In Vitro Insertion of the 22-kD Peroxisomal Membrane Protein into Isolated Rat Liver Peroxisomes

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Abstract. The membrane insertion of the 22-kD integral peroxisomal membrane protein (PMP 22) was studied in a system in which peroxisomes isolated from rat liver were incubated with the [^{35}S]methionine-labeled in vitro translation product of PMP 22 mRNA. Membrane insertion of PMP 22 was demonstrated by protease treatment of peroxisomes in the absence and presence of detergent. Approximately 35% of total in vitro translated PMP 22 became protease resistant after a 1-h incubation at 26°C. Import was dependent on time and temperature, did not require ATP or GTP and was not inhibited by *N*-ethylmaleimide treatment of neither the soluble components of the translation mixture nor of the isolated peroxisomes. In contrast to these results it was recently shown that the import of the peroxisomal marker, firefly luciferase, into peroxisomes of permeabilized cells was dependent on ATP hydrolysis and was blocked by *N*-ethylmaleimide pretreatment of the cytosol-depleted cells (Rapp et al., 1993; Wendland and Subramani, 1993). Therefore, the present data suggest that insertion of PMP 22 into the peroxisomal membrane and translocation of firefly luciferase into peroxisomes follow distinct mechanisms.

At low temperature binding of PMP 22 to the peroxisomal membrane was not influenced whereas insertion was strongly inhibited. Pretreatment of peroxisomes with subtilisin reduced binding to a low level and completely abolished insertion. Therefore it is suggested that binding is prerequisite to insertion and that insertion may be mediated by a proteinaceous receptor.

In 1978 Goldman and Blobel, and Robbi and Lazarow described the synthesis of catalase and urate oxidase on free polyribosomes indicating that the import of these peroxisomal markers proceeds posttranslationally without the implication of the ER. Within the last years several other peroxisomal matrix and membrane proteins were studied and it was found that all of them follow this route of biosynthesis (Fujiki et al., 1984; Miura et al., 1984; Suzuki et al., 1987). There is only one report in the recent literature that a peroxisomal membrane protein (PMP)[1] with apparent molecular mass of 50,000 (PMP 50) may be synthesized on bound polysomes (Bodnar and Rachubinski, 1991). According to the current model of peroxisomal biogenesis the peroxisomal polypeptides after being synthesized are released into the cytosol and subsequently imported into preexisting peroxisomes (for reviews see Lazarow and Fujiki, 1985; Borst, 1989; Just and Soto, 1992). Targeting of some of the matrix proteins is mediated by a consensus targeting signal consisting of SKL or a SKL-related motif (Gould et al., 1988, 1989). 3-Ketoacyl-CoA thiolase lacking the COOH-terminal SKL contains a targeting signal which resides within its NH_2-terminal presequence (Swinkels et al., 1991; Osumi et al., 1991) as it is known for many mitochondrial proteins (for reviews: Verner and Schatz, 1988; Hartl et al., 1989).

The rat liver peroxisomal membrane contains several major proteins (Fujiki et al., 1982; Hashimoto et al., 1986; Hartl and Just, 1987) the functions of which are largely unknown. Their identification as integral membrane constituents is based mainly on the isolation of highly purified peroxisomes, the preparation of their membranes by the carbonate procedure (Fujiki et al., 1982) and analysis by SDS-gel electrophoresis. By treatment of the animals with hypolipidemic drugs which cause peroxisome proliferation in liver, some membrane proteins like PMP 69 and PMP 26 become induced. PMP 69 was recently cloned and sequenced (Kamijo et al., 1989) and found to belong to the class of ATP-binding cassette proteins conferring multidrug resistance to tumor cells. The major constituent of the peroxisomal membrane in livers of uninduced rats is PMP 22. We recently cloned and sequenced the PMP 22 cDNA and deduced its amino acid sequence (Kaldi et al., 1993). PMP 22 belongs to the class of type IIIa integral membrane proteins (Singer, 1990) with four putative transmembrane spanning regions and the NH_2 and COOH terminus facing the cytosol. This hypo-

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1. *Abbreviations used in this paper*: ATP-γ-S, adenosin-5'-O-(3)-thiotriphosphate); GTP-γ-S, guanosin-5'-O-(3-thiotriphosphate); NEM, *N*-ethylmaleimide; PMP, peroxisomal membrane polypeptide; SLO, streptolysin O.

thetical membrane topology suggests a particular embedding of the protein within the peroxisomal membrane which is compatible with the observation that PMP 22 is hardly attacked by the treatment of isolated peroxisomes with proteinase K or subtilisin (Hartl and Just, 1987). It may be worthwhile to mention that the putative membrane topology of PMP 22 is strikingly similar to that of several other interesting membrane proteins such as Mpv 17 (Weiher et al., 1990) and certain transmitter gated ion channels (Schofield et al., 1987; Galzi et al., 1991; and for a review see Barnard, 1992) suggesting that these proteins share common mechanisms of membrane integration.

In the present paper we report on the first in vitro insertion of a peroxisomal membrane protein. The translation product of PMP 22 mRNA was inserted into peroxisomes isolated by Nycodenz density gradient centrifugation. The insertion was highly efficient and dependent on time and temperature. In contrast to the peroxisomal matrix marker firefly luciferase (Rapp et al., 1993; Wendland and Subramani, 1993), PMP 22 insertion did not require the hydrolysis of ATP or GTP and was not influenced by *N*-ethylmaleimide (NEM). The system worked out may be suitable to study particular aspects of PMP 22 insertion such as targeting or the requirement of cytosolic factors.

Materials and Methods

Materials

[^{35}S]Methionine (>1,000 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, FRG). Flexi™ rabbit reticulocyte lysate system was from Promega Biotec (Madison, WI). Antipain and leupeptin were from Bachem (Heidelberg, FRG) and Nycodenz from Immuno (Heidelberg, FRG). Subtilisin, αMEM, luciferin, luciferase, ATP, GTP creatin kinase, and Triton X-100 were purchased from Boehringer Mannheim (Mannheim, FRG) and PMSF, NEM, Adenosin-5′-O-(3-thiotriphosphate) (ATP-γ-S) Guanosin-5′-O-(3-thiotriphosphate), (GTP-γ-S), apyrase (ATPase activity 680 U/mg protein), and deoxycholate from Sigma (Deisenhofen, FRG).

In Vitro Transcription/Translation

The cDNA clone encoding PMP 22 was excised with EcoRI from the phage vector M13mp18 and inserted into the EcoRI site of the pBluescript vector (Stratagene, La Jolla, CA). In vitro transcription was performed as described previously (Kaldi et al., 1993). Cell free in vitro translation in the presence of [^{35}S]methionine was carried out in the rabbit reticulocyte lysate system (Pelham and Jackson, 1976), without further addition of potassium chloride. Usually, the translation mixture contained 2 μl in vitro transcribed mRNA, 2-μl amino acid mixture without methionine as supplied by the manufacturer, 33-μl rabbit reticulocyte lysate and 20 μCi [^{35}S]methionine, and water to give a final volume of 50 μl. After translation, the reaction mixture was centrifuged at 150,000 *g* for 30 min and the supernatant was used for in vitro insertion studies.

Preparation of Subcellular Fractions

Peroxisomes were isolated by differential and isopycnic centrifugation of a homogenate obtained from the liver of a Clofibrate-treated male rat (Wistar strain; Chbb Thomae, Biberach, FRG) as described previously (Hartl et al., 1985; Just et al., 1989).

Mitochondria were purified by rate sedimentation of the postnuclear supernatant of the rat liver homogenate in a 14.5–40% (wt/wt) sucrose gradient at 9,000 *g* for 25 min and subsequent isopycnic centrifugation of the mitochondrial fraction in the same Nycodenz gradient as used for the isolation of peroxisomes.

Isolated peroxisomes and mitochondria were concentrated and cleared from Nycodenz by slowly diluting the organelle fractions with buffered sucrose, pelleting the organelles at 13,000 *g* for 5 min and resuspending them in buffer H (165 mM potassium acetate, 3 mM magnesium acetate, 2.5 mM DTT, 10 mM Hepes/KOH, pH 7.4) at a final concentration of ~10 mg of protein/ml.

Insertion into Isolated Peroxisomes

In a typical insertion experiment 20 μl (200 μg) of purified peroxisomes or mitochondria were added to 10-μl translation mixture and 15 μl of an ATP-regenerating system which consisted of 8 mM ATP, 4 mM GTP, 80 mM creatine phosphate, 5 mg/ml creatine phosphokinase, and 50 mM Hepes/KOH, pH 7.5. Buffer H containing 0.2 mM antipain and 0.02 mM leupeptin was added to a final volume of 100 μl and the total insertion mixture was incubated for 1 h at 26°C. After incubation the mixture was divided into three aliquots and each was diluted with 400 μl of buffer H. To get rid of the protease inhibitors the cell organelles were separated from the soluble components by centrifugation at 13,000 *g* for 5 min. The pelleted peroxisomes were resuspended in 30 μl of buffer H, the first aliquot remained untreated, the second was treated for 30 min at 0°C with 7 μg subtilisin dissolved in buffer H and the third with 7 μg subtilisin in the presence of 1% deoxycholate and 1% Triton X-100, also dissolved in buffer H. Protease treatment was terminated by TCA precipitation and the proteins were analyzed by SDS-PAGE and fluorography or autoradiography (Hyperfilm™-βmax; Amersham). Fluorographs were quantitated by densitometric scanning using the Chromoscan 3 densitometer (Joyce and Loebl, Gateshead, England).

Insertion into Permeabilized Isolated Hepatocytes

Hepatocytes were isolated from a normal fed rat by collagenase perfusion as described previously (Heinemann and Just, 1992). Isolated cells were resuspended in αMEM medium supplemented with 10% FBS, 1×10^{-5} M dexamethasone and 1×10^{-7} M insulin and plated on glass coverslips of 3-cm diam at a density of 5×10^5 cells per plate. Medium was changed 4 h after plating and incubations were continued at 37°C in a humidified atmosphere of 95% air, 5% CO_2 for 20 h. For the membrane insertion of PMP 22 cells on two coverslips were used and the experiment was performed principally as described for the import of firefly luciferase (Rapp et al., 1993). The cells of each coverslip were permeabilized for 5 min at room temperature with 500 hemolytic units of streptolysin O (SLO) in 50 μl buffer H to which was added 10 μl ATP-regenerating system. SLO was a kind gift of Dr. S. Bhakdi (University of Mainz, Mainz, FRG). After permeabilization of the cells insertion was initiated by the addition of 20-μl translation mixture containing in vitro synthesized and [^{35}S]methionine-labeled PMP 22 and 25 I.U. heat-inactivated apyrase. Incubations were carried out at room temperature for 1 h. To test the energy requirements of the PMP 22 insertion cells on two coverslips were treated as described above except the ATP-regenerating system was replaced by an equal volume of buffer H and the translation mixture, after having synthesized PMP 22, was treated with 25 I.U. of apyrase for 10 min at room temperature. After insertion the permeabilized cells were scraped off the coverslips, washed in buffer H and homogenized in the same medium. The homogenate was centrifuged at 1,500 *g* for 2 min in order to prepare a postnuclear supernatant which was divided into three aliquots. One aliquot remained untreated and the other two were treated at 0°C for 30 min with subtilisin (10% of the aliquot's protein content) one in the absence and the other in the presence of Triton X-100 and deoxycholate (each at a concentration of 1%). Proteins were precipitated with TCA and subjected to SDS-PAGE and autoradiography.

Protease Pretreatment of Peroxisomes and Mitochondria

Isolated peroxisomes or mitochondria (200 μg) which were cleared from Nycodenz by dilution with buffer H followed by centrifugation, were resuspended in buffer H containing 20 μg of subtilisin. The digestion was carried out for 30 min at 0°C and terminated by the addition of PMSF. After another 5 min on ice the organelles were pelleted by a short centrifugation at 13,000 *g* and resuspended for the insertion assay in an appropriate volume of buffer H, usually 20 μl. Peroxisomal integrity after protease treatment was assayed by using the same protocol except peroxisomes were incubated with increasing concentrations of subtilisin (0.2–100 μg). Catalase activity was determined in the pelleted peroxisomes as described previously (Hartl et al., 1985).

NEM Treatment of Peroxisomes and the Translation Mixture

Peroxisomes (200 μg) were resuspended in 20 μl of buffer H without DTT but containing 1 mM NEM and were incubated for 10 min at room temperature. The centrifuged translation mixture (10 μl) was treated with NEM for 10 min at room temperature accordingly. Excess NEM was blocked by the addition of 50 μl of buffer H containing 2.5 mM DTT.

ATP Depletion of the Translation Mixture by Apyrase

To study the energetic requirements of the PMP 22 insertion, the ATP-regenerating system normally added to the insertion mixture was replaced by an equal volume of buffer H. However, the ATP present in the commercial preparations of the reticulocyte lysate system had to be deactivated. For this purpose the translation mixture (50 μl) freed from ribosomes by high-speed centrifugation was incubated with 2 I.U. apyrase for 15 min at 30°C and 15 min at 25°C (Pfanner and Neupert, 1986), in order to deplete the mixture on ATP and ADP. For the control experiment the apyrase used was inactivated by prior heating to 95°C for 10 min. ATP concentrations in the translation and insertion mixtures were determined luminometrically (Lundin et al., 1986) using the Biolumat LB 9500 (Berthold, Wildbad, FRG). The measuring device records the light impulses originated by the luciferin/luciferase reaction and presents them as relative light units. The assay mixture usually contained in a final volume of 0.5 ml 15 mM $MgSO_4$, 25 mM glycylglycine, pH 7.8, 0.08 mM luciferin, 5 μl of the diluted luciferase stock solution and 5 μl of the sample. The luciferase stock solution (2 mg/ml in 0.5 M KH_2PO_4, pH 7.5) was stored at −20°C in glass tubes and diluted for the assay 1:100 with 25 mM glycylglycine/1% BSA, pH 7.8.

Results

Insertion of PMP 22 into Isolated Purified Peroxisomes

The most widely used system to import proteins into distinct organelles in vitro consists of the in vitro synthesis of the proteins and their subsequent incubation with isolated cell organelles (Blobel and Dobberstein, 1975). This system has also been used previously to study the import of two peroxisomal matrix proteins, catalase and acyl-CoA oxidase (Fujiki and Lazarow, 1985; Imanaka et al., 1987). In a first approach we probed this system for its capability to import PMP 22 into peroxisomes which were isolated by differential and isopycnic centrifugation as previously described in detail. They represent a homogeneous population at least 95% pure as revealed by marker enzyme measurements (Hartl et al., 1985). Correct membrane integration of PMP 22 is demonstrated by postinsertional treatment of the peroxisomes with exogenously added subtilisin. The use of the protease resistance criterium to ascertain the proper insertion of PMP 22 was made possible by previous observations which identified the mature membrane integrated PMP 22 to be highly resistant to the activity of subtilisin and proteinase K (Hartl and Just, 1987) but not trypsin (Fujiki et al., 1984). The amount of subtilisin necessary to digest PMP 22 was between 5–10% of the protein mass of added peroxisomes and was determined by a titration experiment. Usually the 10% concentration was applied which digested all the newly synthesized protein in the presence of detergent. In some initial experiments the carbonate procedure was used alternatively to discriminate between membrane bound and membrane integrated PMP 22. The amount of carbonate-resistant PMP 22 was consistently higher than that which resisted the protease. The discrepancy most likely is due to those molecules which are integrated into the membrane only partially and therefore withstand the carbonate treatment but are still susceptible to the protease.

The results of the experiments on the in vitro insertion of PMP 22 under our standard conditions are shown in Fig. 1. After incubation with isolated peroxisomes for 1 h at 26°C the major portion of newly synthesized PMP 22, almost 75%, was pelleted together with the organelles (lane *1*) and the remainder of the material was recovered from the supernatant (lane *2*). When the pelleted peroxisomes were resuspended and treated with subtilisin, a consistent amount, ~40–50% of the sedimentable PMP 22, resisted the protease digestion in the absence (Fig. 1, lane *3*) but not in the presence of detergent (lane *4*). Small amounts of pelletable PMP 22 were also found in the absence of isolated peroxisomes (Fig. 1, lane *5*). Under these conditions the major portion of the protein was recovered from the supernatant (Fig. 1, lane *6*). None of this "pelletable" PMP 22 resisted protease digestion (Fig. 1, lanes *7* and *8*). In all the mock import ex-

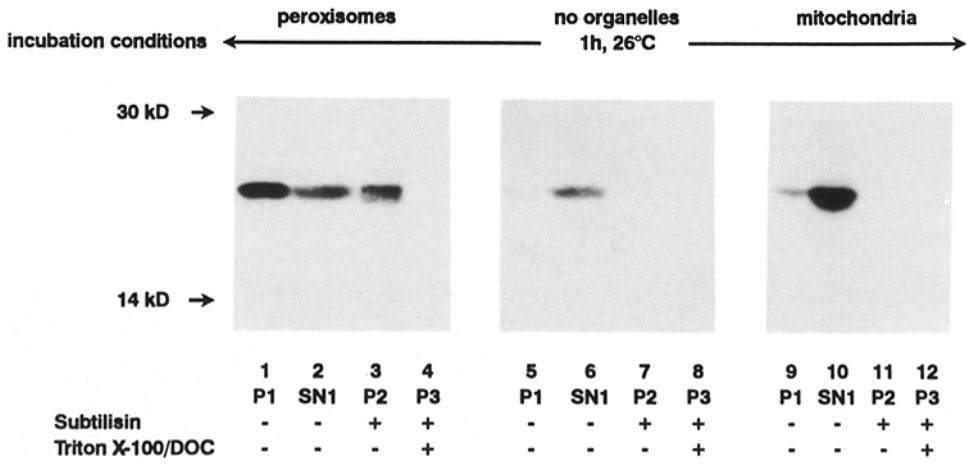

Figure 1. Posttranslational insertion of PMP 22 into the membrane of rat liver peroxisomes. The in vitro translation product of PMP 22 mRNA obtained in the nuclease-treated rabbit reticulocyte lysate system was incubated for 1 h with peroxisomes (200 μg) isolated from the liver of a Clofibrate-treated 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 (−) or 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).

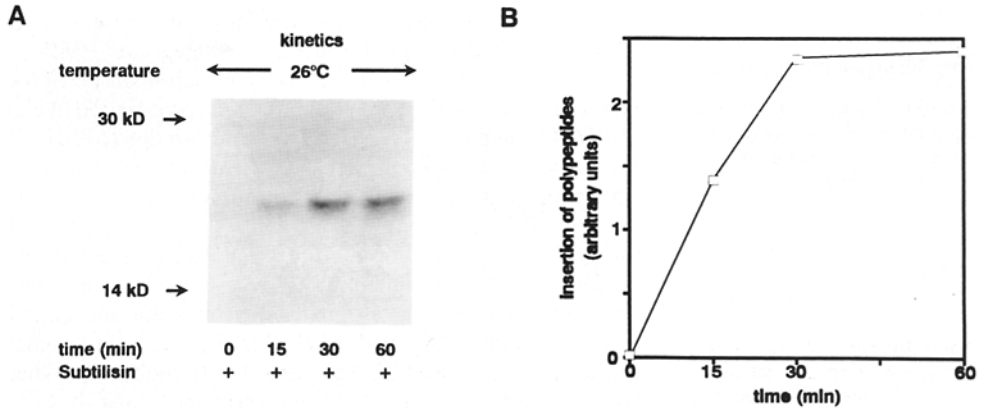

Figure 2. Time-dependent increase of PMP 22 insertion. (*A*) Peroxisomes (50 μg) were incubated with PMP 22 mRNA translation product for various times from 0 to 60 min. At the time points indicated peroxisomes were pelleted and treated with subtilisin (5 μg) at 0°C for 30 min. Protease treatment was terminated by TCA precipitation and protease-resistant PMP 22 was analyzed by SDS-PAGE and fluorography. The arrows at the left represent the molecular weight standards. (*B*) Quantitation of the time dependent insertion of PMP 22 by densitometric evaluation of the fluorograph shown in *A*.

periments in which isolated organelles were lacking, an increased susceptibility of newly synthesized PMP 22 to proteolytic activity present in the reticulocyte lysate was noticed even though protease inhibitors were added. To demonstrate the specificity of import into peroxisomes, PMP 22 was incubated with highly purified rat liver mitochondria isolated by rate sedimentation and subsequent centrifugation in Nycodenz density gradients in a purity of at least 97%. The results were similar to those obtained under mock import conditions. Only low amounts of PMP 22 were pelleted together with the mitochondria (Fig. 1, lane *9*), whereas the major portion of the protein was recovered from the supernatant (lane *10*). None of the PMP 22 bound to mitochondria resisted protease digestion (Fig. 1, lanes *11* and *12*).

Insertion of PMP 22 Is Time and Temperature Dependent

In a kinetic experiment the time-dependent insertion of PMP 22 was followed (Fig. 2, *A* and *B*). The densitometric evaluation of the fluorograph revealed an increase in the amount of membrane integrated PMP 22 up to 30 min with no further increase from 30–60 min.

The insertion of PMP 22 into isolated peroxisomes appeared to be strongly dependent on temperature. Incubation of the organelles at 0°C with in vitro translated PMP 22 (Fig. 3, lanes *5–8*) decreased the amount of sedimentable PMP 22 to ~40% (lane *5*) as compared to 75% at 26°C (lane *1*). Major portions of newly synthesized PMP 22 remained in the supernatant (Fig. 3, lane *6*) and practically all of the sedimentable material was susceptible to the protease (lanes *7* and *8*), hence it was bound to the membrane but not inserted. Compared with the insertion at 26°C (Fig. 3, lanes *1–4*) these observations suggest that the decrease in the amount of sedimentable PMP 22 is due to reduced insertion rather than reduced binding.

The fact that PMP 22 at 0°C binds to the peroxisomal membrane without being integrated into it indicates a two-step process with binding as the first and membrane integration as the second step. To test this idea experimentally, peroxisomes were preincubated (first incubation) with newly synthesized PMP 22 at temperatures of 5° and 10°C for 10 min after which time the peroxisomes were pelleted, resuspended in fresh insertion mixture lacking [^{35}S]methionine and PMP 22 mRNA and incubated (second incubation) for 1 h at 26°C. The results of these experiments are shown in Fig. 4. When peroxisomes were preincubated at 5° and 10°C ~40% of total synthesized PMP 22 was pelleted together with the organelles and about 4 and 7%, respectively, appeared to be inserted such as to resist protease treatment. The subsequent incubation at 26°C appreciably increased the amount of protease resistant PMP 22 to 10 and 20% confirming the assumption of a two-step mechanism.

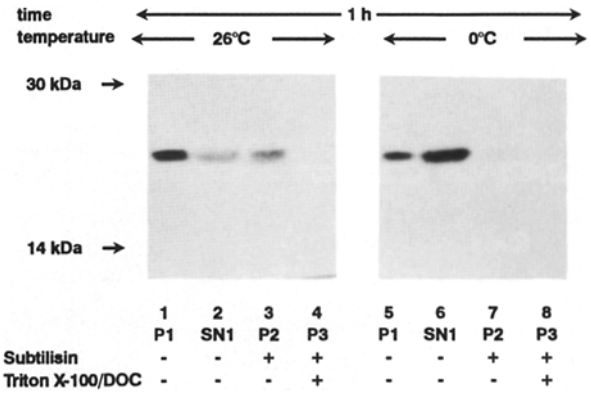

Figure 3. Temperature dependence of PMP 22 insertion. The translation product of PMP 22 mRNA was incubated with isolated peroxisomes (200 μg) at 26°C (lanes *1–4*) and 0°C (lanes *5–8*) for 1 h after which time the incubations were divided into three aliquots. The peroxisomes of each aliquot were separated from the supernatant by centrifugation and the pelleted organelles (lanes *3*, *4*, *7*, and *8*) subjected to treatment with subtilisin (7 μg) at 0°C for 30 min in the absence (−) and the presence (+) of Triton X-100/deoxycholate (each 1%). In lanes *1* and *5* untreated peroxisomes and in lanes *2* and *6* the supernatant of one aliquot was analyzed. The arrows at the left mark the molecular weight standards.

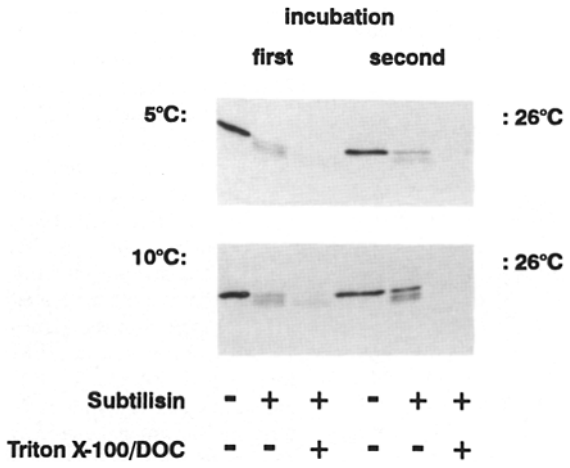

Figure 4. Temperature dependent binding and insertion of PMP 22. (*A*) The translation product of PMP 22 mRNA (20 μl) was incubated with isolated peroxisomes (400 μg) for 10 min at temperatures of 5° and 10°C (first incubation). The organelles were pelleted and one half was preserved and assayed for the protease resistant insertion of PMP 22 in the presence and absence of detergent (see legend to Fig. 1). The other half was resuspended in medium of the same composition as used for the first incubation omitting [^{35}S]methionine and PMP 22 mRNA and incubated for another 60 min at 26°C (second incubation). Subsequently the organelles were analyzed for the protease resistant insertion of PMP 22.

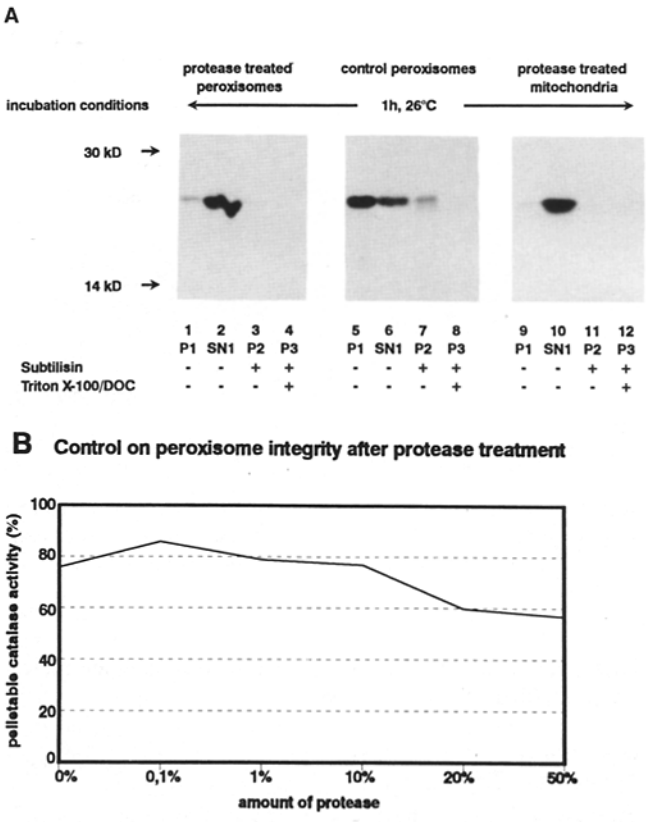

Figure 5. Attempts to insert PMP 22 into protease pretreated peroxisomes and mitochondria. Peroxisomes (200 μg, lanes *1–4*) and mitochondria (200 μg, lanes *9–12*) were pretreated with subtilisin (20 μg) in a volume of 20 μl at 0°C for 10 min before they were used for the insertion experiment. Subtilisin 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. (*B*) The integrity of peroxisomes was investigated by analyzing the release of catalase, a peroxisomal matrix enzyme, following treatment of the organelles by various concentrations of subtilisin. Peroxisomes (200 μg) were incubated with increasing concentrations of subtilisin for 30 min at 0°C and the catalase activity in the pelleted peroxisomes determined. Catalase activity is expressed in % of total present in the peroxisomes and the protease concentrations are given in % of the protein mass of peroxisomes.

Protease Pretreatment of Peroxisomes Prevents Insertion

The common idea of how precursor proteins posttranslationally are imported into the corresponding cell organelle involves binding of the precursor to a receptor prior to its translocation. To study the participation of proteinaceous membrane components on the insertion of PMP 22, isolated peroxisomes were pretreated at 0°C for 30 min with subtilisin in a concentration of 10% of the peroxisomal protein content. As shown in Fig. 5 *A* this protease pretreatment nearly completely abolished the insertion capacity of the peroxisomes. Binding of PMP 22 to the peroxisomes was considerably reduced (Fig. 5, lane *1*) and only trace amounts of protease resistant material were detected (lane *3*). The protein was nearly quantitatively recovered from the supernatant (Fig. 5, lane *2*). The corresponding control experiment (Fig. 5, lanes *5–8*) revealed that the 30-min preincubation at 0°C without protease did not influence the capacity of the peroxisomes to bind PMP 22 but decreased their insertion capacity by ~30% (lane *7*). Purified mitochondria pretreated with protease bound the same small amount of PMP 22 (Fig. 5, lane *9*) as untreated organelles (Fig. 1, lane *9*) suggesting that this binding is unspecific. The overall integrity of the isolated peroxisomes apparently was not affected by the protease pretreatment since the amount of catalase which was sedimentable after the treatment remained fairly constant up to a protease concentration of 10% of the peroxisomal protein mass (Fig. 5 *B*).

Insertion Is Not NEM Sensitive

Free sulfhydryl groups have been found to be crucial for the function of receptors and protein transport across membranes (Siegel and Walter, 1988; Sheffield et al., 1990; Newmeyer and Forbes, 1990). To test such a requirement for free sulfhydryl groups present in cytosolic components, the translation mixture after in vitro synthesis was treated with 1 mM NEM for 10 min. Afterwards excess NEM was blocked by the addition of buffer H containing 2.5 mM DTT (Fig. 6, lanes *1–4*). In the opposite experiment the isolated peroxisomes were treated accordingly with NEM prior to incubation with untreated newly synthesized PMP 22 (Fig. 6, lanes *5–8*). In the third experiment both the translation mixture and the peroxisomes were treated with NEM (Fig. 6, lanes *9–12*). In neither of these experiments an influence of

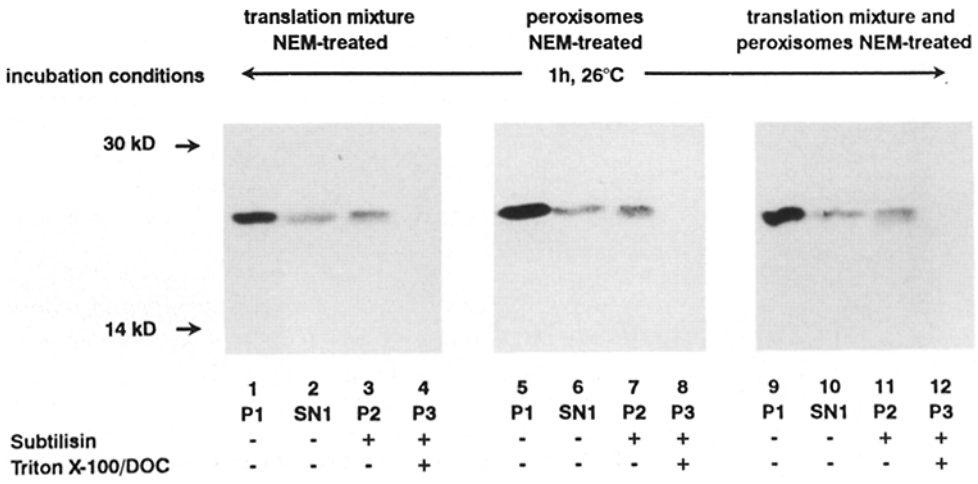

Figure 6. Effect of NEM on the insertion of PMP 22. After in vitro translation of PMP 22 mRNA the translation mixture was treated with NEM (1 mM) for 10 min at room temperature after which time excess NEM was blocked by the addition of DTT (2.5 mM). Subsequently the NEM-treated translation mixture was incubated with untreated isolated peroxisomes (lanes *1–4*). Incubations with NEM treated peroxisomes (1 mM) and untreated translation mixture are shown in lanes *5–8* and those with both peroxisomes and translation mixture treated with NEM (1 mM) are shown in lanes *9–12*. After incubation peroxisomes were recovered by centrifugation (*P*) and subjected to protease treatment in the absence (−) and presence (+) of detergents exactly as described in the legends to previous figures. In lanes *2*, *6*, and *10* the supernatant of one aliquot was analyzed. The arrows on the left mark the molecular weight standards.

NEM treatment on binding to and insertion into the membrane of PMP 22 was observed.

Insertion Is Not Dependent on Nucleoside Triphosphates

The import of many mitochondrial proteins as well as protein translocation across the membrane of the ER as a general rule requires nucleoside triphosphates in the form of ATP and/or GTP (Connolly et al., 1991; and for reviews see Rapoport, 1990; Verner and Schatz, 1988; Hartl et al., 1989), although exceptions to this rule are known, e.g., the import of cytochrome c (Hartl et al., 1989). We therefore were quite interested to investigate the energy requirements for the insertion of PMP 22. Fig. 7 *A* (lanes *1–4*) shows the insertion of PMP 22 under standard conditions except the translation mixture was pretreated with 2 I.U. of heat inactivated apyrase. Under these conditions 45% of the sedimentable PMP 22 remained protease resistant. Surprisingly, no influence on insertion as compared to these standard conditions, was observed when the translation mixture was depleted of ATP by the addition of 2 I.U. of apyrase (Fig. 7 *A*, lanes *5–8*) or when the coenzymatic activity of the nucleotides ATP and GTP was inhibited by adding their non-hydrolyzable analogues ATP-γ-S (lanes *9–12*) or GTP-γ-S (lanes *13–16*) which both were applied at a high concentration of 5 mM. After treatment with apyrase 55% of total pelletable, i.e., 30% of total synthesized PMP 22 resisted the protease (Fig. 7 *a*, lane *7*). The addition of ATP-γ-S (lane *9*) or GTP-γ-S (lane *13*) slightly decreased the amount of sedimentable PMP 22 to ~60%. From these pelletable portions still ~45% became protease resistant (Fig. 7 *A*, lanes *11* and *15*). These data clearly show that neither the presence of the nucleotides ATP or GTP nor their hydrolysis is necessary for the insertion of PMP 22.

Recently the in vitro import of firefly luciferase which is targeted to the peroxisomal matrix was demonstrated in a semi-intact cell system to require the hydrolysis of ATP (Rapp et al., 1993). Therefore, we studied the energy dependence of the PMP 22 insertion comparatively in the same system. Over isolated peroxisomes the semi-intact cell system offers the advantage of largely preserving the integrity of the peroxisomal compartment during insertion and thus enabling studies to be carried out under more physiological conditions. For the experiment primary hepatocytes were cultured on glass coverslips and permeabilized by the bacterial toxin SLO (Bhakdi et al., 1985; Ahnert-Hilger et al., 1989). Subsequently the cells were incubated for 1 h at room temperature with the in vitro translated PMP 22 in the presence and absence of ATP. As shown in Fig. 7 *B* ~50% of the PMP 22 associated with organelles (lanes *1* and *4*) became protease resistant both in the presence (lane *2*) and absence (lane *5*) of ATP. The protease resistance portion of PMP 22, however, became susceptible to the protease by the addition of detergent (Fig. 7 *B*, lanes *3* and *6*) suggesting its correct membrane insertion. Thus in both systems, isolated peroxisomes and permeabilized hepatocytes PMP 22 was inserted without the need of ATP, whereas luciferase import as revealed by immunofluorescence under the same conditions was strictly dependent on ATP (not shown).

Discussion

PMP 22 Is Specifically and Efficiently Inserted into Peroxisomes

Under standard conditions (1-h insertion at 26°C) ~75% of total newly synthesized PMP 22 becomes associated with the peroxisomes and hence is pelleted together with the organelles (Fig. 1). Subsequent treatment of the pelleted peroxisomes with protease demonstrates that not all of the PMP 22 associated with the organelles is correctly inserted into the membrane. At least ~40% of the pelletable PMP 22 (~30% of the total) is resistant to the protease. How specific is this in vitro binding and insertion? Since PMP 22 is a very hydrophobic molecule (Kaldi et al., 1992) we considered the possibility that the newly synthesized polypeptides may form aggregates, and therefore pellet even in the absence of membranes. However, as shown by the mock-insertion experi-

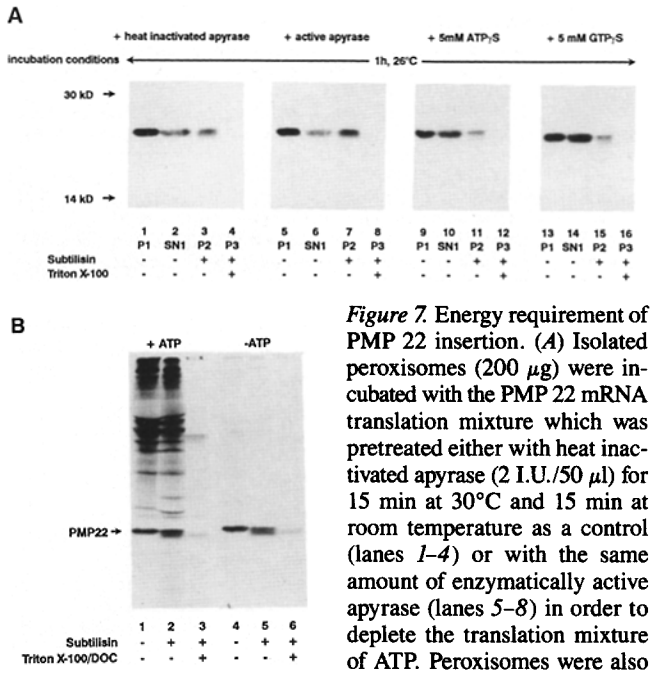

Figure 7. Energy requirement of PMP 22 insertion. (*A*) Isolated peroxisomes (200 μg) were incubated with the PMP 22 mRNA translation mixture which was pretreated either with heat inactivated apyrase (2 I.U./50 μl) for 15 min at 30°C and 15 min at room temperature as a control (lanes *1–4*) or with the 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 previous figures. The arrows at the left mark the molecular weight standards. (*B*) Isolated permeabilized hepatocytes (1×10^6 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 22 was 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 [^{35}S]methionine leads to the translation of many endogenous mRNA species.

ment, this is not the case (Fig. 1). Only trace amounts of PMP 22 were pelleted in the absence of organelles and even in the presence of mitochondria instead of peroxisomes only tiny amounts of PMP 22 were sedimented. Furthermore, the faint amounts of PMP 22 that pellet in the mock-insertion experiment and in the presence of mitochondria are digested completely by protease. Thus binding to and insertion into the peroxisomal membrane of PMP 22 in vitro is a rather specific process. In addition to that protease pretreatment of peroxisomes and mitochondria prior to the incubation with PMP 22 suggests that this process may be mediated by specific proteinaceous structures present in the peroxisomal membrane.

As mentioned above PMP 22 is inserted into isolated peroxisomes in vitro with an overall efficiency of ~35%. Thus, the 75% of total newly synthesized and pelletable PMP 22 are composed of 35% which became integrated into the membrane during the time of incubation in a protease-resistant way and 40% which only were bound to the peroxisomal membrane and not inserted. What is the nature of this relatively large proportion of bound PMP 22? An appreciable amount of PMP 22 bound at low temperature (5–10°C) during the preincubation period inserted into the membrane during a second incubation at 26°C (Fig. 4). Thus at temperatures between 5 and 10°C binding clearly could be separated from membrane integration, suggesting two distinct steps involved in the overall insertion process. A substantial portion of PMP 22 bound at low temperature, however, appeared not to be competent for insertion. The reasons for this at present are not known.

Insertion of PMP 22 and Translocation of Peroxisomal Matrix Proteins Are Mediated by Distinct Mechanisms

So far only the peroxisomal matrix enzyme acyl-CoA oxidase has been reported to be successfully translocated into isolated peroxisomes (Fujiki and Lazarow, 1985; Imanaka et al., 1987). This import has been shown to be dependent on time and temperature, to require ATP hydrolysis and to be independent on a functional membrane potential. The same dependencies recently were also observed with the import of two other peroxisomal markers, firefly luciferase (Soto et al., 1993; Rapp et al., 1993) and bovine serum albumin fused to the peroxisomal targeting sequence SKL (Wendland and Subramani, 1993) although these studies were conducted either by microinjection of the marker into living cells or by the use of selectively permeabilized culture cells. In addition these experiments indicated the involvement of at least one NEM-sensitive membrane component in the translocation process (Wendland and Subramani, 1993). The conditions necessary to insert PMP 22 and those required for the translocation of matrix proteins turn out to be quite different. Insertion of PMP 22 does not need ATP hydrolysis (Fig. 7) and no NEM-sensitive component is involved (Fig. 6). The difference is not attributed to the different experimental systems used to study membrane insertion and translocation since the ATP-independent insertion of PMP 22 was also accomplished in the semi-intact cell system (Fig. 7 *B*). Therefore, these data strongly suggest that insertion of PMP 22 and translocation of firefly luciferase are mediated by distinct mechanisms. Since PMP 22 (like all the other integral peroxisomal membrane proteins) lacks the COOH-terminal SKL targeting signal (Gould et al., 1990; Kaldi et al., 1993) which, however, is present in firefly luciferase (Gould et al., 1987; Gould et al., 1988), it is most likely that these two proteins are recognized by different receptors (McCollum et al., 1993) and hence their pathways diverge already at this early stage of import.

Posttranslational–Preinsertional Events

It was previously shown by Fujiki et al. (1984) that the PMP 22 mRNA is confined to the population of free polysomes and hence the insertion of the protein will occur posttranslationally. How then is this very hydrophobic protein stabilized in the cytosol or lysate before its integration into the membrane? Most probably this may be facilitated by the activity of cytosolic factors acting as molecular chaperones or polypeptide chain binding proteins (for reviews see Ellis and Hemmingsen, 1989; Pfanner and Neupert, 1990). Both protein import into mitochondria and protein translocation into the ER have been demonstrated to be stimulated by 70-kD heat shock–related proteins (Chirico et al., 1988; Murakami et al., 1988; Deshaies et al., 1988). They are believed to play a major role in keeping precursor proteins in a loosely folded

import competent conformation forming complexes with them and preventing their aggregation or malefolding. Their activity is dependent on ATP which causes the release of the precursor proteins from these complexes. For peroxisomal protein import the involvement of heat shock–related proteins is speculative and has not been demonstrated yet, although their implication is most likely. Due to the fact that the insertion of PMP 22 does not require ATP, chaperones like the 90-kD heat shock–related proteins or the small heat shock proteins which do not need nucleoside triphosphates for their activity (Wiech et al., 1992; Jakob et al., 1993; Merck et al., 1993) may be involved rather than the family of the 70-kD stress proteins. The actual insertion process may be driven by biased random thermal motion without the need of ATP hydrolysis as recently proposed by Simon et al. (1992).

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