

## Insertion of the 70-kDa Peroxisomal Membrane Protein into Peroxisomal Membranes *in Vivo* and *in Vitro*\*

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Tsuneo Imanaka<sup>‡§</sup>, Yu'ichi Shiina<sup>‡</sup>, Tatsuya Takano<sup>‡</sup>, Takashi Hashimoto<sup>¶</sup>, and Takashi Osumi<sup>||</sup>

From the <sup>‡</sup>Department of Microbiology and Molecular Pathology, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, the <sup>¶</sup>Department of Biochemistry, Shinshu University School of Medicine, Matsumoto, Nagano 390, Japan, and the <sup>||</sup>Department of Life Science, Himeji Institute of Technology, Kamigori, Hyogo 678-12, Japan

**Biosynthesis and intracellular transport of 70-kDa peroxisomal membrane protein (PMP70) has been studied in rat hepatoma, H-4-II-E cells. Pulse-chase analysis showed that a newly synthesized <sup>35</sup>S-PMP70 first appeared in the cytosolic fraction and was then transported into the peroxisomal fraction. The half-life of <sup>35</sup>S-PMP70 in the cytosolic fraction was approximately 3 min. Integration of <sup>35</sup>S-PMP70 into membranes occurred in the peroxisomal fraction and was completed within 30 min. No proteolytic processing of <sup>35</sup>S-PMP70 was observed. An *in vitro* import system was reconstituted to characterize the insertion mechanism of PMP70 into peroxisomes. Peroxisomes isolated from rat liver were incubated at 26 °C with [<sup>35</sup>S]methionine-labeled *in vitro* translation products of PMP70 mRNA in the presence of the cytosolic fraction. The peroxisomes were reisolated and insertion of <sup>35</sup>S-PMP70 into the membrane was analyzed using a Na<sub>2</sub>CO<sub>3</sub> procedure. The binding and insertion of <sup>35</sup>S-PMP70 were dependent on temperature and incubation time and was specific for peroxisomes. Pretreatment of peroxisomes with trypsin and chymotrypsin almost abolished the binding and insertion of <sup>35</sup>S-PMP70. The translation products contained several truncated <sup>35</sup>S-PMP70s. The NH<sub>2</sub> terminally truncated <sup>35</sup>S-PMP70s, with a molecular mass greater than 50 kDa, bound to and inserted into peroxisomal membranes, whereas truncated <sup>35</sup>S-PMP70s smaller than 45 kDa did not. These results suggest that PMP70 is post-translationally transported to peroxisomes without processing and inserted into peroxisomal membranes by a specific mechanism in which a proteinaceous receptor and a certain internal sequence of PMP70 are involved.**

Peroxisomes are organelles bounded by a single membrane which are present in almost all eukaryotic cells. The peroxisomes are involved in a variety of metabolic processes including peroxide-based respiration, oxidative degradation of purines and fatty acids, and synthesis of plasmalogen and bile acids (1, 2). It has recently been suggested that peroxisomes are formed by post-translational import of newly synthesized proteins into pre-existing peroxisomes, which then divide (3–5). In rat liver, it is known that matrix proteins in peroxisomes are all synthesized on free polysomes in the cytosol. Post-translational im-

port of these proteins into peroxisomes has been shown by *in vivo* pulse-chase (6, 7) and also by *in vitro* import experiments (8–12). We developed an *in vitro* system based on the import of radiolabeled acyl-CoA oxidase (AOx)<sup>1</sup> into purified rat liver peroxisomes and showed that ATP hydrolysis was required for the translocation of AOx through peroxisomal membranes (9). At least two types of peroxisomal targeting sequences (PTSs) have been found. PTS1 consists of the sequence Ser-Lys-Leu (or closely related) at the carboxyl terminus (10, 13) and PTS2, which in 3-ketoacyl-CoA thiolase is found in the 11 amino acids of the amino-terminal leader sequence (14, 15).

The membranes of rat liver peroxisomes contain several integral membrane proteins not found in other organelles. Polypeptides of 69/70 and 22 kDa have been identified as major components and polypeptides of 57, 53, 35/36, and 26/27 kDa have been identified as minor components of peroxisomal membranes (16–19). The 70-kDa peroxisomal membrane protein (PMP70) is markedly induced by administration of hypolipidemic agents and parallels peroxisome proliferation (17, 18). We cloned and sequenced PMP70 and found that PMP70 belonged to a superfamily called an ATP-binding cassette protein, conferring multidrug resistance to tumor cells (20). Recently PMP70 was suggested to be essential for peroxisome formation (21). PMP22 was also cloned and sequenced and it was suggested that the topology of PMP22 was similar to those of Mpv 17 and certain transmitter gated ion channels, although the function of PMP22 has not yet been described (22). Recently peroxisome assembly factor 1, a peroxisomal integral membrane protein with molecular mass of 35 kDa was also cloned and sequenced (23).

A number of studies have been carried out to further investigate the biogenesis of peroxisomal membrane proteins (PMPs). We, and other laboratories, have shown that PMP70, -26, and -22 were synthesized on free polysomes (24–26), suggesting that the PMPs are likely to be inserted post-translationally into peroxisomal membranes. However, the intracellular transport of any PMP has not yet been investigated. The occurrence of this event should be confirmed before establishing an *in vitro* import system.

As a preliminary step to understanding the molecular mechanism of assembly of PMPs, we carried out pulse-chase experiments to investigate the kinetics of intracellular transport of newly synthesized PMP70 in rat hepatoma H-4-II-E cells. From these results, we have been able to develop an *in vitro* import system using isolated rat liver peroxisomes and *in vitro* translation products of PMP70 mRNA. We obtained experi-

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§ To whom all correspondence should be addressed: Dept. of Microbiology and Molecular Pathology, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan. Tel.: 81-426-85-3739; Fax: 81-426-85-3738.

<sup>1</sup> The abbreviations used are: AOx, acyl-CoA oxidase; NEM, N-ethylmaleimide; PMP, peroxisomal membrane protein; PMP70, 70-kDa peroxisomal membrane protein; PMP22, 22-kDa peroxisomal membrane protein; PNS, postnuclear supernatant; PTS, peroxisome targeting sequence; PAGE, polyacrylamide gel electrophoresis.

mental evidence showing that PMP70 was post-translationally transported to peroxisomes and then inserted into their membranes without processing. In addition we found that the binding and insertion of PMP70 was mediated by a specific mechanism in which a proteinaceous receptor and a certain internal sequence of PMP70 may be involved.

#### EXPERIMENTAL PROCEDURES

**Materials**—EXPRE<sup>35S</sup>S (46.3 TBq/mmol for Met) and <sup>125</sup>I-protein A (2.60–3.70 TBq/g) were purchased from DuPont NEN and ICN Biochemicals Inc. (Irvine, CA), respectively. Protein A-agarose, trypsin, chymotrypsin, apyrase, soybean trypsin inhibitor, leupeptin, antipain, chymostatin, pepstatin A, and phenylmethylsulfonyl fluoride were obtained from Sigma. Rabbit reticulocyte lysate and *N*-ethylmaleimide (NEM) were from Wako Pure Chemicals (Osaka, Japan). A synthetic peptide corresponding to the COOH-terminal 15 amino acids of rat PMP70 was supplied from Dr. S. Ohkuma (Kanazawa University). Antibodies against synthetic peptide were prepared by immunization of rabbits with the peptide coupled to keyhole limpet hemocyanin (Calbiochem, La Jolla, CA). Antibodies against rat PMP70 were prepared according to the method of Hashimoto *et al.* (17).

**Subcellular Fractionation of H-4-II-E Cells**—Rat hepatoma H-4-II-E cells were grown with Dulbecco's modified Eagle's medium containing 20% fetal calf serum and 100  $\mu$ M non-essential amino acids. Confluent cells in 75-cm<sup>2</sup> culture flasks were washed 3 times with phosphate-buffered saline and harvested with the aid of a rubber policeman in phosphate-buffered saline. The cell suspension was centrifuged at 800  $\times g$  for 10 min at room temperature. The cell pellet was suspended in 0.25 M sucrose, 1 mM EDTA, and 0.1% (v/v) ethanol, pH 7.4 (SVE), 0.25 M sucrose containing 1 mM EDTA, and 0.1% (v/v) ethanol, containing 3 mM imidazole and centrifuged at 1,000  $\times g$  for 10 min at 4  $^{\circ}$ C. The cells were resuspended in the above solution at approximately  $1 \times 10^8$  cells/ml and leupeptin, antipain, chymostatin, and pepstatin A were added to a final concentration of 10  $\mu$ g/ml of each. The cells were homogenized with a Teflon/glass Potter-Elvehjem homogenizer and a postnuclear supernatant (PNS) fraction was obtained by centrifugation at 2,700  $\times g$  for 10 min. Heavy and light mitochondrial, microsomal, and cytosolic fractions were obtained by differential centrifugation according to the method of de Duve *et al.* (27). A light mitochondrial fraction (~10 mg of protein in 0.5 ml) was further subjected to equilibrium density centrifugation in a 10-ml linear sucrose gradient (1.15–1.27 g/ml) in a Hitachi RP 55VF rotor. The gradient rested on 1.0 ml of 1.30 g/ml sucrose. All sucrose solutions contained 1 mM EDTA, 3 mM imidazole, and 0.1% (v/v) ethanol. Centrifugation was carried out at 50,000 rpm (193,000  $\times g$ ) for 60 min at 4  $^{\circ}$ C. Membranes from each subcellular fraction were prepared by the Na<sub>2</sub>CO<sub>3</sub> procedure (28). In the case of density gradient fractions, some of which contained very little protein, rabbit red blood cell membrane ghosts (50  $\mu$ g of protein) were added as carrier to obtain membranes quantitatively as pellets (29).

**Pulse-Chase Studies**—Confluent H-4-II-E cells, in 60-mm dishes, were washed 3 times with phosphate-buffered saline and incubated with methionine-free modified Eagle's medium for 1 h and then pulsed for 5 min with [<sup>35</sup>S]methionine (3.7 MBq/ml) in the above medium. After the cells were washed with the medium containing cold methionine, the radioactivity was chased for up to 60 min. The cells were harvested in chilled 0.25 M SVE containing 3 mM imidazole and PNS fractions were prepared as described above. The PNS fractions were further separated into supernatant and particulate fraction by centrifugation at 16,000  $\times g$  for 20 min. In some experiments, the PNS fraction was further fractionated by sucrose gradient centrifugation as described above. <sup>35</sup>S-PMP70 in subcellular fractions were immunoprecipitated with anti-PMP70 antibodies by the method previously described (30). The immunoprecipitates were subjected to SDS-PAGE, followed by fluorography at -80  $^{\circ}$ C with Kodak X-Omat AR film with intensifying screens for 2–7 days. The band of <sup>35</sup>S-PMP70 was quantified by densitometric scanning (9).

**In Vitro Transcription and Translation**—The cDNA clone encoding PMP70 was inserted between the *Eco*RI and *Hind*III sites of pTZ18U vector (Toyobo, Osaka, Japan). The plasmid was linearized by digestion with *Eco*T22I downstream of the cDNA sequence and was transcribed *in vitro* using T7 RNA polymerase. In a typical experiment, 1  $\mu$ g of plasmid DNA was transcribed in 25  $\mu$ l of transcription mixture, of which 1  $\mu$ l was used for translation in a rabbit reticulocyte lysate cell-free protein synthesis system using [<sup>35</sup>S]methionine. After translation, the reaction mixture was centrifuged for 1 h at 10,000  $\times g$  and the supernatant was used in the import assay.

**Preparation of Subcellular Fractions from Rat Liver**—Peroxisomes

and mitochondria were prepared by isopycnic centrifugation in Nycodenz and sucrose, respectively (19). To remove the Nycodenz and concentrate the peroxisomes, the gradient fractions were pooled, slowly diluted by the dropwise addition of 5 volumes of ice-cold 0.25 M sucrose containing 0.1% (v/v) ethanol and 5 mM Hepes/KOH, pH 7.4 (0.25 M SEH), and centrifuged for 20 min at 12,000 rpm (17,000  $\times g$ ) (9). Mitochondria were similarly diluted and concentrated. Cytosolic fraction was prepared by centrifugation of a rat liver PNS at 10,000  $\times g$  for 1 h in a Hitachi S100AT5 rotor.

**In Vitro Insertion of PMP70 into Rat Liver Peroxisomes**—In a typical experiment 50  $\mu$ l of concentrated peroxisomes (100–200  $\mu$ g) was mixed with 50  $\mu$ l of cytosolic fraction (1.2–1.5 mg), 150  $\mu$ l of 0.25 M SEH containing 1.25 mM ATP, 3.75 mM MgCl<sub>2</sub>, 62.5 mM KCl, and 5  $\mu$ l of *in vitro* translation products. After incubation at 26  $^{\circ}$ C for 20–60 min, a final concentration of 2 mM methionine, 10  $\mu$ g/ml leupeptin, antipain, chymostatin, pepstatin A, and 100  $\mu$ M phenylmethylsulfonyl fluoride were added. The samples were then centrifuged for 20 min in a microcentrifuge (16,000  $\times g$ ; Kubota 1700) to isolate peroxisomes, and then were suspended in 250  $\mu$ l of 0.25 M SEH containing 2 mM methionine and protease inhibitors described above. The supernatant (20  $\mu$ l) and the pellet (20  $\mu$ l) were subjected to SDS-PAGE and fluorography. Aliquots of the pellets (125  $\mu$ l) were treated with Na<sub>2</sub>CO<sub>3</sub> and separated into membrane and the resulting soluble fractions. The proteins in the soluble fractions were precipitated by trichloroacetic acid. The soluble and membrane fractions were subjected to SDS-PAGE and fluorography. To quantitate the binding and insertion of <sup>35</sup>S-PMP70, 10–50% of the adjusted translation products with peroxisomes were used and fluorograms were quantitated by densitometric scanning (9).

**Other Methods**—Post-translational import of AOx into peroxisomes *in vitro* was assayed as described previously (9, 31). Protein, catalase, *N*-acetyl- $\beta$ -D-glucosaminidase, and cytochrome *c* oxidase were assayed as described previously (19). NADPH cytochrome *c* reductase was assayed by the method of de Duve *et al.* (27). Lactate dehydrogenase was assayed using the LDH-UV test kit (Wako, Osaka, Japan). ATP content was measured with a bioluminescence ATP assay kit using luciferin-luciferase (Kikkoman, Osaka, Japan) after deproteinization with trichloroacetic acid. Immunoblotting was done by the method of Small *et al.* (32).

#### RESULTS

**Localization of PMP70 in H-4-II-E Cells**—In order to investigate the intracellular transport of PMP70 in cultured cells, we first examined whether H-4-II-E cells contain PMP70 in the peroxisomes. Isolated subcellular fractions were subjected to immunoblot analysis. A band corresponding to PMP70 was enriched in a light mitochondrial fraction and the distribution of the polypeptide was similar to that of catalase, a peroxisomal marker enzyme (Fig. 1, A and B). Furthermore, as shown in Fig. 1, C and D, PMP70 was recovered mainly in fractions 6–8 on sucrose gradient centrifugation and the peak of PMP70 corresponded to that of peroxisomal catalase. These results suggest that PMP70 is located in the peroxisomes of H-4-II-E cells.

**Intracellular Transport and Sorting of PMP70 in H-4-II-E Cells**—H-4-II-E cells were labeled for 5 min with [<sup>35</sup>S]methionine, and the radioactivity was chased for up to 60 min. The cells were homogenized and fractionated by differential or sucrose gradient centrifugation. As shown in Fig. 2A, immediately after labeling (chase, 0 min) <sup>35</sup>S-PMP70 was initially detected in the postperoxisomal supernatant fraction (lane 2). After chase periods of 2.5–10 min, the amount of <sup>35</sup>S-PMP70 decreased in the postperoxisomal fraction and increased in the particulate fraction (lanes 3–10). After a 15-min chase, <sup>35</sup>S-PMP70 was detected only in the particulate fractions (lanes 11–18). The molecular size of <sup>35</sup>S-PMP70 did not change during a chase period of 1 h. The localization of <sup>35</sup>S-PMP70 was further analyzed by sucrose gradient centrifugation (Fig. 2B). After 5 min of labeling, <sup>35</sup>S-PMP70 was mainly recovered in fractions 1–2, which corresponded to a cytosolic marker enzyme, lactate dehydrogenase (Fig. 1C), suggesting that the <sup>35</sup>S-PMP70 was located in the cytosol. After 7.5 min of chase, <sup>35</sup>S-PMP70 in fractions 1–2 disappeared, but it was detected in fractions 7–9.

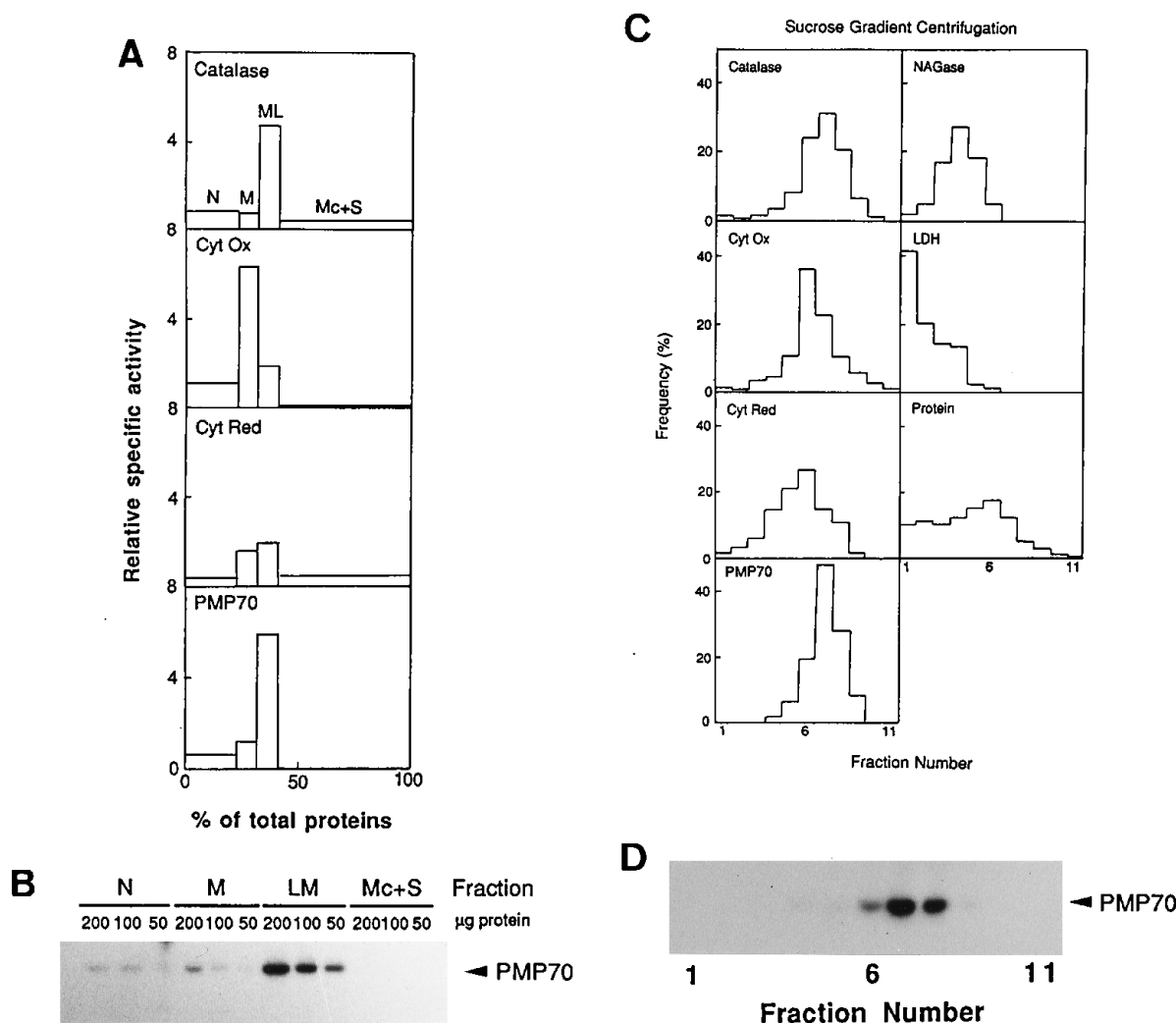


FIG. 1. **Subcellular localization of PMP70 in H-4-II-E cells.** A, H-4-II-E cells were fractionated by differential centrifugation into nuclear fraction (N), mitochondrial fraction (M), light mitochondrial fraction (LM), and microsomal and cytosolic fraction (Mc+S). Catalase, cytochrome *c* oxidase (Cyt Ox), and NADPH-cytochrome *c* reductase (Cyt Red) were measured as marker enzymes of peroxisomes, mitochondria, and microsomes, respectively. The recoveries varied between 80 and 115%. Distribution of PMP70 was calculated from scanning densitometry of the autoradiogram shown in B. B, immunoblot analysis of PMP70. Membranes in each subcellular fraction were prepared by a  $\text{Na}_2\text{CO}_3$  procedure. Applied samples for immunoblot analysis were derived from 50 to 200  $\mu\text{g}$  of protein of each subcellular fraction. C, a light mitochondrial fraction from H-4-II-E was fractionated by sucrose gradient centrifugation. N-Acetyl- $\beta$ -D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH) were measured as marker enzymes of lysosomes and cytosol, respectively. The recoveries varied between 70 and 120%. D, immunoblot analysis of PMP70. Membranes were prepared from 100  $\mu\text{l}$  of each fraction from the top (No. 1) to the bottom (No. 11) of the gradient.

The peak of  $^{35}\text{S}$ -PMP70 in the gradient corresponded to that of catalase. After a 15- or 60-min chase, the peak of  $^{35}\text{S}$ -PMP70 also corresponded to that of catalase.

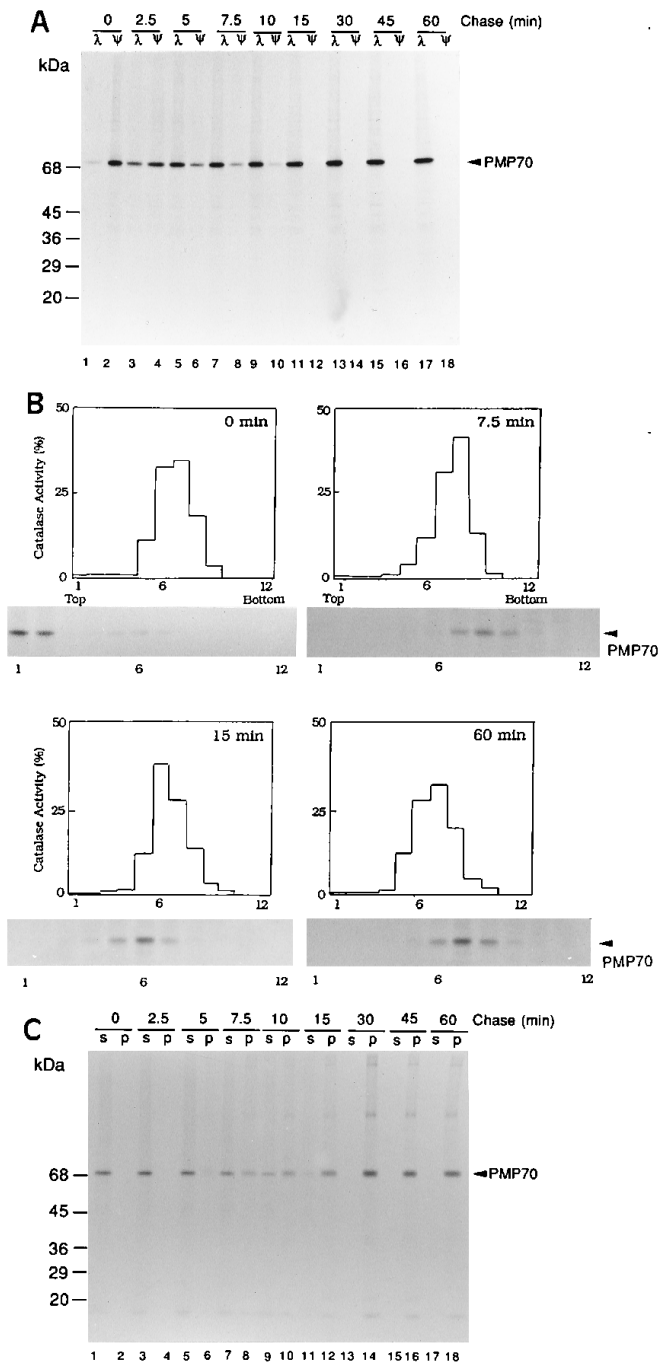
To analyze the kinetics of  $^{35}\text{S}$ -PMP70 integration into membranes, PNS fractions isolated after different chase periods were separated into membrane and soluble fractions by a  $\text{Na}_2\text{CO}_3$  procedure. As shown in Fig. 2C, lanes 1 and 2, immediately after labeling the cell almost all the  $^{35}\text{S}$ -PMP70 was recovered in the soluble fraction, suggesting that the protein had not been integrated into any membrane. After chase periods of 2.5–15 min, the amount of  $^{35}\text{S}$ -PMP70 increased gradually in the membrane fraction (lanes 3–12) and almost 100% of  $^{35}\text{S}$ -PMP70 was recovered in the membrane fraction after 30 min (lanes 13 and 14).

Quantification of the radioactivity was achieved by densitometric scanning of the autoradiographs in Fig. 2. As shown in Fig. 3, newly synthesized  $^{35}\text{S}$ -PMP70 appeared first in the cytosol, after which it transferred to the peroxisomes with a half-time of approximately 3 min. The time course of  $^{35}\text{S}$ -PMP70 integration into membranes was delayed in comparison to that of  $^{35}\text{S}$ -PMP70 association with peroxisomes. These re-

sults suggest that PMP70 is associated post-translationally to peroxisomes and then integrated into their membranes.

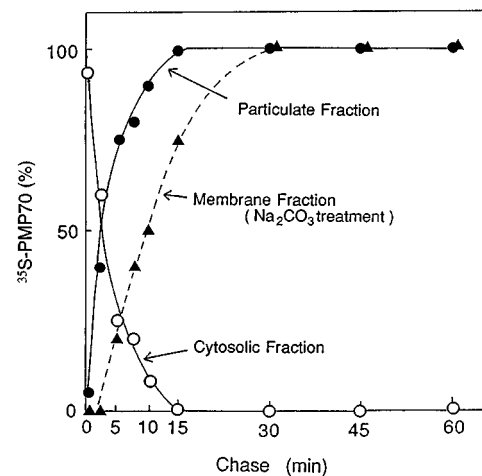
**In Vitro Translation of PMP70 mRNA**—The PMP70 cDNA was transcribed with T7 RNA polymerase and the transcript translated *in vitro* in a rabbit reticulocyte lysate in the presence of [ $^{35}\text{S}$ ]methionine.  $^{35}\text{S}$ -Labeled polypeptide, with a molecular mass of 70 kDa, and several smaller polypeptides were detected in the translation mixture (Fig. 4, lane 1). In order to determine whether these polypeptides were translation products derived from PMP70 mRNA, the mixture was immunoprecipitated with anti-PMP70 or antibodies to the COOH-terminal 15 amino acids of PMP70. As shown in lanes 3 and 4, a  $^{35}\text{S}$ -labeled polypeptide of 70 kDa and polypeptides greater than 30 kDa were immunoprecipitated with both antibodies, whereas these polypeptides were not immunoprecipitated with preimmune serum (lane 2). These results indicate that the 70-kDa polypeptide is an intact PMP70. Other immunoprecipitable polypeptides are thought to be translated from any of 20 internal methionine residues in the PMP70 cDNA.

**In Vitro Insertion of PMP70 to Peroxisomes**—In a typical experiment,  $^{35}\text{S}$ -PMP70 synthesized in a cell-free system was

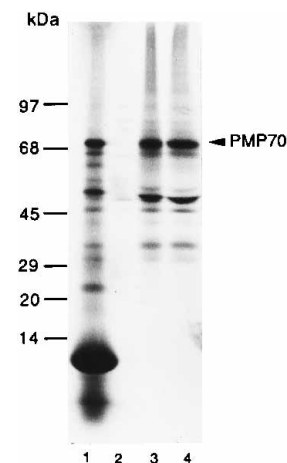


**FIG. 2. Synthesis and intracellular transport of PMP70 in H-4-II-E cells.** H-4-II-E cells were pulsed for 5 min and chased for 0–60 min. The cells at each chase period were homogenized and PNS fractions were prepared. **A**, each PNS fraction was fractionated into supernatant (post-peroxisomal supernatant) ( $\psi$ ) and particulate ( $\lambda$ ) fractions by centrifugation at  $16,000 \times g$  for 20 min. Aliquots (200  $\mu$ l) of each fraction (1.0 ml) were subjected to immunoprecipitation. **B**, PNS fractions prepared in a separate chase experiment were fractionated by sucrose density centrifugation. A portion (500  $\mu$ l) of each fraction was subjected to immunoprecipitation. **C**, portions of the PNS fractions obtained in **A** were fractionated into soluble (S) and membrane (P) fractions by  $\text{Na}_2\text{CO}_3$  treatment. Both fractions were subjected to immunoprecipitation.

incubated for 60 min at 0 °C or 26 °C in the presence or absence of purified rat liver peroxisomes (Fig. 5A). A significant amount (~50%) of mature size  $^{35}\text{S}$ -PMP70 was associated with peroxisomes at 26 °C (lanes 7 and 9). Truncated  $^{35}\text{S}$ -PMP70s, with a molecular mass greater than ~50 kDa, were also associated with peroxisomes, whereas truncated  $^{35}\text{S}$ -PMP70s smaller



**FIG. 3. Kinetics of intracellular transport of PMP70 after pulse-chase labeling.** The points represent the radioactivities of immunoprecipitates of Fig. 2. ●, particulate fraction; ○, supernatant fraction; ▲, membrane fraction after  $\text{Na}_2\text{CO}_3$  treatment.



**FIG. 4. *In vitro* synthesis of PMP70.** PMP70 was synthesized from a cDNA by coupling *in vitro* transcription and translation (lane 1). The *in vitro* translation products were immunoprecipitated with preimmune serum (lane 2), anti-PMP70 (lane 3), or anti-COOH-terminal 15 amino acids of PMP70 (lane 4).

than 45 kDa were not. No  $^{35}\text{S}$ -PMP70 was recovered in pellets in the absence of peroxisomes (lanes 3 and 5). Association of  $^{35}\text{S}$ -PMP70s with peroxisomes was less than 5% of input  $^{35}\text{S}$ -PMP70s at 0 °C (lanes 15 and 17). When the pelleted peroxisomes were re-suspended in 0.25 M SVE and separated by a  $\text{Na}_2\text{CO}_3$  procedure into soluble and membrane fractions, almost all the intact PMP70 and truncated PMP70s greater than 50 kDa were recovered in the membrane pellets (Fig. 5B, lanes 3 and 5), suggesting that these polypeptides were inserted into peroxisomal membranes. In a control experiment in the absence of peroxisomes, almost no  $^{35}\text{S}$ -PMP70 was recovered in the membrane fraction following the  $\text{Na}_2\text{CO}_3$  procedure (data not shown). The insertion of  $^{35}\text{S}$ -PMP70 into peroxisomes increased with time up to 40 min (Fig. 6). These results suggest that  $^{35}\text{S}$ -PMP70 is associated with and inserted into peroxisomes *in vitro*. In addition, an internal sequence(s) of PMP70 seems to be important to target PMP70 to peroxisomes, since the intact PMP70 and the truncated PMP70s greater than 50 kDa were associated with peroxisomes.

**Organelle Specificity of PMP70 Insertion**— $^{35}\text{S}$ -PMP70 was incubated with isolated peroxisomes or mitochondria. As shown in Fig. 7, A and B, intact  $^{35}\text{S}$ -PMP70, as well as truncated  $^{35}\text{S}$ -PMP70 greater than 50 kDa were associated with,

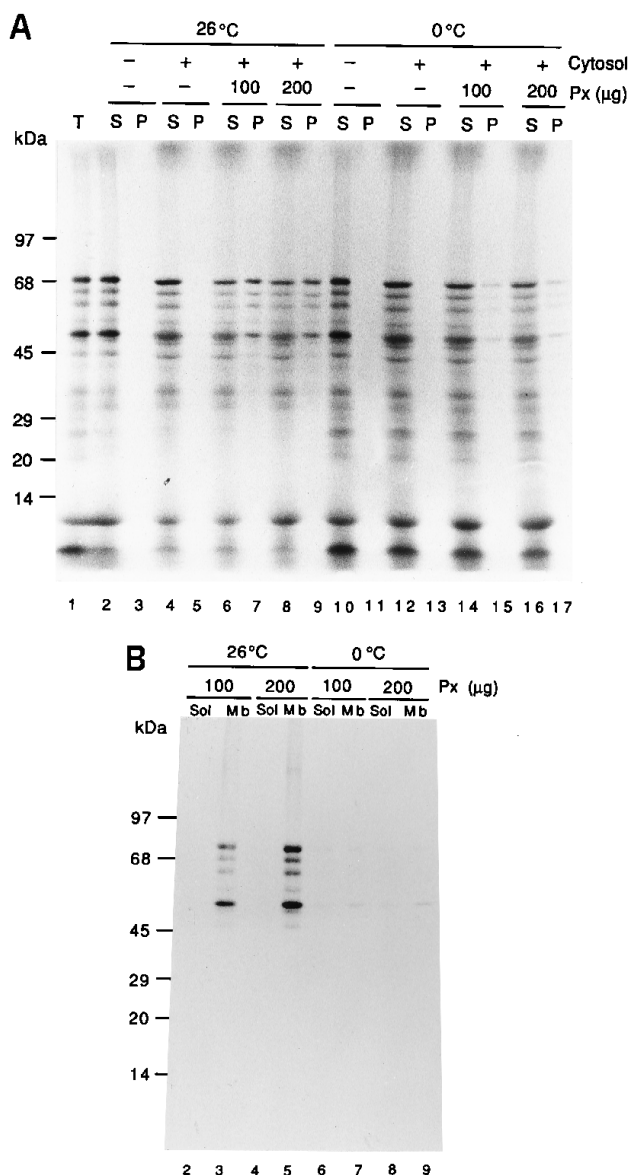


FIG. 5. *In vitro* import of PMP70 to rat liver peroxisomes. The *in vitro* translation products of PMP70 mRNA were mixed at 0 °C with or without rat liver peroxisomes (100 or 200  $\mu$ g) in a volume of 250  $\mu$ l of SEH containing 1 mM ATP, 3 mM  $MgCl_2$ , 50 mM KCl, and 1.5 mg of cytosolic proteins as described under "Experimental Procedures." After incubation for 60 min at 26 or 0 °C, the peroxisomes were separated into the supernatant (S) and pellet (P) by centrifugation at  $14,000 \times g$  for 20 min. *A*, *in vitro* association of PMP70 to peroxisomes. Aliquots of P and S fractions were analyzed by SDS-PAGE and fluorography. *T*, total translation products corresponding to 50% of the input radioactivity. *B*, peroxisomal pellets after *in vitro* import experiment were fractionated in soluble (Sol) and membrane (Mb) fractions by  $Na_2CO_3$  treatment. Aliquots of both fractions were analyzed by SDS-PAGE and fluorography.

and integrated into, peroxisomes. On the other hand,  $^{35}S$ -PMP70 neither associated with nor integrated into mitochondrial membranes. The major portion of  $^{35}S$ -PMP70 was recovered in the supernatant.

**Protease Pretreatment of Peroxisomes Inhibits Association and Insertion of PMP70**—Peroxisomes were treated with trypsin and chymotrypsin, and then re-isolated. This protease pretreatment resulted in the disappearance of intact PMP70 and PMP22, whereas many matrix proteins inside the peroxisomes were not degraded (Fig. 8A). Furthermore, the latency of catalase remained unchanged (about 80%). This mild protease treatment resulted in the binding of  $^{35}S$ -PMP70s to peroxi-

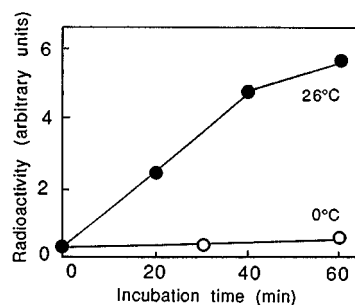


FIG. 6. **Time dependent insertion of PMP70.** The insertion assay was carried out with 200  $\mu$ g of peroxisomal protein and peroxisomal membranes were prepared by  $Na_2CO_3$  treatment. Radioactivity is expressed in arbitrary units as the area of each peak obtained by densitometric scanning of 70-kDa protein band.

somes being almost completely abolished (Fig. 8B) and the  $^{35}S$ -PMP70s quantitatively recovered in the supernatant fraction (lanes 4 and 5 versus lanes 2 and 3). These results suggest that a proteinaceous component(s) on peroxisomal membranes is involved in the insertion of  $^{35}S$ -PMP70.

**Effect of NEM and ATP on Insertion of PMP70**—It has been suggested that a NEM-sensitive factor(s) associated with peroxisomes may be involved in the import of human serum albumin cross-linked with the PTS1 in a permeabilized cell system (33). In order to test whether it is necessary to have free sulfhydryl groups present, both peroxisomal or cytosolic fractions, together with translation products, were treated with 1 mM NEM for 10 min and the excess NEM was blocked by 2.5 mM dithiothreitol. As shown in Fig. 9, *A* and *B*, association and insertion of  $^{35}S$ -PMP70s to peroxisomes was inhibited slightly (~20%) by this treatment. On the other hand, import of AOx into isolated peroxisomes was inhibited approximately 90% by pretreatment of peroxisomes with NEM (Fig. 9C, lanes 4 and 5 versus lanes 2 and 3).

The translocation of AOx, firefly luciferase, and human serum albumin with PTS through peroxisomal membranes has been shown to require ATP hydrolysis both *in vitro* (9) and in a permeabilized cell system (33, 34). As shown in Fig. 10, depletion of ATP with apyrase did not reduce association and insertion of  $^{35}S$ -PMP70. Under this condition, the ATP content of the import mixture was less than 5  $\mu$ M, a concentration too small to enable the translocation of AOx into peroxisomes (9). Treatment of the import mixture with heat inactivated enzyme also had no effect.

## DISCUSSION

**Intracellular Transport of PMP70 in H-4-II-E Cells**—First, the presence of PMP70 in H-4-II-E cells was investigated, since a peroxisomal localization of PMP70 has been confirmed only in rat liver (16, 17), human liver (35), and human fibroblasts (36, 37). The following evidence suggested that PMP70 is an integral membrane protein of peroxisomes in H-4-II-E cells. First, following differential centrifugation, PMP70 was enriched in the light mitochondrial fraction as catalase was enriched (Fig. 1, *A* and *B*). Second, the distribution of PMP70 in a sucrose gradient was similar to that of catalase (Fig. 1, *C* and *D*), and third, PMP70 was recovered entirely in the membrane fraction following after a  $Na_2CO_3$  procedure (data not shown).

In light of these experiments, pulse-chase studies were carried out. It was shown that PMP70 was synthesized by free polysomes in the cytosol, and then rapidly transferred post-translationally to peroxisomal membranes. The initial cytosolic location of PMP70 was shown by the fact that the newly synthesized PMP70 was mainly in the cytosolic fraction at chase 0 min (Figs. 2 and 3). The kinetics of PMP70 transport suggest that PMP70 has a half-life in the cytosol of approximately 3

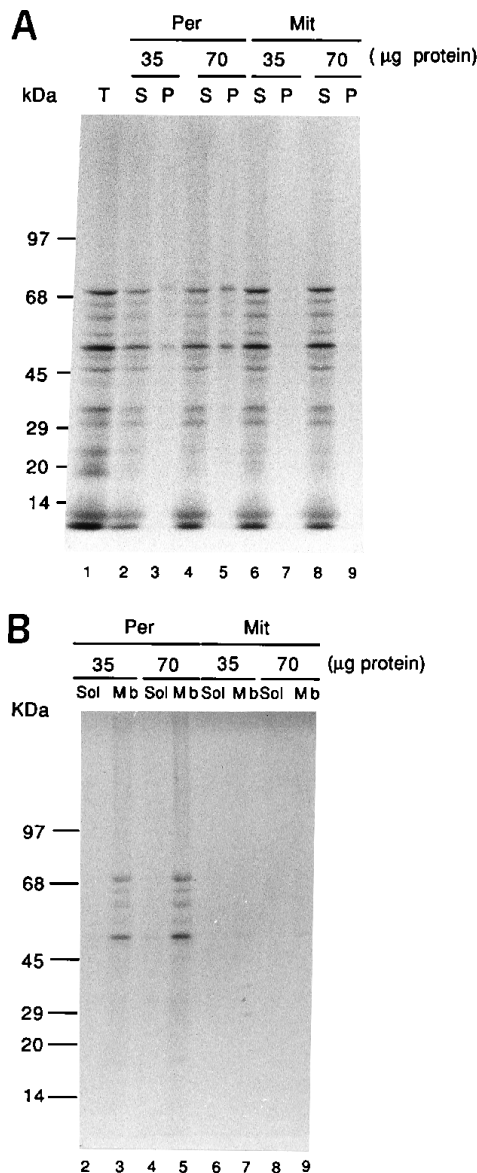


FIG. 7. **Organelle specificity of the import of PMP70.** The import assay was carried out with equal amounts of peroxisomal and mitochondrial protein. **A**, association of PMP70 with peroxisomes or mitochondria. **T**, total translation products corresponding to 50% of the input radioactivity. **B**, insertion of PMP70 into peroxisomal membranes. **S**, supernatant; **P**, pellet; **Sol**, soluble; **Mb**, membrane.

min. In previous studies of the post-translational import of peroxisomal matrix proteins, rat liver urate oxidase and hydratase-dehydrogenase have a half-life in the cytosol of <5 min, catalase has 14 min (6) and yeast *Candida boidinii* alcohol oxidase 20 min (38). Thus PMP70 is rapidly transported to peroxisomes as on a similar time scale to urate oxidase and hydratase-dehydrogenase. In addition, insertion of PMP70 into membranes was delayed slightly compared to the binding of PMP70 to peroxisomes (Fig. 3), suggesting that newly synthesized PMP70 is transported to peroxisomes and then integrated into their membranes. Additional studies showed that PMP70 is synthesized at the mature size with no detectable proteolytic processing occurring upon transfer into the peroxisomal membranes. This is in agreement with previous studies which showed that the size of the translation product of PMP70 was indistinguishable from PMP70 present in peroxisomes (25, 26).

It seems unlikely that newly synthesized PMP70 is first transported to the endoplasmic reticulum and then buds as

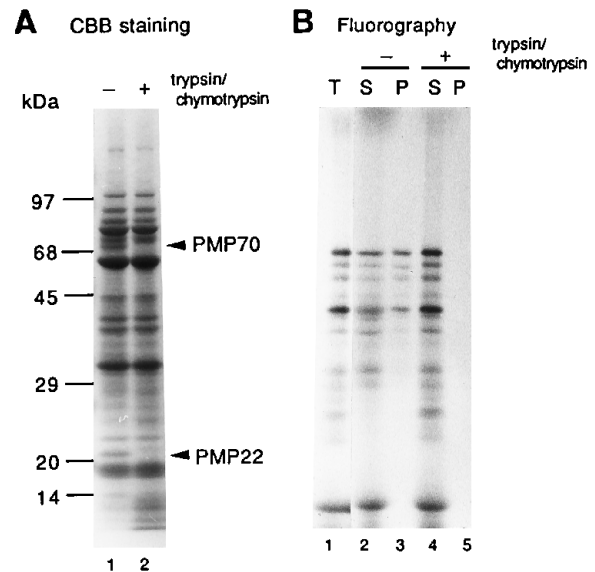


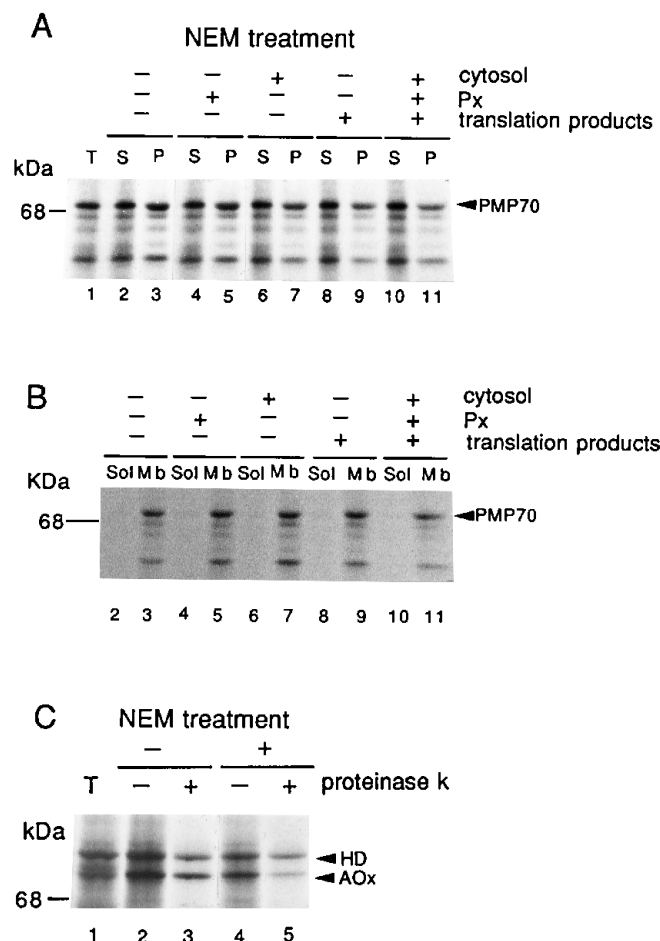
FIG. 8. **Effect of mild trypsin and chymotrypsin pretreatment on the ability of peroxisomes to import PMP70.** Peroxisomes (600  $\mu$ g) were treated with trypsin and chymotrypsin (6  $\mu$ g each) in 100  $\mu$ l of 0.25 M SVE containing 5 mM Hepes/KOH, pH 7.4, at 0  $^{\circ}$ C for 10 min. After incubation, protease inhibitors (30  $\mu$ g of soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml leupeptin, chymostatin, antipain, and pepstatin A) were added to the reaction mixture. The samples were diluted 10-fold with the above solution and centrifuged at  $16,000 \times g$  for 20 min and the peroxisomal pellets were resuspended in 0.25 M SEH. **A**, peroxisomal proteins stained with Coomassie Brilliant Blue (CBB). **B**, association of PMP70 to peroxisomes. Import assay was carried out with 150  $\mu$ g of peroxisomal protein. **T**, total translation products corresponding to 50% of the input radioactivity. **S**, supernatant; **P**, pellet.

small membrane vesicles targeted to peroxisomes. Since  $^{35}$ S-PMP70 in the postperoxisomal fraction at 0 min chase was not sedimented by centrifugation at  $100,000 \times g$  for 1 h, while almost all the microsomes were recovered in the pellet (data not shown). Furthermore, *in vitro* translation products of PMP70 mRNA were neither associated with nor inserted into microsomal membranes in an *in vitro* import assay (data not shown).

**In Vitro Insertion of PMP70 in Purified Rat Liver Peroxisomes**—An *in vitro* import system was reconstituted to characterize the insertion mechanism of PMP70. Under standard conditions, ~50% of newly synthesized PMP70 with a molecular mass greater than 50 kDa becomes associated with the peroxisomes and ~80% of it is inserted into their membranes (Fig. 5). Evidence from our *in vitro* assay seems to reflect a specific process of association and insertion of PMP70 into peroxisomal membrane. 1) The association and insertion of PMP70 were temperature dependent and no such association occurred at 0  $^{\circ}$ C. 2) This association and insertion was also dependent upon time (Fig. 6) and peroxisome concentration (data not shown). 3) Both the association and insertion were organelle specific and association and insertion was not observed in the mitochondrial fraction (Fig. 7). 4) In the absence of peroxisomes only trace amounts of PMP70 were pelleted, excluding the possibility that newly synthesized polypeptides might form aggregates to be pelleted, even in the absence of peroxisomes. In addition, the association of PMP70 was cytosol dependent, if cytosol was omitted from the import assay, the association was reduced to one-half (data not shown).

The organelle specificity suggest that specific factor(s) on peroxisomal membranes may participate in binding and/or insertion of PMP70. The existence of a protein which mediates the binding and insertion is supported by the present finding

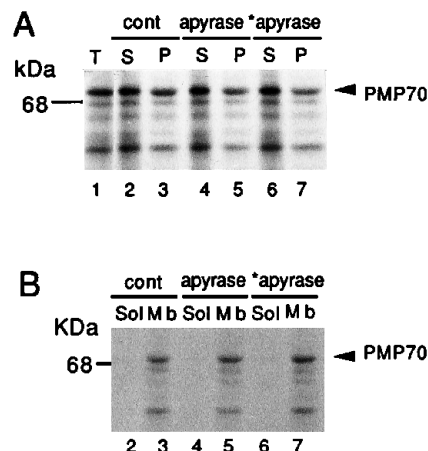




**FIG. 9. Effect of NEM on the import of PMP70.** Isolated peroxisomes, cytosol, and translation products of PMP70 mRNA were treated with 1 mM NEM for 10 min at 26 °C. After incubation excess NEM was blocked by the addition of 2.5 mM dithiothreitol. **A**, association; and **B**, insertion. **Lanes 2 and 3**, control (no treatment of NEM); **lanes 4 and 5**, NEM-treated peroxisomes; **lanes 6 and 7**, NEM-treated cytosol; **lanes 8 and 9**, NEM-treated translation products; **lanes 10 and 11**, NEM-treated peroxisomes, cytosol, and translation products. **T**, total translation products corresponding to 50% of the input radioactivity. **C**, import of AOx into peroxisomes. Import assay was carried out with control or NEM-treated peroxisomes. At the end of import assay, one of duplicated samples (+) was treated with 4  $\mu$ g of proteinase K at 0 °C for 15 min. The peroxisomes were pelleted by centrifugation and analyzed by SDS-PAGE and fluorography according to the method described in Ref. 31. **HD**, hydratase-dehydrogenase. **T**, total translation products corresponding to 20% of input radioactivity.

that mild proteolytic pretreatment of the peroxisomes abolished both binding and insertion of PMP70 into the peroxisomal membranes (Fig. 8). We do not yet know which proteins(s) is responsible, since even under the mild conditions employed a number of PMPs were damaged. However, it appears unlikely that the essential protein is one of the small PMPs such as 22, 26/27, and 35/36 kDa, since these remained undamaged under conditions where PMP70 insertion was markedly reduced (data not shown).

Another important point addressed by the present study is the topogenic sequence of PMP70. It is suggested that PMP70 contains six putative transmembranes and the ATP-binding motif at the carboxyl-terminal is exposed to the cytosol, although the exact location of the amino terminus has not yet been determined (20). PMP70 must use a targeting signal other than PST1 or PST2, since PMP70 has PST1- or PST2-like sequences at the COOH or NH<sub>2</sub> terminus (20). As shown in the case of mitochondrial outer membrane proteins (39), the transmembrane domains of PMP70 will be necessary to anchor it in



**FIG. 10. Effect of ATP on the import of PMP70.** Depletion of ATP from the import assay. Peroxisomes were incubated with translation products and cytosol which were treated either with apyrase (1.4 units/250  $\mu$ l) at 26 °C for 10 min or with heat inactivate apyrase in order to deplete ATP from translation products and cytosol. **A**, association; **B**, insertion. **Lanes 2 and 3**, control; **lanes 4 and 5**, active apyrase; **lanes 6 and 7**, inactive apyrase. **T**, total translation products corresponding to 50% of the input radioactivity. **S**, supernatant; **P**, pellet; **Sol**, soluble; **Mb**, membrane.

the membranes and a signal sequence near the stop-anchor sequence will be required for specific association with only the peroxisomal membranes. The truncated <sup>35</sup>S-PMP70s greater than 50 kDa bound to and inserted into peroxisomal membranes, whereas the truncated <sup>35</sup>S-PMP70s smaller than 45 kDa did not (Fig. 5). This association of several truncated PMP70s to peroxisomes gives a clue as to the position of the signal sequence of PMP70. Furthermore, the efficiency of association and insertion of truncated <sup>35</sup>S-PMP70s greater than 50 kDa seem to be essentially the same (Figs. 5 and 7–10). These results suggest that an internal region of PMP70, especially the region around 20–25 kDa from the NH<sub>2</sub>-terminal is necessary for peroxisomal targeting and insertion. This region roughly corresponds to the predicted third transmembrane sequences. Recently McCammon *et al.* (40) suggested that PMP47 of the methylotrophic yeast, *C. boidinii*, possess a peroxisomal targeting sequence in an internal region, which nearly corresponds to the fourth of six putative transmembrane regions.

The insertion mechanism of PMP70 into peroxisomal membranes seems to be different from the translocation mechanism of several peroxisomal matrix proteins. In the case of the matrix protein import, ATP hydrolysis (9, 33, 34) and NEM-sensitive component(s) of peroxisomal membranes (33) are required. However, insertion of PMP70 into peroxisomal membranes did not need ATP (Fig. 10) and NEM-sensitive component(s) (Fig. 9). It remains, however, unresolved whether ATP or the NEM-sensitive factor is required for correct folding of PMP70.

Very recently Diestelkotter and Just (41) demonstrated that PMP22 was inserted into peroxisomal membranes of liver prepared from clofibrate-treated rat *in vitro*. The insertion of <sup>35</sup>S-PMP22 into peroxisomes seems to be mediated by a proteinaceous receptor, did not require ATP, and was not inhibited by NEM treatment. The insertion of PMP70 seems to be facilitated by a similar mechanism to PMP22. However, the association of PMP22 to peroxisomal membranes occurred at 0 °C and 26 °C with the same efficiency, whereas the association of PMP70 did not occur at 0 °C, suggesting that binding of PMP70 and PMP22 have different features. Different types of receptor(s) or cytosolic factor(s) may facilitate binding of PMP70 and PMP22. A detailed comparison of both insertion mechanisms will be the subject of future research.

In this study, we confirmed that PMP70 was post-translationally transported to and inserted into peroxisomal membranes without processing. Furthermore, we suggest that a proteinaceous receptor and a certain internal sequence of PMP70 might be involved in the binding and the insertion of PMP70 into the peroxisomal membranes. Identification of the receptor as well as the minimum topogenic sequence are now under investigation.

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