The absence of Pmp47, a putative yeast peroxisomal transporter, causes a defect in transport and folding of a specific matrix enzyme

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Abstract. Candida boidinii Pmp47, an integral peroxisomal membrane protein, belongs to a family of mitochondrial solute transporters (e.g., ATP/ADP exchanger), and is the only known peroxisomal member of this family. However, its physiological and biochemical functions have been unresolved because of the difficulties in the molecular genetics of C. boidinii. In this study, we first isolated the PMP47 gene, which was the single gene encoding for Pmp47 in a gene-engineerable strain S2 of C. boidinii. Sequence analysis revealed that it was very similar to PMP47A and PMP47B genes from a polyploidal C. boidinii strain (ATCC32195). Next, the PMP47 gene was disrupted and the disruption strain (pmp47AΔ) was analyzed. Depletion of Pmp47 from strain S2 resulted in a retarded growth on oleate and a complete loss of growth on methanol. Both growth substrates require peroxisomal metabolism. EM observations revealed the presence of peroxisomes in methanol- and oleate-induced cells of pmp47AΔ, but in reduced numbers, and the presence of material of high electron density in the cytoplasm in both cases. Methanol-induced cells of pmp47AΔ were investigated in detail. The activity of one of the methanol-induced peroxisome matrix enzymes, dihydroxyacetone synthase (DHAS), was not detected in pmp47AΔ. Further biochemical and immunocytochemical experiments revealed that the DHAS protein aggregated in the cytoplasm as an inclusion body, while two other peroxisome matrix enzymes, alcohol oxidase (AOD) and catalase, were active and found in peroxisomes. Two peroxisome-deficient mutants, strains M6 and M13 (described in previous studies), retained DHAS activity although it was mislocalized to the cytoplasm and the nucleus. We disrupted PMP47 in these peroxisome-deficient mutants. In both strains, M6-pmp47AΔ and M13-pmp47AΔ, DHAS was enzymatically active and was located in the cytoplasm and the nucleus. We suggest that an unknown small molecule, which PMP47 transports, is necessary for the folding or the translocation machinery of DHAS within peroxisomes. Pmp47 does not catalyze folding directly because active DHAS is observed in the M6-pmp47AΔ and M13-pmp47AΔ strains. Since both AOD and DHAS have the PTS1 motif sequences at their carboxyl terminal, our results first show that depletion of Pmp47 could dissect the peroxisomal import pathway (PTS1 pathway) of these proteins.

Eukaryotic cells are compartmentalized into several organelles that have single, double, or triple membranes. Organelle matrix enzymes are translocated into the targeted organelle by its cis-targeting signal and trans-acting translocation machinery, and they must fold properly and often obtain coenzymes to acquire enzymatic activity. From the organelle's side, each organelle membrane has to import and export not only proteins, but also small molecules between the cytoplasm and the organelle matrix to activate enzymes, maintain ion gradients, and often provide energy. These processes are often linked with protein translocation.

The peroxisome is a class of ubiquitous eukaryotic organelles where various kind of oxidative metabolisms are executed. Current interest in peroxisomes comes not only from their functions, but also from their importance regarding human genetic diseases. Notably, human peroxisomal transporters, Pmp70 and ALDP, both belonging to the ABC transporter family, were reported to be responsible for genetic disorders, Zellweger syndrome (ZS), and X-linked adrenoleukodystrophy (ALD; Kamiyo et al., 1990; Gärtnert et al., 1992; Mosser et al., 1993), respectively. Both are severe disorders that usually lead to death within several years of birth. In ZS patients, normal peroxisomes are absent, but peroxisomal enzymes are usually active and are mislocalized in the cytoplasm. ALD pa-

1. Abbreviations used in this paper: ALD, adrenoleukodystrophy; AOD, alcohol oxidase; CAT, catalase; DHAS, dihydroxyacetone synthase; KPB, potassium phosphate buffer; ZS, Zellweger syndrome.
patients are deficient in a peroxisomal enzyme, very long chain fatty acyl CoA synthase (VLCFAS) (Van den Bosch et al., 1992). However, the roles of these transporters in peroxisome assembly or in VLCFAS enzyme activity are not clear.

It is known that there are at least three independent pathways of protein import into peroxisomes (Subramani, 1993; Purdie and Lazarow, 1994). Many peroxisomal proteins contain a carboxyl-terminal–targeting signal, PTS1, represented by the sequence -SKL or its derivatives, 3-ketoacyl-CoA thiolase and several others contain an NH₂-terminal targeting signal, PTS2, a sequence of 25–36 amino acids in length (Swinkels et al., 1991). Recently, another targeting signal for an integral peroxisome membrane protein, Pmp47 for Candida boidinii (McCannon et al., 1990, 1994), has been found to exist. Pichia pastoris Pas8p is considered to be the PTS1 receptor (McCollum et al., 1993; Terlecky et al., 1995). In the pas8 mutant, thiolase is competent for peroxisomal protein import, but other PTS1-containing proteins are mislocalized into the cytoplasm. Recently, the human homologue of Pas8p, PXRI, has been cloned, and it has been shown to complement some ZS cell lines (Dodd et al., 1995; Wiemer et al., 1995).

There are both biochemical and genetic advantages to study peroxisomal assembly in methylotrophic yeasts, P. pastoris, Hansenula polymorpha, and C. boidinii (Bellion and Goodman, 1987; Hansen et al., 1992; Subramani, 1993; Heyman et al., 1994): (a) peroxisomal proliferation is robust, such that peroxisomes can comprise most of the cytoplasmic volume; (b) proliferation can be easily detected and studied by EM; and (c) only two enzymes, alcohol oxidase (AOD) and dihydroxyacetone synthase (DHAS), comprise most of the matrix mass.

In respect to applied fields, both AOD and DHAS have biotechnological importance. The use of the AOD promoter for the expression of heterologous genes in methylotrophic yeasts is now a commonly used technology in molecular biology and in the production of heterologous proteins (Gellissen et al., 1991; Cregg, 1993; Sakai et al., 1994, 1994c). AOD can be used to produce various useful aldehydes (Sakai and Tani, 1987, 1988), and recently, we described a new enzymatic method to prepare 13C-labeled dihydroxyacetone phosphate by the combined reaction system of AOD and DHAS (Yanase et al., 1995). Therefore, revealing the mechanism of translocation and folding of these enzymes will give us useful information to overproduce these enzymes in peroxisomes of methylotrophic yeast cells.

Pmp47 of C. boidinii is an integral membrane protein that belongs to a protein family of mitochondrial solute carriers (e.g., ATP/ADP exchanger; Jank et al., 1993; Kuan and Saier, 1993). Pmp47 is induced on divergent peroxisome-inducing carbon sources (methanol, oleate, and D-alanine), suggesting its importance in basic peroxisomal matrix proteins (Gellissen et al., 1991; Cregg, 1993; Sakai et al., 1990, 1994), has been found to exist. Pichia pastoris Pas8p is considered to be the PTS1 receptor (McCollum et al., 1993; Terlecky et al., 1995). In the pas8 mutant, thiolase is competent for peroxisomal protein import, but other PTS1-containing proteins are mislocalized into the cytoplasm. Recently, the human homologue of Pas8p, PXRI, has been cloned, and it has been shown to complement some ZS cell lines (Dodd et al., 1995; Wiemer et al., 1995).

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Materials and Methods

Strains, Media, and Cultivation

C. boidinii strains TK62 (ura), Sakai et al., (1991), M6 (ura3), and M13 (ura3) were used as hosts for transformation. The latter two strains were derived from strain TK62 as nonutilizers of multiple peroxisomal proliferating carbon sources (methanol, oleate, and D-alanine), and were characterized as peroxisome-deficient strains in a previous study (Sakai et al., 1995b). C. boidinii transformant GC (Sakai et al., 1995c) was used as the wild-type strain. Escherichia coli XL-1 Blue (Ausubel et al., 1987) was used for plasmid propagation.

Synthetic MI media (Sakai et al., 1995) were used for growth experiments and for the preparation of cells for EM. The concentrations of the carbon sources used were 0.7% (vol/vol) methanol, 0.5% (vol/vol) oleate, 0.6% (wt/vol) D-alanine, 2% (wt/vol) glycerol, and 2% (wt/vol) glucose. Tween 80 was added to the oleate medium at a concentration of 0.05% (vol/vol). The initial pH of the media were adjusted to 6.0. Cultivation was aerobic at 28°C with shaking, and the growth was followed by measuring the OD at 610 nm. Determination of the growth on oleate was described previously (Sakai et al., 1995b) using a diluted sample of the same medium as the reference. The semisynthetic MI YE medium (MI + 0.5% yeast extract) containing 0.7% (vol/vol) methanol and 2% (wt/vol) glycerol were used for the induction of methanol-induced enzymes and for peroxisome purification for both wild-type and pmp47Δ cells.

DNA and RNA Methods

Southern blotting to a Biodyne nylon membrane (Pall Bio Support, New York, NY) and hybridization were done under high stringent conditions as previously described (Sakai et al., 1995a). Gel-purified DNA was 32P-labeled according to the method of Feinberg and Vogelstein (1983). Total RNA and formaldehyde-denatured gels for Northern analysis were prepared as described previously (Sakai and Tani, 1992). PolyA RNA was purified using the BIOMAG™ mRNA purification kit (PerSeptive Diagnostics, Inc., Cambridge, MA).

Protein Methods and Antibody Preparations

Standard 9% Laemmli gels (Laemmli, 1970), with the separating gel at pH 9.2, were used. Immunoblotting was performed by the method of Towbin et al. (1979) using the ECL detection kit (Amersham, Arlington Heights, IL). The IVA7 monoclonal anti-PMP47 antibody and anti-AOD were kindly provided by Dr. J.M. Goodman (University of Texas Southwestern Medical Center, Dallas, TX). DHAS was purified from the cell-free extract of C. boidinii No. 2201 to an apparent homogeneity on SDS-PAGE as described previously (Kato et al., 1982). Anti-DHAS polyclonal antibody was raised in rabbits, and the antisera were purified using Econo-Pac Serum IgG purification column (Bio Rad Laboratories, Hercules, CA). For immunocytochemical experiments, the material that cross-reacts to the cell wall was removed as follows: C. boidinii cell wall fraction was prepared from a 9–12% dextrin continuous gradient centrifugation of the cell lysate. 10 µl cell wall fraction (prepared from ~1.5 OD610 units cells) was added to a 100-µl anti-AOD antiserum or anti-DHAS IgG fraction. After an overnight incubation at 4°C, the suspension was centrifuged at 20,000 g to remove the cross-reactive material, and the resultant supernatant was used for immunocytochemistry experiments.

Cloning and Sequencing of PMP47 from C. boidinii S2

The probe harboring PMP47 gene from C. boidinii ATCC32195, a 1.2-kb PsI-HindIII fragment (Moreno et al., 1994), was used to clone the PMP47 gene of C. boidinii S2. A Southern analysis of EcoRV-digested genomic DNA of C. boidinii S2 probed with PMP47A fragment revealed a single 3.9-kb band. A pool of EcoRV-digested genomic DNA of approximately this size was gel purified and ligated into the EcoRV site of pBlue- script II SK -. E. coli transformants were transferred onto a Biodyne nylon
DNA was bound to the nylon membrane, and these blots were then used to detect membrane (Pall Bio Support). After the lysis of bacteria, the liberated DNA was performed at 65°C overnight and then the membranes were washed three times in 0.3× SSC at the same temperature. Three clones that showed strong positive signals were found to harbor a recombinant 5.9-kb EcoRV fragment. The total TAM47 gene was read using the synthetic oligos for sequencing primers, and the nested deletion mutants were derived as described previously (Yanisch-Perron et al., 1985). The sequenced region was read on both strands using a 7-deaza sequencing kit from Takara Shuzo (Kyoto, Japan) and PRISM DyeDeoxy Terminator Cycle Sequencing Kit and DNA sequencer model 373A (Applied Biosystems, Inc., Foster City, CA).

Construction of the Disruption Cassette and One-step Gene Disruption of PMP47

The 3.3-kb HincII-HindIII fragment of pMP471 DNA (Fig. 2) containing the truncated COOH-terminal coding region and 3' flanking region of C. boidinii PMP47 was cloned into the multiple cloning site of pBluescript II KS+, yielding pMP47. Next, the 746-bp SspI-EcoRV fragment of pMP47 DNA containing the 5' flanking and truncated NH2-terminal coding region was blunt-ended with T4 polymerase, then ligated to the unique EcoRV site of pMP473, yielding pMP4732. Finally, the BamHI-PstI fragment of C. boidinii URA3 DNA (Sakai and Tani, 1992b) and the HindIII digest of pMP4732 were blunt-ended and subjected to ligation, yielding the fragment PMP47 disruption vector, pMP47BP. This vector had the C. boidinii URA3 DNA as the selectable marker and the truncated C. boidinii PMP47-flanking sequences (Fig. 1). Transformation of C. boidinii strains was performed with the modified lithium acetate method (Sakai et al., 1993).

Preparation of Cell-free Extract and Enzyme Assays

Cells were harvested by centrifugation at 500 g, washed twice with ice-cold distilled water, suspended in 0.1 M potassium phosphate buffer (KPB), pH 7.5, and disrupted with an sonicator (model 201M; Kubota, Co. Ltd., Tokyo, Japan) (2 MHz for 35 min). The cell debris was removed by centrifugation at 20,000 g for 20 min at 4°C. The resultant supernatant fluid was immediately assayed for enzyme activity.

DHAS (EC 2.2.1.3) activity was determined as described previously using β-hydroxyoxypyrurate (lithium salt; Sigma Chemical Co., St. Louis, MO) as the substrate (Yanase et al., 1995). Formaldehyde disappearance, which was dependent on the addition of the substrate, was measured by the method of Nash (1953). 1 U was defined as the amount of enzyme catalyzing the conversion of 1 μmol substrate per minute.

The enzyme activities of alcohol oxidase (EC 1.1.3.13; Tani et al., 1985), catalase (CAT; EC 1.11.1.6; Bergmeyer, 1955), and cytochrome c oxidase (EC 1.9.3.1; Tolbert, 1974), were assayed by the described procedures. Protein was measured by the method of Bradford (1976) with a protein assay kit (Bio Rad Laboratories) using BSA as the standard.

Subcellular Fractionation

Wild-type and pmp47Δ cells, grown on YPD medium for 24–30 h, were washed once and transferred to the semisynthetic glycerol plus methanol medium (2 liters) at an initial OD600 of 0.2–0.4 and grown for 10–16 h. The induced cells were harvested by centrifugation, treated with 0.1 M Tris-HCl, pH 9.3, 10–15 mM DTT for 15 min, and converted to spheroplasts in 1.6 M KCl–10 mM MOPS–5 mM Na2SO4, pH 7.2, containing Zymolyase 100T (~1 mg/150 OD600 U cells) for 60–90 min.

Subfractionation was performed as follows, essentially according to the method of Goodman (1984). All subsequent steps were performed at 2°C. Spheroplasts were harvested by centrifugation at 500 g and suspended in Sorbitol buffer (1.0 M sorbitol, 5 mM MES, pH 5.5, containing 1 mM PMSF), and then osmotically lysed by the addition of 1.0–1.5 vol of ice-cold lysis buffer (0.25 M sorbitol, 5 mM MES, pH 5.5, containing 1 mM PMSF). Lysis was monitored microscopically. The lysate was osmotically adjusted back to 1.0 M sorbitol by the addition of equilibrium buffer (1.75 M sorbitol, 5 mM MES, pH 5.5, containing 1 mM PMSF). Unlysed cells, large organelles, and other cell debris were removed carefully from the lysate by repeating centrifugation at 500 g. The resulting supernatant was subjected to a centrifugation at 20,000 g for 20 min to obtain a crude pellet consisting mainly of peroxisomes and mitochondria.

The obtained organellar suspension was fractionated on a semiconsinuous sucrose gradient. The organellar suspension (~2.0 ml) was layered on top of a 36-mL semiconsinuous gradient (2.0 ml of 30, 36, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52, 54, 56, 60, 63, 66, and 68% (wt/wt) sucrose), and centrifuged at 2°C for 5 h 30 min at 27,000 rpm (100,000 g) in a rotor (SW28; Beckman Instruments, Inc., Fullerton, CA). The gradient was drained by pippetting into 19 fractions from the top (fraction 1) to the bottom (fraction 19). Fractions were assayed for CAT and cytochrome c oxidase activities. For immunoblotting, proteins from the selected fractions (100 μl) were precipitated by the addition of TCA to a final concentration of 10%, washed twice with cold acetone, and resuspended in 100 μl of 1× Laemmli sample buffer. These suspensions were boiled, subjected to SDS-PAGE, and immunoblotted with anti-AOD (×10,000 dilution) and anti-DHAS (×20,000 dilution) antibodies.

To a portion (~100 μl) of the peak fraction of peroxisomes (fraction 13 for wild type and fraction 14 for pmp47Δ), 1 M Tris-Cl buffer, pH 8.0, was added to a final concentration of 30 mM, and the samples were incubated on ice overnight, then spun at 20,000 g to obtain supernatant and pellet fractions (Fig. 5 C). An identical volume of a portion of the pellet suspension was examined with or without the addition of 1 M Tris-Cl buffer. Equivalent portions of pellet and supernatant fractions were loaded on SDS-PAGE and analyzed by immunoblotting.

EM and Immunocytochemistry

Whole cells were fixed in 2.0% glutaraldehyde in 0.1 M KP B, pH 7.2, at 4°C for 2 h. After washing with 0.1 M KP B, the cells were postfixed with 1.5% (wt/vol) KMnO4 at 4°C for 16 h and poststained in 1.5% (wt/vol) aqueous uranyl acetate at room temperature overnight. Afterwards, they were pelleted and dehydrated in a graded acetone series, and were infiltrated in a Spur resin (hard) series (Spur resin/proplylene oxide 1:1, 3:1, 7:1, and 100% Spur resin). Polymerization was performed at 37°C for 24 h, 45°C for 24 h, and 60°C for 2 d.

Cells were fixed in a solution containing 4.0% paraformaldehyde and 1.0% glutaraldehyde in 0.1 M KP B, pH 7.2, at 4°C for 4 h. After fixation, cells were washed once with 0.1 M KP B, pH 7.2, containing 8% (wt/vol) sucrose and 0.05 M NH4Cl, then three times with 0.1 M KP B, pH 7.2, containing 8% sucrose. They were thereafter pelleted and dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, 90%, twice, 100% twice for 10 min) with gentle shaking. The cells were then infiltrated in graded LR White series (The London Resin Co. Ltd., Hampshire, U.K.; LR White/ethanol 1:1 for 60 min, 2:1 for 60 min, and 100% LR White overnight). After replacing 100% LR White by centrifugation, polymerization was performed at 60°C for 3 d.

Immunogold labeling was performed on ultrathin sections using anti-AOD and anti-DHAS antibodies. Sections were quenched in 50 mM glycine in PBS (2 mM KH2PO4, 8 mM Na2HPO4, 150 mM NaCl, pH 7.4) for 10 min. After washing in PBS three times, they were blocked with 5% normal goat serum in the washing buffer (PBS containing 0.8% BSA, 0.1% IGSS quality gelatin, 2 mM Na2SO4) for 30 min, washed in the washing buffer three times, and incubated with primary antibody at the dilution rate of 1/20,000 for anti-AOD and 1/10,000 for anti-DHAS in an incubation buffer (1.0% normal goat serum in the washing buffer) for 2 h. After 5× 10 min washes in the washing buffer, sections were incubated with 15 nm gold-labeled goat anti-rabbit IgG (H+L) (AuroProbe™EM GAR G15; Amersham) diluted 1/30 for 2 h. After washing in PBS twice and distilled H2O for 1 min, sections were stained with 2.0% aqueous uranyl acetate at 45°C for 30 min and then with lead citrate at room temperature for 3 min. Ultrathin sections were cut with a diamond knife on a Reichert-Jung Ultracut E (Reichert-Jung Optische Werke, AG, Vienna, Austria). "Gold colored" sections were transferred to nickel grids and observed under an EM (model 100C, Jeol Ltd., Tokyo, Japan).

Results

C. boidinii S2 Contains a Sole Gene Encoding for Pmp47

Previously, two closely related genes, termed PMP47A and PMP47B, were isolated from C. boidinii ATCC32195.
(Moreno et al., 1994). To determine if strain S2, a haploid strain of C. boidinii, also contained two PMP47 family genes, genomic Southern analysis of C. boidinii S2 DNA was performed using PMP47A or PMP47B as probes. Only one fragment from each digestion bound to the PMP47A probe, and an identical banding pattern was seen with the PMP47B probe. In contrast, genomic DNA from strain ATCC32195 gave doublet or triplet bands on Southern analysis (data not shown). These results indicate that C. boidinii S2 contains only one gene encoding for Pmp47. Similar results were seen with the C. boidinii Pmp30 (Sakai et al., 1995a), i.e., Pmp30 was encoded by only one transporter family (data not shown). An internal region 419-amino acid sequence of Pmp47 was consistent with the flanking regions were also highly conserved. The deduced coding region was 99% identical to PMP30. Differences in amino acid sequences are also shown in bold type. Dashes signify deleted bases. The six-membrane-spanning model for the mitochondrial peroxisomal function because they could not grow on ole-, o-alanine, or methanol as a sole carbon source. C. boidinii PMP30A contains only one gene encoding for Pmp47.

The sequence was submitted to GenBank and was assigned the accession number U53145.

Figure 1. Nucleotide sequences and their deduced amino acid sequences of PMP47 from C. boidinii S2 and PMP47B from C. boidinii ATCC32195. Upper sequence represents the PMP47 gene. The nucleotide and amino acid sequence of PMP47B is shown only when it is different from PMP47. Differences in amino acid sequences are also shown in bold type. Dashes signify deleted bases. The PMP47 sequence was submitted to GenBank and was assigned the accession number U53145.

**Growth Characteristics of C. boidinii pmp47Δ**

Peroxisome-deficient mutants (strains M6 and M13) (Sakai et al., 1995b) of C. boidinii are severely deficient in peroxisomal function because they could not grow on ole-, o-alanine, or methanol as a sole carbon source. C. boidinii PMP47-disruption vector pMP47BP was constructed (Fig. 2 A). C. boidinii TK62, the ura3 derivative of strain S2 (Sakai et al., 1991), was transformed with pMP47BP. The obtained disruptant was named pmp47Δ. Proper gene disruption in pmp47Δ was confirmed by Southern analysis with EcoRI-digested DNA from the transformant (Fig. 2 B). The DNA from the host strain gave a doublet band of 3.7 and 1.6 kb. Only the 3.7-kb band shifted to 4.0 kb in pmp47Δ, as expected for a disruption caused by homologous recombination (Fig. 2 A). In addition, immunoblotting with an anti-Pmp47 revealed the loss of the signal in pmp47Δ (Fig. 2 C). These results confirmed that the haploid strain C. boidinii S2 contained only one gene coding for Pmp47.
Figure 2. One-step gene disruption of PMP47 gene in C. boidinii. (A) Restriction map of the cloned fragment and its disruption strategy. (B) Genomic Southern analysis from EcoRI-digested total DNA (∼3 μg) from various C. boidinii strains with the radiolabeled probe shown in A. (C) Immunoblot analysis of cell-free extract from 10-h methanol-induced cells using anti-Pmp47 monoclonal antibody. About 3 μg protein was loaded on each lane.

dininii mutant defective in the PTS1 receptor homologue gene (PSR1) also showed the same growth phenotype on these carbon sources (Sakai, Y., H. Matso, and N. Kato, unpublished data). In contrast, pmp47Δ was able to grow on oleate and d-alanine plates, although it did not grow on methanol. This result suggested that functional peroxisomes, at least in oleate- and d-alanine–grown cells, were present in the absence of Pmp47. We compared the growth rate in several liquid media between the wild-type and pmp47Δ strains (Fig. 3). Both grew the same in glucose. In contrast, the disruption caused the loss of growth in methanol and a marked defect of growth in oleate. The disruption caused a small but reproducible (in three experiments) inhibition of growth in glycerol, acetate, and d-alanine (d-alanine was used as a single carbon and nitrogen source). In other words, effects of growth in “peroxisomal substrates” caused by PMP47 disruption varied from little (d-alanine) to severe (methanol).

DHAS Is Inactive and Aggregated in pmp47Δ

Because the most severe growth defect was observed on methanol, the phenotype of pmp47Δ grown on methanol-glycerol was studied further; activities of peroxisomal enzymes in the cell-free extracts of methanol-induced cells were determined. Since pmp47Δ could not grow on methanol, glycerol was added to the methanol medium for both wild-type and pmp47Δ strains. The addition of glycerol did
Figure 3. The disruption of PMP47 affects growth in methanol- and oleate-media severely. Strains were grown on synthetic MI medium with the indicated carbon sources. Open symbols, C. boidinii pmp47Δ; closed symbols, wild-type strain.

not repress the methanol-induced enzymes (Sakai et al., 1995c). We could detect no DHAS activity in pmp47Δ, while AOD and CAT were active at levels comparable to the wild-type strain (Fig. 4 A). Immunoblot analysis with anti-DHAS and anti-AOD was performed with the extracts from pmp47Δ. Although anti-AOD gave a strong band of 74 kD, no bands were detected with anti-DHAS (soluble fraction; designated as S in Fig. 4 B, left panel). In this experiment, the cell-free extract was prepared by sonicating the cells, and unbroken cells and insoluble materials were removed by centrifugation at 20,000 g. However, when the aggregated material in the precipitate was solubilized by boiling in Laemmli sample buffer (containing 0.1% SDS) (Laemmli, 1970), a strong 78-kD band cross-reacting with anti-DHAS appeared (Fig. 4 B, left panel; pellet fraction designated as P). This indicated that DHAS was synthesized in pmp47Δ, but was in an inactive and insoluble form in these cells. In contrast, AOD existed in a soluble form in pmp47Δ.

Peroxisomal enzyme activities were also examined with cell-free extracts from oleate- and d-alanine-induced cells. The peroxisomal enzymes tested (CAT, acyl CoA oxidase, isocitrate lyase, thiolase, and d-amino acid oxidase) were active in levels comparable to the wild-type strain (data not shown). However, oleate-induced pmp47Δ accumulated two- to threefold higher levels of unmetabolized oleate within the cells than the wild-type strain, indicating an effect of the gene disruption on the β-oxidation pathway.

Purification of Peroxisomes on Sucrose Gradient Ultracentrifugation

We then tried to purify the peroxisomal fraction from methanol plus glycerol–grown cells of pmp47Δ and wild type on sucrose gradients (Fig. 5) to get information on the subcellular localization of insoluble DHAS in these cells. Cells were spheroplasted, gently disrupted by osmotic lysis, and unbroken cells, nuclei, and cell debris were removed by low speed centrifugation at 500 g twice. This pellet from pmp47Δ contained a large amount of aggregated DHAS (>90% of total amount), whereas that from wild-type cells contained less DHAS (~45–60%). Organelle pellets mainly containing peroxisomes and mitochondria (for both strains) and aggregated DHAS (for pmp47Δ) were prepared by differential centrifugation. These pellets were fractionated on discontinuous sucrose gradients.

Fig. 5 A summarizes the experiments performed on sucrose gradient fractions from wild-type cells. CAT activity was seen primarily in fractions 10–14, and a mitochondrial marker, cytochrome c oxidase, was seen in fractions 3–8. CAT seen in the top fraction may have been caused by the leakage of peroxisomes during manipulation. Immunoblots with antibodies against AOD and DHAS showed that these peroxisomal proteins also colocalized with CAT within the gradient. To confirm that AOD and DHAS were inside peroxisomes, fraction 13 was subjected to 30 mM Tris-Cl, pH 8.0, which causes peroxisomes to rupture and release matrix enzymes (Goodman et al., 1984). As shown in Fig. 5 C, these conditions caused release of AOD and DHAS, confirming that they were indeed compartmented in peroxisomes. Under the conditions used, Pmp47 remained in the pellet fraction showing a strong interaction with the membrane.

Fig. 5 B summarizes the parallel experiments performed for the pellet fraction from pmp47Δ cells. This pellet frac-
Figure 4. DHAS was inactive and was present as insoluble material in \textit{pmp47Δ} but was in a soluble and active form in \textit{pmp47Δ} derivatives from two peroxisome-deficient strains M6 and M13. (A) Methanol-induced enzyme activities after induction on methanol plus glycerol for 16 h. \textit{n.d.}, not detected. (B) Immunoblot of the cell-free extract from 16-h methanol plus glycerol induced cells with anti-AOD or anti-DHAS. Cells of the indicated strains were disrupted by sonication, and were separated into supernatant (S) and pellet fraction (P) after centrifugation at 20,000 g. The samples were boiled in Laemmli sample buffer (0.1% SDS) and subjected to SDS-PAGE.

EM Observations

The subcellular morphology of the wild-type and \textit{pmp47Δ} cells grown on methanol plus glycerol was compared with KMnO4-fixed cells and by immunogold EM using antibodies raised against AOD or DHAS.

Typically, wild-type cells grown on methanol plus glyc-
Figure 5. Semicontinuous sucrose gradient fractionation of organelle pellets from (A) wild-type and (B) pmp47Δ strains. Cells were grown on methanol plus glycerol medium for 16 h, and then organelar pellets were prepared and fractionated on a sucrose gradient as described in Materials and Methods. Dotted line represents sucrose concentration. CAT (closed triangle) and cytochrome c oxidase (closed circle) activity are expressed in relative values to the maximum activity in the fractions. The relative activity 1.0 for CAT corresponds to 77.8 U/ml for wild type and 86.5 U/ml for pmp47Δ, and that for cytochrome c oxidase corresponds to 1.44 U/ml for wild type and 1.61 U/ml for pmp47Δ. (C) DHAS was not released by treatment with 30 mM Tris-C1, pH 8.0, from peroxisomal fractions of pmp47Δ. The peak of peroxisomal fraction for wild type (fraction 13 in A) and that for pmp47Δ (fraction 14 in B), were divided into a pellet fraction (P) and supernatant fraction (S) by 20,000 g centrifugation after treatment with (+) and without (-) 30 mM Tris-C1, pH 8.0. Immunoblot was performed with anti-DHAS polyclonal, anti-AOD polyclonal, and anti-Pmp47 monoclonal.

erol (or methanol alone) had spheroids of ~3 μm in diameter, composed of three or four tightly packed peroxisomes (Fig. 6 A). In the wild-type cells, AOD and DHAS localized differently within peroxisomes. Anti-AOD cross-reacted preferably with the core of peroxisomes (Fig. 6 B), while those labeled with anti-DHAS dominantly cross-reacted with the peripheral part of peroxisomes (Fig. 6 C). In contrast to the wild-type cells, pmp47Δ had only one or two peroxisomes when induced on methanol plus glycerol medium (Fig. 6 D). The most striking feature in methanol-induced cells of pmp47Δ was cytoplasmic irregularly shaped regions of high electron density, suggesting proteinaceous aggregates (Fig. 6 D). These suggested the involvement of Pmp47 in the translocation of some matrix enzymes, since similar aggregates were also observed for the peroxisome-deficient mutant strains (Sakai et al., 1995b). Indeed, anti-DHAS reacted with aggregates in the cytoplasm (Fig. 6 F). This shows that the cytoplasmic aggregates that we observed in KMnO4-fixed cells contained DHAS. Also, anti-DHAS did not react with the peroxi-
some matrix while anti-AOD reacted exclusively with the peroxisomal matrix (Fig. 6 E). From these immuno-EM and fractionation experiments, DHAS aggregated within the cytoplasm whereas AOD (and we assume CAT) translocated properly in the absence of Pmp47. Concomitation of a part of aggregated DHAS with peroxisomal and mitochondrial fraction in sucrose gradient centrifugation experiments with pmp47Δ may be caused by the nonspecific binding of aggregated DHAS to the organelle membranes through hydrophobic interaction.

We also observed an high electron density region in oleate-induced cells of pmp47Δ, suggesting aggregation of some oleate-induced protein. While 9 or 10 separated peroxisomes of small sizes (0.1–0.7 μm) were observed in the wild-type cells (Fig. 6 G), there were only 3 or 4 in oleate-induced cells of pmp47Δ (Fig. 6 H). Thus, both methanol- and oleate-induced cells of pmp47Δ had a smaller number of peroxisomes than those of the wild-type strain. Previously, we observed the decreased number of peroxisomes in pmp30Δ and suggested that Pmp30 is responsible for peroxisome proliferation (Sakai et al., 1995). Not only protein translocation but also peroxisome proliferation seems to be inhibited in pmp47Δ. D-alanine-induced cells of both wild-type and pmp47Δ had a very few number of small peroxisomes (0.1–0.2 μm) compared with methanol- and oleate-induced cells (data not shown). Areas of high electron density were not observed in D-alanine- and glucose-grown cells (data not shown).

**DHAS Is Active in pmp47Δ Strains in the Context of Peroxisome-deficient Mutants**

Our experiments show that Pmp47 is necessary for the translocation and proper folding of DHAS. Then, is Pmp47 required for the folding per se of DHAS? In a previous study, we derived two mutant C. boidinii strains deficient in peroxisomes, strain M6 and strain M13 (Sakai et al., 1995). These strains, which were originally isolated as non-utilizers of multiple peroxisome-proliferating carbon sources, mislocalize several peroxisomal enzymes (AOD, CAT, D-amino acid oxidase, thiolase, and acyl-CoA oxidase) to the cytoplasm. Morphologically, these mutants lacked peroxisomes, and areas of high electron density were seen in the cytoplasm and the nuclei of methanol-induced cells. Meanwhile, another C. boidinii peroxisome assembly mutant defective in the PTS1 receptor (PSRIΔ) had small methanol-induced peroxisomes (Sakai, Y., unpublished results).

To explore the relationship between Pmp47 and these mutants, we first confirmed the mislocalization of DHAS in strains M13 and M6 by differential centrifugation and immunocytochemical experiments. Protoplast of methanol-induced cells was osmotically ruptured, and the cell debris was removed by low speed centrifugation at 500 g, as described above. The organelar pellet fraction (containing mainly peroxisomes and mitochondria) was pelleted by centrifugation at 20,000 g. The supernatant fraction was considered as the cytoplasmic fraction. In the wild-type strain, 75% of the activity of DHAS, 90% of the activity of AOD, and 60% of the activity of peroxisomal catalase were found in the organelar pellet fraction (Fig. 7 A). These pelletable enzyme activities were released into the supernatant fraction by the addition of Triton X-100 before the 20,000 g centrifugation, indicating that these enzymes are enclosed in a membranous structure. In contrast to the wild-type strain, both strains M6 and M13 had only a trace of pellettable activities of AOD, CAT, and DHAS, with or without the addition of Triton X-100. DHAS mislocalization in strain M6 and M13 was also confirmed by immunoblot analysis (Fig. 7 A). Immunoblot (Fig. 7, B and C) showed that AOD and DHAS were observed in both the cytoplasm and the nucleus of strain M13. A similar labeling pattern was observed for strain M6 (data not shown). The mislocalization of AOD into the nucleus in pas mutants of P. pastoris and H. polymorpha have also been reported (van der Klei et al., 1991; McCollum et al., 1993). Thus, both M6 and M13 that had been induced on methanol contained active and soluble forms of DHAS, AOD, and CAT (Fig. 4, A and B, right panel) in the cytoplasm. Also, both M6 and M13 retained the ability to synthesize Pmp47 (Fig. 2 C).

To determine whether Pmp47 was required for DHAS activity in these mutants, we disrupted Pmp47 in these mutant strains, yielding M6-pmp47Δ and M13-pmp47Δ (Fig. 2), and enzyme activities were followed after methanol induction. As shown in Fig. 4 A, DHAS activity, as well as AOD and CAT activities, were induced in both M6-pmp47Δ and M13-pmp47Δ to comparable levels of their host strain. Also, soluble fractions of cell-free extracts from M6-pmp47Δ and M13-pmp47Δ yielded DHAS-reactive bands by immunoblot analysis, in contrast to pmp47Δ (Fig. 4 B, right panel). Next, the localization of DHAS and AOD in these strains were examined. Differential centrifugation experiments of M6-pmp47Δ and M13-pmp47Δ gave the same results as their host strain (Fig. 7 A). Also by immuno-EM, AOD and DHAS were mislocalized in the cytoplasm and the nucleus of M13-pmp47Δ (Fig. 7, D and E), similar to the host strain M13 (Fig. 7, B and C). The same labeling patterns were also observed with strains M6 and M6-pmp47Δ (data not shown). From these experiments, we conclude that Pmp47 itself was not essential for the folding of DHAS.

Since Pmp47 belongs to a transporter family, we assumed that a compound normally transported by Pmp47 into peroxisomes was necessary for the translocation and folding process of DHAS. Also, another factor necessary for this process, which is the direct acceptor of the solute, must exist inside peroxisomes.

**Induction of mRNA for Pmp47, AOD, and DHAS during the Early Stage of Peroxisome Proliferation**

In the experiments described above, Pmp47 was found to be necessary for the transport and folding process of DHAS. Therefore, at the early stage of peroxisomal proliferation, induction of Pmp47 should precede or be concomitant with the induction of DHAS, as was observed in C. boidinii ATCC32195 (Veenhuis and Goodman, 1990). First, we confirmed this observation in strain S2, where Pmp47 was encoded by a single gene. We also determined the mRNA concentrations for Pmp47, DHAS, and AOD to see whether the induction of Pmp47 was regulated at the mRNA level. In detail, wild-type cells precultured on glucose were transferred to methanol MI medium, and at
Figure 6. Subcellular morphology and immunocytochemical experiments of (A–C and G) the wild-type and (D–F and H) pmp47A cells grown on (A–F) methanol plus glycerol for 10 h, and (G and H) oleate for 16 h. Overall morphology with (A, D, G, and H) KMnO₄-fixed cells and immunocytochemical experiments using (B and E) anti-AOD and (C and F) anti-DHAS. Note the high electron density regions in the cytoplasm of (D) methanol- and (H) oleate-induced cells of pmp47A. (E) Anti–AOD-antibodies reacted with the peroxisome matrix in pmp47A. (F) On the other hand, anti-DHAS antibodies did not react with the peroxisome matrix, but with aggregates in the cytoplasm in pmp47A. P, peroxisome; N, nucleus; V, vacuole; M, mitochondrion. Bar, 1 μm.

Discussion

During the gene disruption studies of a putative peroxisomal transporter Pmp47 of C. boidinii, we found that pmp47Δ cells lost the activity of DHAS, an important enzyme for methanol metabolism. This observed phenotype is analogous to the phenotype of cell lines from ALD patients. In both cases, the loss of a matrix enzyme activity came from the loss of a peroxisomal transporter.

Methanol-induced cells were effected more severely by PMP47-disruption than oleate- or D-alanine–induced cells. Therefore, in this study, we have focused on the function of Pmp47 in relation to the translocation and folding process of two major peroxisomal matrix enzymes, AOD and DHAS. The carboxyl terminal sequence of AOD and DHAS of C. boidinii has a PTS1-like motif of ARY (Sakai and Tani, 1992) and NHL (Sakai, Y., unpublished data), respectively. In the case of H. polymorpha, these proteins had a carboxyl-terminal sequence of ARF and NKL, respectively (Hansen et al., 1992), and both tripeptide sequences were shown to be sufficient for targeting nonperoxisomal proteins to peroxisomes, indicating that they function as PTS1. In our experiments described here, depletion of Pmp47 in the wild-type cells resulted in the mislocalization of only DHAS and not AOD. Our results first suggested a diversity of the PTS1 pathway (or peroxisomal protein import), which originates from the difference of the solute requirement of these PTS1 proteins. Recent studies showed that some folded proteins are competent for peroxisomal transport, and suggested that folding machineries are not necessary within peroxisomes (Glover et al., 1994; McNew and Goodman, 1994; Walton et al., 1995).
However, these reports do not exclude the existence of some other protein that is folded after its import into peroxisomes. In our case with DHAS, the results suggest that DHAS folds within peroxisomes. If the folding of DHAS occurs in the cytoplasm, it is hard to explain the observed phenotypes of pmp47Δ strains. Another observation to support this idea is that a small but significant fraction of DHAS is resistant to extraction from peroxisomal membranes (Goodman et al., 1986; Sakai, Y., unpublished data). Under conditions where AOD, Pmp20, and Pmp30 were extracted (90 mM NaOH for 1 h), a considerable amount of DHAS (approximately equimolar to Pmp47) remained in the membrane fraction together with the integral Pmp47. This biochemical result suggests that DHAS was trapped in the peroxisomal membrane as a hydrophobic form.

In pmp47Δ-cells, DHAS protein could not be folded properly and accumulated as aggregates in the cytoplasm. However, in peroxisome-deficient strains, M6 and M13 and their pmp47Δ-derivatives, DHAS was located in the cytoplasm in a soluble form and DHAS aggregates were not observed. A soluble and active form of DHAS was also observed in the cytoplasm of the PTS1 receptor-deficient strains, P. pastoris pas8 (McCollum, et al., 1993) and C. boidinii psr1Δ (Sakai, Y., unpublished results). To explain these observed phenotypes, we hypothesize a relationship between the biochemical function of Pmp47 and the translocation-folding process of DHAS (Fig. 9). At least four components are thought to be necessary for this process: DHAS, Pmp47, the unknown solute transported by Pmp47, and the peroxisomal factor necessary for DHAS folding (the peroxisomal chaperone model) or DHAS translocation (the translocation machinery model). According to the peroxisomal chaperone model, this is explained as follows: although DHAS and the solute transported by Pmp47 are both mislocalized in the cytoplasm of pmp47Δ, the absence of peroxisomal chaperone in the cytoplasm prohibits DHAS to fold properly. This problem might be amplified by the large concentration of DHAS in the cytoplasm. However, peroxisome-deficient strains M6 and M13 allowed folding of DHAS in the cytoplasm probably because the peroxisomal chaperone was also mislocalized there. On the other hand, according to the translocation machinery model, the solute is primarily required for the translocation of DHAS through the peroxisomal membrane. For example, the solute may be necessary for releasing DHAS from the translocation machinery within the membrane to the matrix. In this case, some interaction between DHAS and the membrane component prevents DHAS from folding into an active state in pmp47Δ. Since inhibition by membrane components of the translocation machinery will not occur in peroxisome-deficient mutants, DHAS could fold into an active form. We think that the peroxisomal chaperone model seems to be more consis-
Figure 7. DHAS is active but mislocalized in peroxisome-deficient mutant strains (M6 and M13) and their pmp47Δ derivatives (M6-pmp47Δ and M13-pmp47Δ). (A) Activities of peroxisomal enzymes in the organellar pellet after differential centrifugation in the (open box) presence and (shadow box) absence of 0.5% Triton X-100. Cells were induced on methanol for 12–16 h to see the localization of peroxisomal enzymes. Total activities (U) in each experiment (the total activity in the pellet fraction plus the total activity in the supernatant fraction) were as follows: AOD; 0.225 for wild-type strain (−T), 0.208 for wild-type strain (+T), 0.118 for strain M6 (−T), 0.126 for strain M6 (+T), 0.098 for strain M6-pmp47Δ (−T), 0.105 for strain M6-pmp47Δ (+T), 0.176 for strain M13 (−T), and 0.198 for strain M13 (+T), 0.190 for strain M13-pmp47Δ (−T), and 0.182 for strain M13-pmp47Δ (+T). Catalase; 8230 for wild-type strain (−T), 7603 for wild-type strain (+T), 3550 for strain M6 (−T), 3920 for strain M6 (+T), 3602 for strain M6-pmp47Δ (−T), 3650 for strain M6-pmp47Δ (+T), 3720 for strain M13 (−T), 4310 for strain M13 (+T), 3950 for strain M13-pmp47Δ (−T), and 4050 for strain M13-pmp47Δ (+T). DHAS; 0.512 for wild-type strain (−T), 0.437 for wild-type strain (+T), 0.125 for strain M6 (−T), 0.110 for strain M6 (+T), 0.135 for strain M6-pmp47Δ (−T), 0.133 for strain M6-pmp47Δ (+T), 0.618 for strain M13 (−T), and 0.552 for strain M13 (+T), 0.550 for strain M13-pmp47Δ (−T), and 0.527 for strain M13-pmp47Δ (+T). DHAS activities were not detected in pellet fractions except the case for the wild-type strain (−T). Equivalent portion of the (P) organellar pellet and (S) supernatant fraction from 20,000 g centrifugation was loaded on SDS-PAGE and analyzed by immunoblot analysis using anti-DHAS. The absence or presence of 0.5% Triton X-100 was shown by − or +, respectively. (B–E) Immunocytochemical experiments using (B and D) anti-DHAS and (C and E) anti-AOD antibodies and immunogold on ultrathin sections of strain M13 cells (B and C), and strain M13-pmp47Δ cells (D and E). In both peroxisome-deficient strains M13 and M13-pmp47Δ, anti-AOD or anti-DHAS-labeled gold particles reacted with the cytoplasm and the nucleus, but not with mitochondria. Symbols are the same as Fig. 6.

(a) In the translocation machinery model, DHAS aggregation in pmp47Δ is caused by interaction between DHAS and membrane component of the translocation machinery. However, such interaction will not occur when the PTS1 motif is deleted from DHAS. Hansen et al. (1992) reported that H. polymorpha DHAS depleted of its PTS1 motif aggregated in the cytoplasm. While deletion of the PTS1 tripeptide might directly lead to misfolding, the inaccessibility of the peroxisomal chaperone to this molecule is
the more likely explanation. In peroxisome-deficient mutants, the peroxisomal chaperone will also be mislocalized in cytoplasm together with DHAS. (b) In methanol-induced cells, a large amount of both DHAS and AOD are produced in the cells (>50% of total soluble proteins). These amounts are sure to exceed the amount of the translocation machinery (e.g., PTS1 receptor), since the machinery molecules are to be reused in other cycles of translocation. As a result, some part of the produced DHAS in pmp47A will have to exist free from the translocation ma-

Figure 8. Induction of Pmp47, AOD, and DHAS, and their mRNAs during the early stage of peroxisomal induction. (A) Immunoblot analysis. (B) Northern analysis. Proteins (3.0 μg) and mRNAs (1.0 μg) extracted from each time point after transfer to methanol medium were loaded on to each lane. The bands from immunoblot analysis and Northern analysis were quantified by densitometric analysis as shown in right figures. Squares, Pmp47 and its mRNA; closed circles, AOD and its mRNA; triangles, DHAS and mRNA; open circles, actin mRNA. Northern filters were labeled with radiolabeled DNA fragments from coding sequence of AOD1 (0.7-kb BgII-Sal I fragment of pMOX33) (Sakai and Tani, 1992), PMP47 (1.6-kb HindII-HindIII fragment from pMP471) (this study), DAS1 (1.8-kb EcoRV-Bgl II fragment) (Sakai, Y., unpublished data), and C. boidinii ACT1 DNA fragment (0.6-kb ClaI-HindIII fragment; Komeda, T., unpublished data).
We wish to acknowledge Dr. Joel M. Goodman, University of Texas Southwestern Medical Center at Dallas, for his generous contribution of the PMP47 gene, antibodies, and helpful discussion. We thank Prof. Yoshiki Tani (Nara Institute of Science and Technology) for his continuous encouragement throughout the work. And we also acknowledge Dr. Kazushi Fujimoto and professor emeritus, Kazuo Ogawa, Department of Anatomy, Faculty of Medicine, Kyoto University, for their generous help and suggestions on EM work during the initial stage of this study. We thank Dr. Hideshi Yanase (Faculty of Engineering, Tottori University) for purification of DHAS, to Tomoyuki Nakagawa and Hideaki Matsuo for their skillful assistance, and Hiromi Sakai for her critical reading of the manuscript.

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Figure 9. The diversity of peroxisomal protein import pathway (the PTS1 pathway) and a working hypothesis for the mechanism of DHAS import and folding. Both DHAS and AOD have a PTS1 motif sequence, NHL and ARY, respectively. However, only DHAS seems to demand a solute transported by Pmp47 for showing its activity. The solute may be necessary for the folding of DHAS in peroxisomes (the peroxisomal chaperone model) or for the translocation of DHAS (the translocation machinery model). According to the peroxisomal chaperone model, in pmp47Δ cells, although both DHAS and the solute are present in the cytoplasm, DHAS could not fold into an active form because of the lack of peroxisomal factor. In peroxisome-deficient strains, all of these molecules are within the cytoplasm, and so DHAS could fold into an active form. According to the translocation model, DHAS could not fold into an active form in pmp47Δ because of the inhibition of folding by the membrane component. Peroxisome-deficient strains had an enzyme activity since DHAS folding was not inhibited by the membrane component.

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the cytoplasm and could enable DHAS-folding in the PTS1 receptor-deficient strain. The development of a direct transport assay with purified peroxisomes has been greatly hindered by the extreme fragility of peroxisomes. However, further analysis of pmp47Δ and reconstitution of DHAS activity in vitro (i.e., determination of factors involved in the folding of DHAS into an active form from aggregated DHAS) will give us information on the compound transported into peroxisomes by Pmp47.

Our present studies have been limited to methanol-induced cells of C. boidini. However, the function of Pmp47 was not restricted to methanol-induced cells, since growth was retarded, electron-dense aggregates were seen, the number of peroxisomes was decreased, and unmetabolized oleate accumulated in oleate-induced pmp47Δ cells. As Pmp47 is related to oleate metabolism, homologues of Pmp47 may be present in other organisms, e.g., S. cerevisiae and other higher organisms. From these observations, we speculated that the role of Pmp47 in vivo is a general function in peroxisomal transport and organelle proliferation rather than a specific function in methanol metabolism. This was also supported by the fact that a larger amount of Pmp47 was produced in oleate-induced cells than in methanol- and d-alanine-induced cells.

In conclusion, our results show that the loss of an organelle transporter (not a protein translocator) causes a severe defect in the translocation and folding of an organelle matrix protein and in organelle proliferation. Transporters of this sort may not be restricted to peroxisomes and may exist in other organelles. Although our understanding of the role of transporters has been focused on metabolites and drug transport, a defect in an organelle transporter was shown to result directly in severe cellular disorders. Revealing the molecular mechanism of these defects will help us to understand the molecular basis of genetic diseases such as ALD.

We wish to acknowledge Dr. Joel M. Goodman, University of Texas Southwestern Medical Center at Dallas, for his generous contribution of the PMP47 gene, antibodies, and helpful discussion. We thank Prof. Yoshiki Tani (Nara Institute of Science and Technology) for his continuous encouragement throughout the work. And we also acknowledge Dr. Kazushi Fujimoto and professor emeritus, Kazuo Ogawa, Department of Anatomy, Faculty of Medicine, Kyoto University, for their generous help and suggestions on EM work during the initial stage of this study. We thank Dr. Hideshi Yanase (Faculty of Engineering, Tottori University) for purification of DHAS, to Tomoyuki Nakagawa and Hideaki Matsuo for their skillful assistance, and Hiromi Sakai for her critical reading of the manuscript.

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