomal matrix proteins either are to be translocated in their mature forms, or converted into an import competent conformation prior to their translocation. Up to now there is no experimental evidence that would favour one of these alternatives.

2. Peroxisomal protein import in the permeabilized cell system

After selective permeabilization of the plasma membrane by the bacterial toxin streptolysin O (SLO) peroxisomal matrix as well as membrane proteins are efficiently imported into peroxisomes [2,33]. Protein translocation into the matrix again was studied in CHO cells with FL as the import marker. Import was demonstrated by the immunofluorescent colocalization of FL with catalase, the marker for the peroxisomal compartment (Fig. 4). FL import under these conditions was both time-dependent linearly for up to 30–45 min and temperature-dependent with no import below 14° C and optimal import at 26° C [32].

Since the cell permeabilization results in a considerable decrease of the level of cellular ATP, FL import has to be carried out in the presence of added ATP and an ATP-regenerating system. The need for this exogenous ATP offered the possibility to study the energy requirements of the import in more detail than by the microinjection studies. ATP alone at 2 mM still promoted import. However, omission of both ATP and the energy regenerating system completely abolished it (Fig. 5). ATP alone or in the presence of GTP-γ-S, a nonhydrolysable GTP analog, allowed import, whereas GTP alone or in the presence ATP-γ-S, a nonhydrolyzable ATP analog, was unable to do so. From these data we concluded that FL import is strictly energy dependent, that ATP-hydrolysis is the principle source of energy, and that the presence and hydrolysis of GTP is of no particular importance in the overall import process.

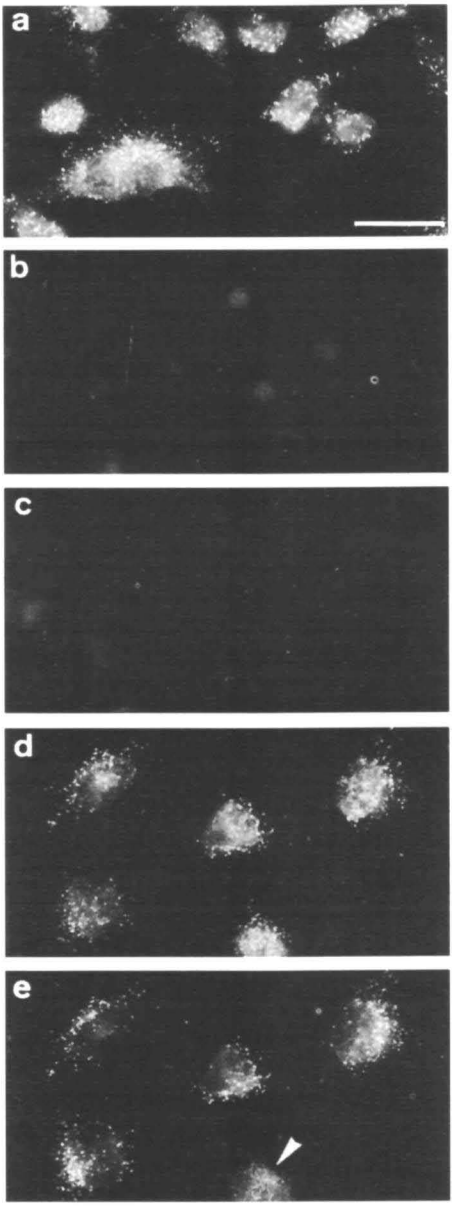


Fig. 4. Import of FL into peroxisomes of SLO-permeabilized CHO cells. SLO-permeabilized CHO cells were incubated under standard conditions at 26° C in the presence of 1 μg FL for 60 min. Following immunostaining of the cells with anti-FL antiserum, FL reveals a punctate fluorescence pattern, suggesting its intraparticulate localization (a). Omission of FL from the import mix (b) and of the Trition X-100 permeabilization step from the immunostaining protocol (c) abolishes the appearance of the punctate staining. Cells stained for both catalase (d) and FL (e) by double immunofluorescence show colocalization of both enzymes, suggesting import of FL into peroxisomes. Note that the cell in (e) marked with an arrowhead reveals weak FL immunostaining due to insufficient SLO permeabilization. Bar, 25 μm. From ref. 32 with permission.

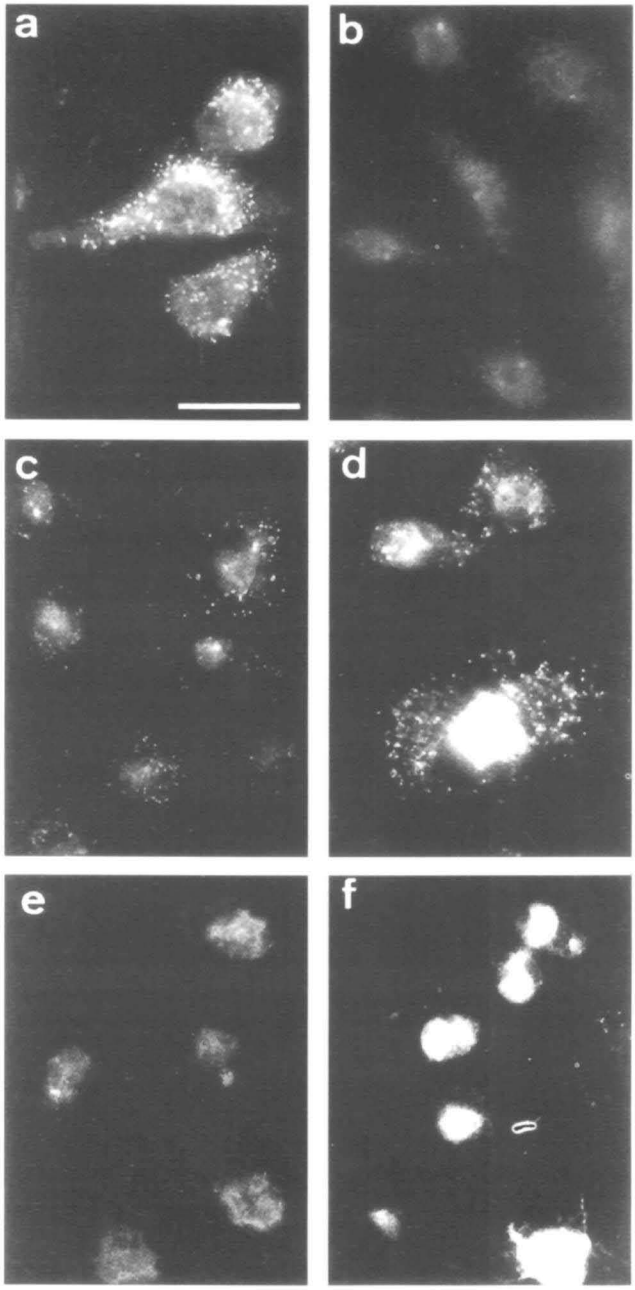


Fig. 5. Energy dependence of FL import. Under standard conditions for FL import, both nucleotides ATP and GTP are contained in the import mix together with an ATP-regenerating system (a). Omission of the NTPs and the ATP-regenerating system from the import mix completely abolished FL import (b). ATP in a final concentration in the import mix of 2 mM (c) and 6 mM (d) and in the absence of the ATP-regenerating system promotes import. Both GTP (e) and ATP-γ-S (f) in final concentrations of 6 and 10 mM, respectively, were unfavourable, and no import was observed under these conditions. Bar, 25 μm. From ref. 32 with permission.

At which step in the overall import is ATP required? Since FL, the import substrate in these experiments, is a mature, completely folded molecule, its unfolding prior to import might be prerequisite. Recently a TCP-1 ring complex (TRiC) which resembles the GroEL double ring has been demonstrated to mediate FL folding [7]. However, this complex seems to be active cotranslationally on the nascent polypeptide chain and may not be involved in FL import [13]. Thus unfolding of mature FL before import into peroxisomes may be

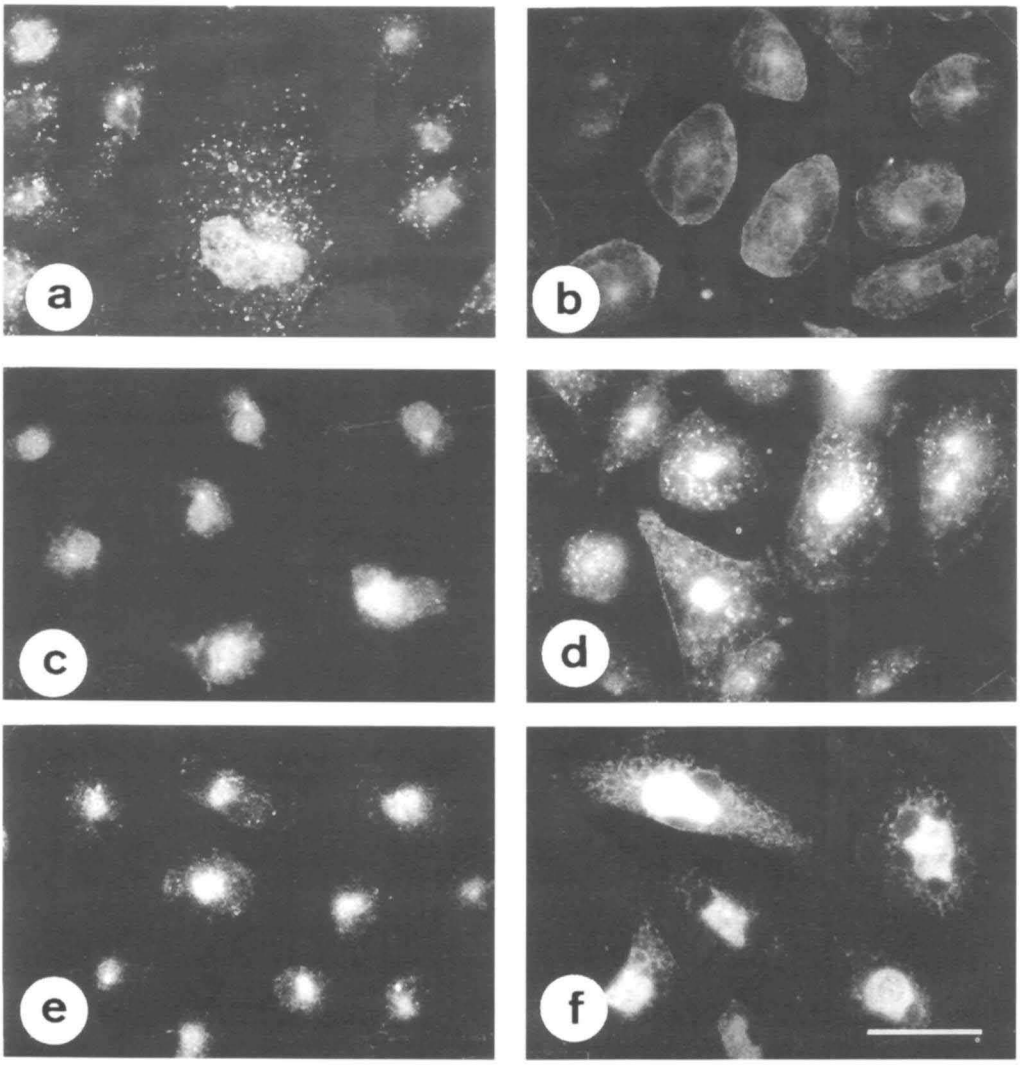


Fig. 6. Import of FL in permeabilized CHO cells (a). Inhibition by RNase A (10 Kunitz units, b), dependence of cytosol (c, d) and inhibition by NEM (10 μM, e; 100 μM, f). Cytosol dependence of import was analyzed after depleting the cells on cytosol by washing with buffer (e) and reconstituting the import by adding CHO cytosol to the cytosol depleted cells (f). Bar, 25 μm.

catalysed by cytosolic chaperones other than TRiC or may occur during the peroxisomal translocation process. For that reason we investigated the requirement of cytosol in FL import, and found that FL import is strictly dependent on the presence of cytosol (Fig. 6) and that a proteinaceous factor is responsible for this activity [36, see also ref. 44]. In cytosol depleted CHO cells we could restore import by the readdition of either CHO cytosol or rabbit reticulocyte lysate. These observations are favourable preconditions which we hope will allow us to characterize possible cytosolic components required for import.

With the assumption that one of the cytosolic components mediating import may be a ribonucleic acid-protein complex, the cytosol used for reconstitution of import was pretreated with RNase A. This treatment completely abolished import (Fig. 6). However, the use of RNase S-protein which is a protease modified RNase A devoid of any RNase activity also prevented import. Thus, the mechanism by which RNase A interferes with FL import might be based on the interaction of RNase A with the import apparatus rather than on its enzymatic activity [36]. Several sulfhydryl reactive reagents including NEM also blocked import (Fig. 6). Further analysis of this observation revealed that only treatment of the cytosol-depleted CHO cells but not of the cytosol itself caused the inhibition [see also ref. 44]. Thus, free sulfhydryl groups are actively involved in the import process and the components carrying them most likely are located in the peroxisomal membrane.

3. Insertion of PMPs into isolated peroxisomes

The insertion of integral membrane proteins into membranes of different subcellular origin has been studied with great detail and several underlying

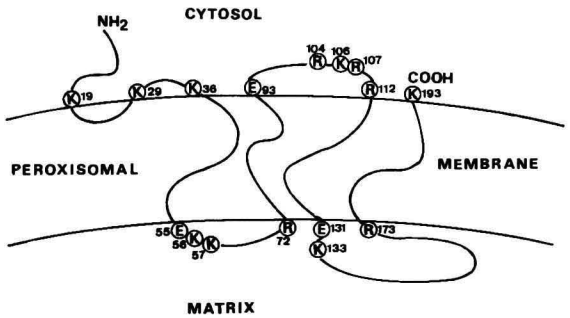


Fig. 7. Hypothetical topology of PMP 22 in the peroxisomal membrane. The model is distinguished by the presence of 4 hydrophobic transmembrane segments spanning the peroxisomal membrane and the orientation of both the amino- and the carboxy-terminus to the cytosol. Between Lys19 and Lys29 there is a hydrophobic stretch of 9 amino acids which might be loosely associated with the bilayer. Polar amino acid residues are limiting the transmembrane segments. Most interestingly the positively charged amino acids lysine (K) or arginine (R) and the negatively charged amino acid glutamic acid (E) are alternately flanking the membrane spanning segments. According to the model one loop of the polypeptide chain between Glu93 and Arg112 faces the cytosol and contains additional positively charged residues (Arg104, Lys106, Arg107) permitting tryptic attack. From ref. 20 with permission.

mechanisms have been proposed [23,35]. However, nothing was known on the transfer of peroxisomal membrane components into peroxisomal membranes. We therefore cloned and sequenced the cDNA of PMP22, the major peroxisomal membrane protein in the liver of uninduced rats, and established its membrane topology [20]. Hydropathy analysis of the predicted amino acid sequence revealed 4 transmembrane spanning regions each composed of approximately 20 hydrophobic residues flanked by charged ones. Both the N- and the C-terminus of the protein face the cytosol (Fig. 7). Thus, the putative membrane topology of PMP22 based on the nomenclature of Singer [35] is of type IIIa which designates polytopic molecules with more than one transmembrane stretch having their NH2-termini exposed on the cytoplasmic side.

In order to study in vitro the insertion of PMP22 the in vitro translated polypeptide was incubated with isolated peroxisomes purified by differential and isopycnic centrifugation [11,37]. After a 1 h incubation approximately 75% of total in vitro translated PMP22 was pelletable together with the organelles. Since the membrane integrated form of PMP22 is practically not susceptible to exogenously added subtilisin, protease treatment of the pelleted organelles was used as a suitable criterium in order to discriminate between membrane attached and membrane integrated PMP22. About 40–50% of pelletable PMP22, i.e. 30–35% of the total synthesized, resisted the protease digestion and is considered to be inserted into the membrane indistinguishable from the mature PMP22 [3], (Fig. 8).

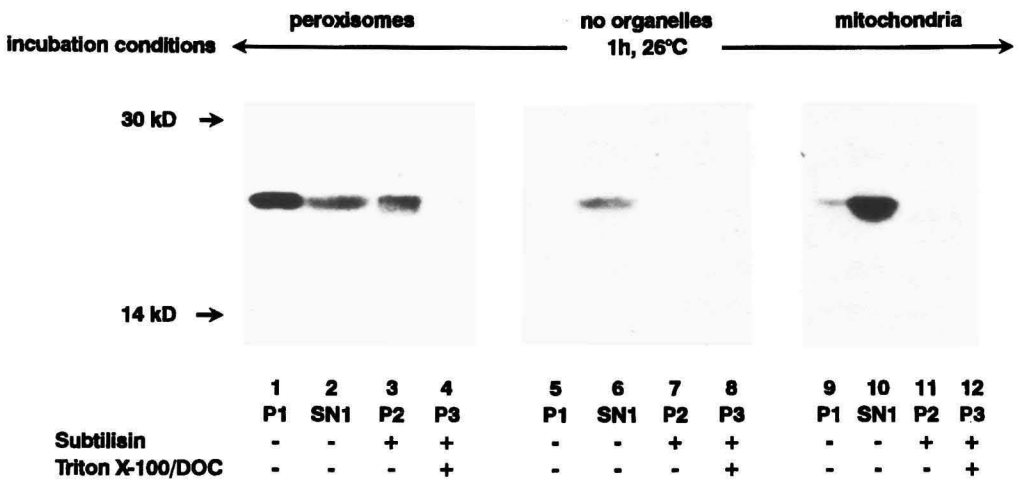


Fig. 8. Posttranslational insertion of PMP 22 into the membrane of rat liver peroxisomes. The in vitro translation product of PMP 22 mRNA obtained in the nucleasetreated rabbit reticulocyte lysate system was incubated for 1 h with peroxisomes (200 μg) isolated from the liver of a Clofibrate-treated male rat (lanes 1–4), without organelles (lanes 5–8) and with isolated rat liver mitochondria (200 μg, lanes 9–12). After 1 h each incubation assay was divided into three aliquots and the organelles (P) were separated from the supernatant (SN) by centrifugation at 13,000 g for 10 min. Pellets remained untreated (−) or were treated (+) with subtilisin (7 μg) at 0° C for 30 min either in the absence (−) of presence (+) of Triton X-100/deoxycholate (each 1%). The arrows at the left indicate the molecular weight standards carboanhydrase (30 kD) and lysozyme (14 kD). From ref. 3 with permission.

The amount of inserted polypeptide increased linearly for about 30 min and was strongly dependent on temperature. Insertion was optimal at 26° C whereas at 0° C the pelletable PMP22 decreased to about 40% and was completely digested by the protease. However, when the pelleted organelles preincubated at 0° C were resuspended and further incubated at 26° C, a considerable proportion of pelletable PMP22 became protease resistant. Thus, two steps of the transfer of PMP22 to peroxisomes, the binding to and the insertion into the membrane, were clearly distinguished [3].

Is the PMP22 insertion energy dependent? The translation mixture after synthesis of PMP22 and prior to its incubation with peroxisomes was depleted of ATP and GTP by apyrase. In vitro insertion assays were also run in the presence of ATP-γ-S and GTP-γ-S, and in permeabilized hepatocytes instead of isolated peroxisomes (Fig. 9). All these experiments revealed that neither ATP nor GTP is required for the insertion of PMP22 into the peroxisomal membrane [3]. Likewise in contrast to the translocation of FL which was inhibited by rather low concentrations of NEM (see above), NEM was without effect on the insertion of PMP22. Therefore, these data strongly suggest that translocation of FL and membrane insertion of PMP22 are mediated by distinct mechanisms.

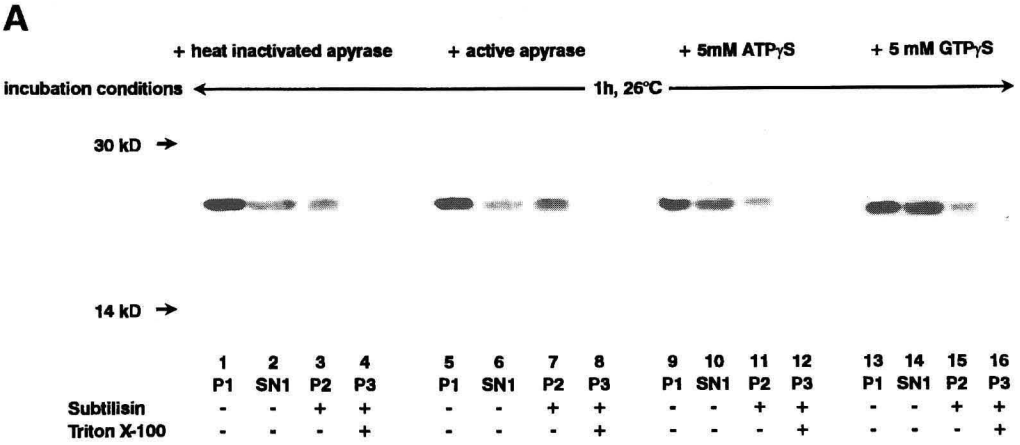
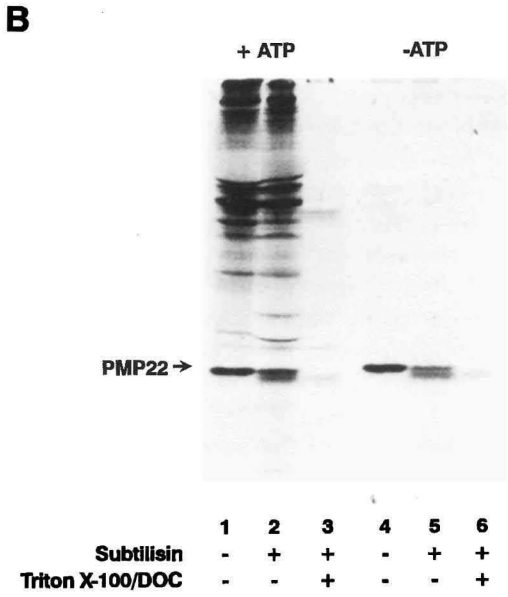


Fig. 9. Energy requirement of PMP 22 insertion. (A) Isolated peroxisomes (200 μg) were incubated with the PMP 22 mRNA translation mixture which was pretreated either with heat inactivated apyrase (2 I.U./50 μl) for 15 min at 30° C and 15 min at room temperature as a control (lanes 1–4) or with the same amount of enzymatically active apyrase (lanes 5–8) in order to deplete the translation mixture of ATP. Peroxisomes were also incubated with PMP 22 mRNA translation product containing the non-hydrolyzable nucleotide analogues ATP-γ-S (5 mM, lanes 9–12) and GTP-γ-S (5 mM, lanes 13–16). Subsequent pelleting of peroxisomes by centrifugation (P) and protease treatment in the absence (−) or presence (+) of detergents was carried out exactly as described in the legends to figure 8. The arrows at the left mark the molecular weight standards. (B) Isolated permeabilized hepatocytes ($1x10^6$ cells) were incubated for 1 h at room temperature with PMP 22 mRNA translation product treated with heat inactivated apyrase (+ATP, lanes 1–3) and with enzymatically active apyrase (−ATP, lanes 4–6). Insertion of PMP 22was analyzed in postnuclear supernatants of the cells by protease treatment as described in A. Note that the incubation of the permeabilized cells with energized translation mixture (lanes 1–3) still containing [35S]methionine leads to the translation of many endogenous mRNA species. From ref. 3 with permission.



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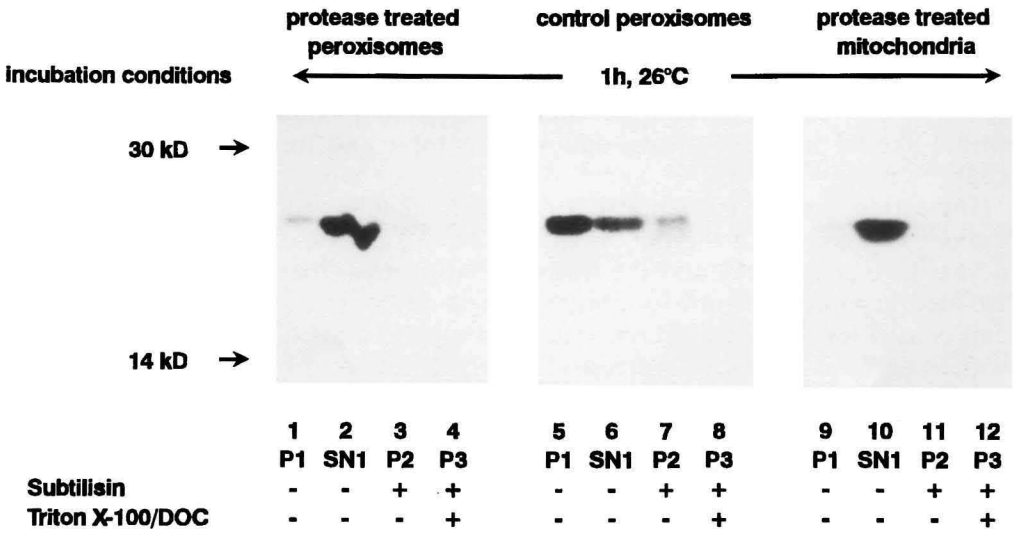

Fig. 10. Attempts to insert PMP 22 into protease pretreated peroxisomes and mitochondria. Peroxisomes (200 μg, lanes 1–4) and mitochondria (200 μg lanes 9–12) were pretreated with subtilisin (20 μg in a volume of 20 μl at 0° C for 10 min before they were used for the insertion experiment. Subtilisin digestion was stopped by the addition of PMSF (10 mM) and the organelles were recovered by centrifugation at 13,000 g for 5 min. The corresponding control experiment was carried out with PMSF-deactivated subtilisin and is shown in lanes 5–8. At the end of the incubation (1 h) organelles were again divided into three aliquots and analyzed for the protease resistant insertion of PMP 22 in the presence (+) and absence (−) of detergent. In lanes 2, 6, and 10 the supernatant (SN) of one aliquot was analyzed. The arrows at the left mark the molecular weight standards. From ref. 3 with permission.

As mentioned above, FL is targeted to peroxisomes by means of the tripeptide targeting signal SKL located at the C-terminal end of the polypeptide that is recognized by a specific receptor. Analogous to the matrix proteins, membrane proteins may also carry distinct targeting signals other than SKL for their specific insertion into peroxisomes, although none of them has been identified so far. Accordingly these targeting signals may be recognized by specific receptors. First indications for the existence of a proteinaceous receptor mediating the insertion of PMP22 were based on the fact that protease pretreatment of peroxisomes completely abolished its insertion (Fig. 10). The observation that PMP22 at low temperature binds to the peroxisomal membrane without being inserted may facilitate the identification of this receptor polypeptide [29].

4. Homogeneity vs heterogeneity of the peroxisomal compartment

The current model of peroxisome biogenesis suggested by Lazarow and Fujiki in 1985 [24] predicts firstly that newly synthesized peroxisomal proteins are imported posttranslationally, and secondly that new peroxisomes are formed by segregation from preexisting ones. Besides a heterogeneity in peroxisome size, this model argues for a complete homogeneity of the population with respect to its matrix and membrane composition and function. However, within the last years several reports appeared that indicate a greater heterogeneity within the peroxisomal compartment [33]. We will not discuss these reports here in detail but would like to emphasize that for most of them further experimental work is required in order to substantiate their conclusions [1,6,25,30,42]. Here we would like to present our own data on this topic and to discuss their pros and cons.

Indications for the existence of a peroxisomal subpopulation in rat liver were obtained by following the import in vivo of two peroxisomal matrix proteins, acyl-CoA oxidase [26] and the trifunctional protein, that both carry the PTS1. Isolated hepatocytes from Clofibrate-treated rats were pulse labeled for 7.5 min and chased for up to 1 h. The postnuclear supernatants of the cells were treated with exogenous protease to digest all newly synthesized unimported proteins and were separated on a Nycodenz density gradient. The subcellular distribution of the newly synthesized imported proteins was determined by immunoprecipitation. Both proteins studied in this system principally behaved similar in that after the pulse they were detected in higher concentration in fractions of intermediate density, clearly separated from the major peroxisomal population, and only after the chase they were distributed like their mature copies (Fig. 11). We have interpreted this observation as an indication for the existence of a peroxisomal subpopulation especially competent for the import of newly synthesized polypeptides. It is known from the work of Gorgas [9] that the peroxisomal compartment appears as a rather complex morphological structure which upon homogenization may give rise to various peroxisomal populations. Within the cell, the subpopulation identified by the pulse labeling experiments

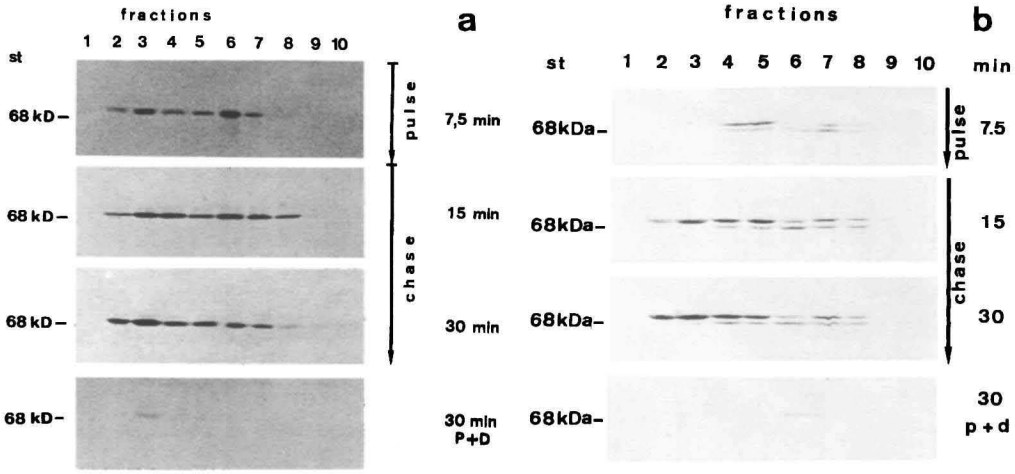

Fig. 11. Distribution of newly synthesized acyl-CoA oxidase (a) and peroxisomal trifunctional protein (b) in 14–45% Nycodenz density gradients after pulse labeling of isolated hepatocytes of a Clofibrate treated rat for 7.5 min with 35S-methionine followed by a 15 and 30 min chase in the presence of 2 mM unlabeled amino acid. After labeling the postnuclear supernatant of the cell homogenate was centrifuged and an aliquot of each fraction was treated with subtilisin in order to digest nonimported protein. Another aliquote of the 30 min chase was also treated with subtilisin plus Triton X-100/deoxycholate (each 1%, p + d) in order to digest imported and nonimported material. The proteins were isolated by immunoprecipitation and visualized by SDS-PAGE and fluorography.

may be part of the peroxisomal reticulum but may represent an import active section at which newly synthesized polypeptides enter before they are distributed along the organelle. The major portion of the compartment that represents the major population which is distinguished by its well known high equilibrium density may still possess some import competence but its main function may be peroxisomal substrate metabolism. Although this model sounds attractive because it fits with several observations made by others, at this time its experimental evidence entirely relies on the criterium of protease resistance to distinguish imported from unimported molecules. Therefore, it will be necessary to provide additional evidence for the existence of peroxisomal subpopulations, to isolate them and to demonstrate biochemical differences to the main population.

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