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 unrevealed 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 (*pmp47*Δ) 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 *pmp47*Δ, but in reduced numbers, and the presence of material of high electron density in the cytoplasm in both cases. Methanol-induced cells of *pmp47*Δ were investigated in detail. The activity of one of the methanol-induced peroxisome matrix enzymes, dihydroxyacetone synthase (DHAS), was not detected in *pmp47*Δ. 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-*pmp47*Δ and M13-*pmp47*Δ, 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-*pmp47*Δ and M13-*pmp47*Δ 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 genetical disorders, Zellweger syndrome (ZS)[1], and X-linked adrenoleukodystrophy (ALD; Kamijo et al., 1990; Gärtner 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-

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1. *Abbreviations used in this paper*: ALD, adrenoleukodystrophy; AOD, alcohol oxidase; CAT, catalase; DHAS, dihydroxyacetone synthase; KPB, potassium phosphate buffer; ZS, Zellweger syndrome.

tients 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; Purdue 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 NH_2-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* (McCammon 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, PXR1, has been cIoned, and it has been shown to complement some ZS cell lines (Dodt 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, 1995*c*). AOD can be used to produce various useful aldehydes (Sakai and Tani, 1987, 1988), and recently, we described a new enzymatic method to prepare ^{13}C-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 function rather than in a specific metabolic pathway (Goodman et al., 1990). However, its function was not investigated because of the lack of molecular genetics of the polyploidal strain, *C. boidinii* strain (ATCC32195), used in the study. As the first step to understand the function of Pmp47, we have cloned and disrupted the corresponding gene in the haploid strain S2. The phenotype of the resulting strain *pmp47*Δ indicates an important role of Pmp47 in the translocation and folding of DHAS. We suggest here that the folding and translocation process of some matrix protein depends not only on its targeting signal and translocation machinery, but also on some solute factor within peroxisomes.

Materials and Methods

Strains, Media, and Cultivation

C. boidinii strains TK62 (*ura3*; 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., 1995*b*). *C. boidinii* transformant GC (Sakai et al., 1995*c*) 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., 1995*b*) 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 *pmp47*Δ 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., 1995*a*). Gel-purified DNA was ^{32}P-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 OD_{610} 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 *PMP47A* gene from *C. boidinii* ATCC32195, a 1.2-kb PstI-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 5.9-kb band. A pool of EcoRV-digested genomic DNA of approximately this size was gel purified and ligated into the EcoRV site of pBluescript II SK$^+$. *E. coli* transformants were transferred onto a Biodyne nylon

membrane (Pall Bio Support). After the lysis of bacteria, the liberated DNA was bound to the nylon membrane, and these blots were then used for colony hybridization under high stringency hybridization conditions using Church-Gilbert buffer (1% BSA, 1 mM EDTA, 0.25 M NaCl, 0.25 M Na_3PO_4, pH 7.2, 7% SDS) (Church and Gilbert, 1984). Hybridization 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 reactive 5.9-kb EcoRV fragment. The total *PMP47* 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 pMP473. Next, the 746-bp SspI-EcoRV fragment of pMP471 DNA containing the 5′ flanking and truncated NH_2-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, 1992*b*) and the HindIII digest of pMP4732 were blunt-ended and subjected to ligation, yielding the *C. boidinii 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 were 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 insonator (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-hydroxypyruvate (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 OD_{610} of 0.2–0.4 and grown for 10–16 h. The induced cells were harvested by centrifugation, treated with 0.1 M Tris-H_2SO_4 (pH 9.3)–10 mM DTT for 15 min, and converted to spheroplasts in 1.6 M KCl–10 mM MOPS–5 mM Na_2SO_3, pH 7.2, containing Zymolyase 100T (~1 mg/150 OD_{610} U cells) for 60–90 min.

Subcellular fractionation 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 Suspend 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 semicontinuous sucrose gradient. The organellar suspension (~2.0 ml) was layered on top of a 36-ml semicontinuous gradient (2.0 ml of 30, 36, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52, 54, 57, 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 KPB, pH 7.2, at 4°C for 2 h. After washing with 0.1 M KPB, the cells were postfixed with 1.5% (wt/vol) $KMnO_4$ 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/propylene 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 KPB, pH 7.2, at 4°C for 4 h. After fixation, cells were washed once with 0.1 M KPB, pH 7.2, containing 8% (wt/vol) sucrose and 0.05 M NH_4Cl, then three times with 0.1 M KPB, pH 7.2, containing 8% sucrose. They were thereafter pelleted and dehydrated in a graded ethanol series (30%, 50%, 70%, 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 2 d.

Immuno-gold labeling was performed on ultrathin sections using anti-AOD and anti-DHAS antibodies. Sections were quenched in 50 mM glycine in PBS (2 mM KH_2PO_4, 8 mM Na_2HPO_4, 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 NaN_3) 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 3× 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 in incubation buffer for 2 h. After washing in the washing buffer, sections were postfixed with 2.0% glutaraldehyde in PBS for 10 min, washed twice in PBS and twice in distilled H_2O. Finally, 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., 1995*a*), i.e., Pmp30 was encoded by only one gene (*PMP30*) in strain S2, while strain ATCC32195 had two genes *PMP30A* and *PMP30B*.

The gene encoding for Pmp47 in *C. boidinii* S2 (*PMP47*) was isolated by colony hybridization using *PMP47A* as the probe. Restriction mapping and sequence analysis showed near perfect identity to *PMP47B* (Fig. 1). DNA within the coding region was 99% identical to *PMP47B*, resulting in changes in only two amino acid residues. It was also 92% identical to *PMP47A*. The sequenced portions of 5′ and 3′ flanking regions were also highly conserved. The deduced 419–amino acid sequence of Pmp47 was consistent with the six-membrane–spanning model for the mitochondrial transporter family (data not shown). An internal region (amino acids 199–267), which was recently shown to be essential for the sorting of the protein to peroxisomes (McCammon et al., 1994), was completely conserved among the three genes coding for Pmp47. From these results, we conclude that *PMP47A* and *PMP47B* are probably allelic in the polyploid strain ATCC32195.

Next, the *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.

Growth Characteristics of C. boidinii pmp47Δ

Peroxisome-deficient mutants (strains M6 and M13) (Sakai et al., 1995*b*) of *C. boidinii* are severely deficient in peroxisomal function because they could not grow on oleate, D-alanine, or methanol as a sole carbon source. *C. boi-*

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-545                                                    GATATCTTTCA   -535
-534 CAGTATGAATGTGATTTTTAGCCTATCTTGAATATACTTCTTCTCCACTTTTTATTTTAT   -475
-474 TTATAATGATTCACGCTGAATTTTTTGAAAATTATATTGTAATTTAACATTGGTTTACCG   -415
-414 TTCTTGATTGTTAACTTAACAAACTGGTTTGATTGAATTCATATATCGAATTTTATTATA   -355
          C
-354 TTTATCATATGAGATTTTTATTCTTTTTTATATATTCTTTTAAATCTGAAATCGGCGAAT   -295
-294 AGATTCAAATAATTATTTACCATTTCCATATTCATATTCATATTCATATAGATTCACTCT   -235
                              ------                          A
-234 TTTTGAAAACAATCTTTAATAAATCACGGCTCATTTAAAATTCATTGTTTTCATTTGAAT   -175
                    C
-174 CAAGTCGGAATATATAATTTTTTATCTTCTTATCTTGACACTTGC------TCAAACAAG   -121
                                                  CCTATA
-120 CATATATATATATATTCATATATCTACAAATAGTTATATAGATACATAGAATTAATTATA    -61
 -60 TGAAAAGAAAGTTCACTATATCCATATACTAAACTATCACACAAGAAACAACTCACATCA     -1

   1 M S S R E Y D D L S H A F A G A G G G L                        20
   1 ATGTCTTCAAGAGAATACGACGATTTATCCCATGCTTTTGCAGGGGCTGGTGGTGGATTA    60
                                          A

  21 L S M T L T Y P L V T L T T H A Q T M V                        40
  61 TTGTCAATGACTTTAACTTATCCATTAGTTACTTTAACAACTCATGCACAAACTATGGTT   120

  41 R L K K N E E E E E K E N S N E D G S L S                      60
 121 AGATTGAAAAAAAATGAAGAAGAAGAAAAAGAAAATTCAAATGAAGATGGTTCGTTATCT  180

  61 P K S S N T S N I S Q K K I S Q F E I L                        80
 181 CCAAAATCATCAAATACAAGTAATATTTCTCAAAAGAAAATTTCACAATTTGAAATATTG  240

  81 K K I L K D Q G A K G L Y N G L E S A L                       100
 241 AAAAAAATATTAAAAGATCAAGGTGCAAAAGGTTTATATAATGGTTTAGAAAGTGCTTTA  300

 101 F G I A V T N F V Y Y Y F Y E L T G K T                       120
 301 TTTGGTATTGCAGTTACAAATTTTGTTTACTATTATTTTTATGAATTAACAGGTAAAACT  360

 121 L S R R S N P Q T T S G S K K V T L K K                       140
 361 TTAAGTAGAAGATCAAATCCACAAACAACATCAGGTTCTAAAAAAGTTACTTTGAAAAAA  420

 141 G L S V W Q S M A A G A V A G T I S R V                       160
 421 GGTTTATCTGTTTGGCAAAGTATGGCTGCTGGTGCAGTTGCTGGTACAATTTCACGGGTC  480

 161 A T N P I W V A N T R M T I L S K N Q G                       180
 481 GCAACAAATCCAATTTGGGTTGCTAATACAAGAATGACAATCTTATCTAAAAATCAAGGT  540

 181 K L G K L N T I E A I I Y I L K N E G W                       200
 541 AAATTAGGCAAATTAAATACAATTGAAGCCATTATTTATATCTTAAAGAATGAAGGTTGG  600

 201 Q K L F T G I V P A L F L V L N P I I Q                       220
 601 CAAAAATTATTTACAGGTATTGTACCTGCATTATTTTTGGTTTAAATCCAATTATTCAA   660

 221 Y T I F E Q L K S F I V K I K K R N V T                       240
 661 TACACAATTTTTGAACAATTAAAATCATTTATTGTTAAAATTAAAAAGAGAAATGTTACA  720

 241 P V D A L L L G A F G K L I A T I I T Y                       260
 721 CCTGTTGATGCTTTATTATTAGGTGCTTTTGGTAAATTAATTGCCACAATTATTACATAT  780

 261 P Y I T L R S R M H V K S M T E N N E D                       280
 781 CCTTACATTACTTTACGTTCTAGAATGCACGTTAAAAGTATGACTGAAAATAATGAAGAT  840

 281 S E K E R T D S V Q S L P E D G S D E D                       300
 841 TCTGAAAAGGAAAGAACAGATAGCGTTCAATCTTTACCTGAAGATGGATCAGATGAAGAT  900
                                                          C

 301 N S K E N P Y A E T I N K I I S K L P S                       320
 901 AACTCGAAAGAAAATCCTTACGCTGAAACAATCAATAAAATTATATCTAAATTACCATCT  960

 321 P I V S M F T I G Y K M Y K E E G V S S                       340
                  I
 961 CCAATTGTATCAATGTTTACAATAGGTTATAAAATGTATAAAGAAGAAGGTGTAAGTTCA 1020
                    T

 341 F Y R G L S V K L L Q S I L N A A F L F                       360
1021 TTTTATCGTGGTTTATCTGTGAAATTATTACAAAGTATATTGAATGCTGCATTCTTATTT 1080

 361 Y F K E E L L I L S D G I I K S T K R V                       380
                            A
1081 TATTTCAAAGAAGAATTATTAATCTTATCTGATGGAATAATAAAATCAACTAAAAGAGTT 1140
                                  C

 381 T G L A N N P Y N A K D V I H S F E K A                       400
1141 ACTGGATTAGCTAATAATCCTTATAATGCAAAAGATGTTATTCATTCATTTGAAAAAGCT 1200

 401 L C M R N P R S R T T T V P Q T N E E *                       420
1201 TTATGTATGCGTAATCCTCGTTCACGTACTACAACAGTTCCTCAAACTAATGAAGAATAA 1260

1261 ATTCAGAAAAAGCAAATTTAAATTTAAATTTTAATAAGTATCTGTTTCACTACTATTTAC 1320

1321 TTTTTTTTAGTTTTTTTGTTTTTCCCTTTTGTAATTCCCCATCAA                 1365
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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.

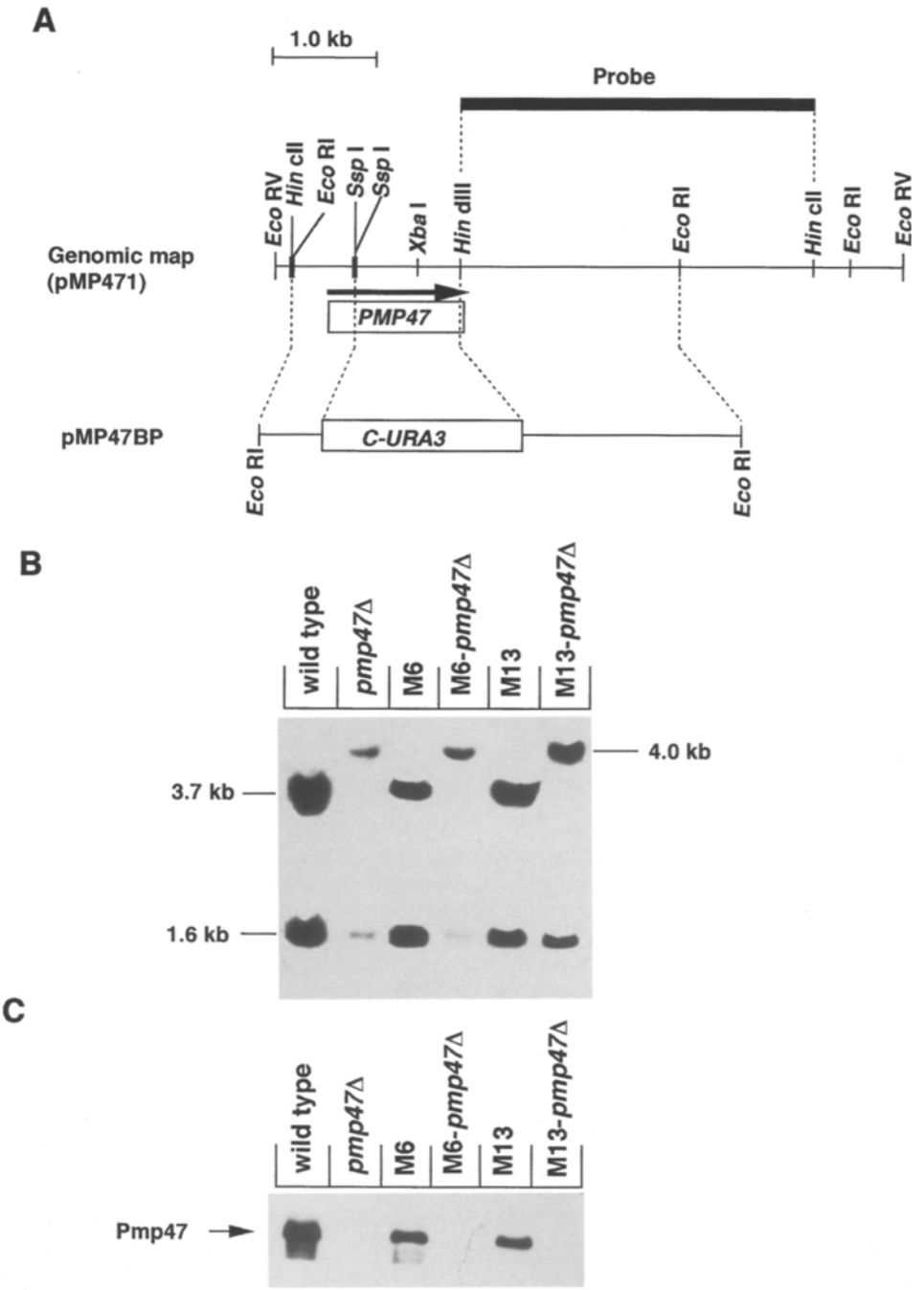


Figure 2. One-step gene disruption of *PMP47* gene in *C. boidinii.* (*A*) Restriction map of the cloned fragment and its disruption strategy. (*B*) Genomic Southern analysis from EcoRI-digested total DNA (~3 μg) from various *C. boidinii* strains with the radiolabeled probe shown in *A*. (*C*) Immunoblot 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.

dinii 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

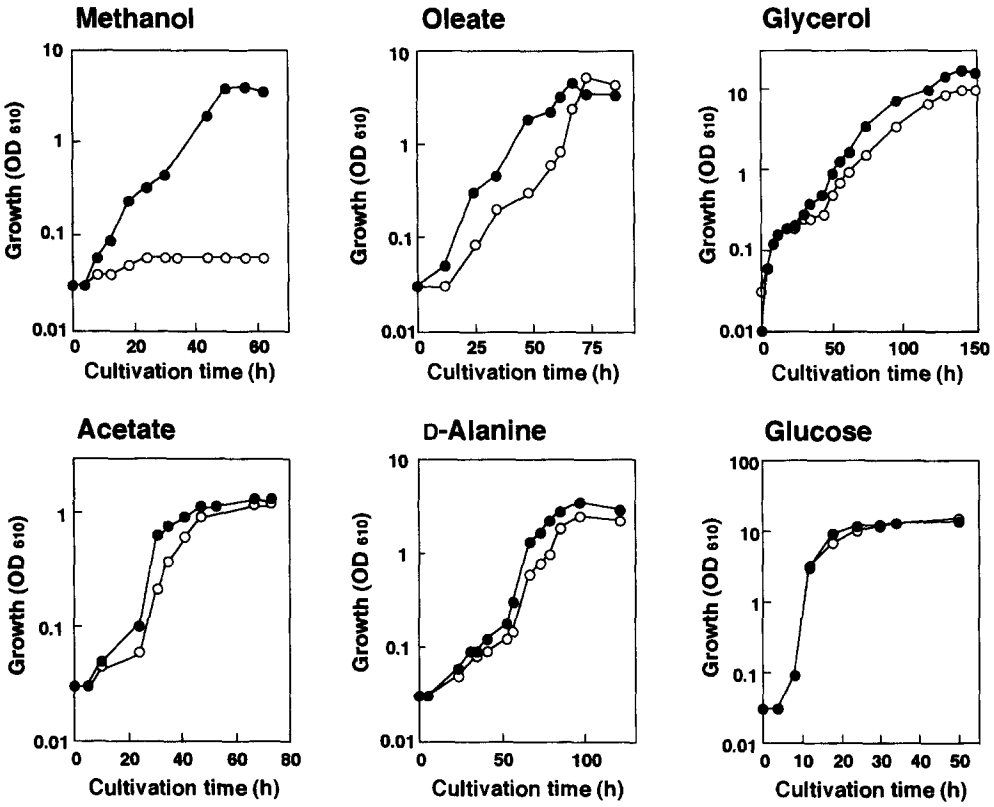


Figure 3. The disruption of *PMP47* affects growth in methanol- and oleate-media severely. Strains were grown on synthetic MI medium with the indicated carbon sources. *Open symbols*, *C. boidinii pmp47Δ*; *closed symbols*, wild-type strain.

not repress the methanol-induced enzymes (Sakai et al., 1995*c*). 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-

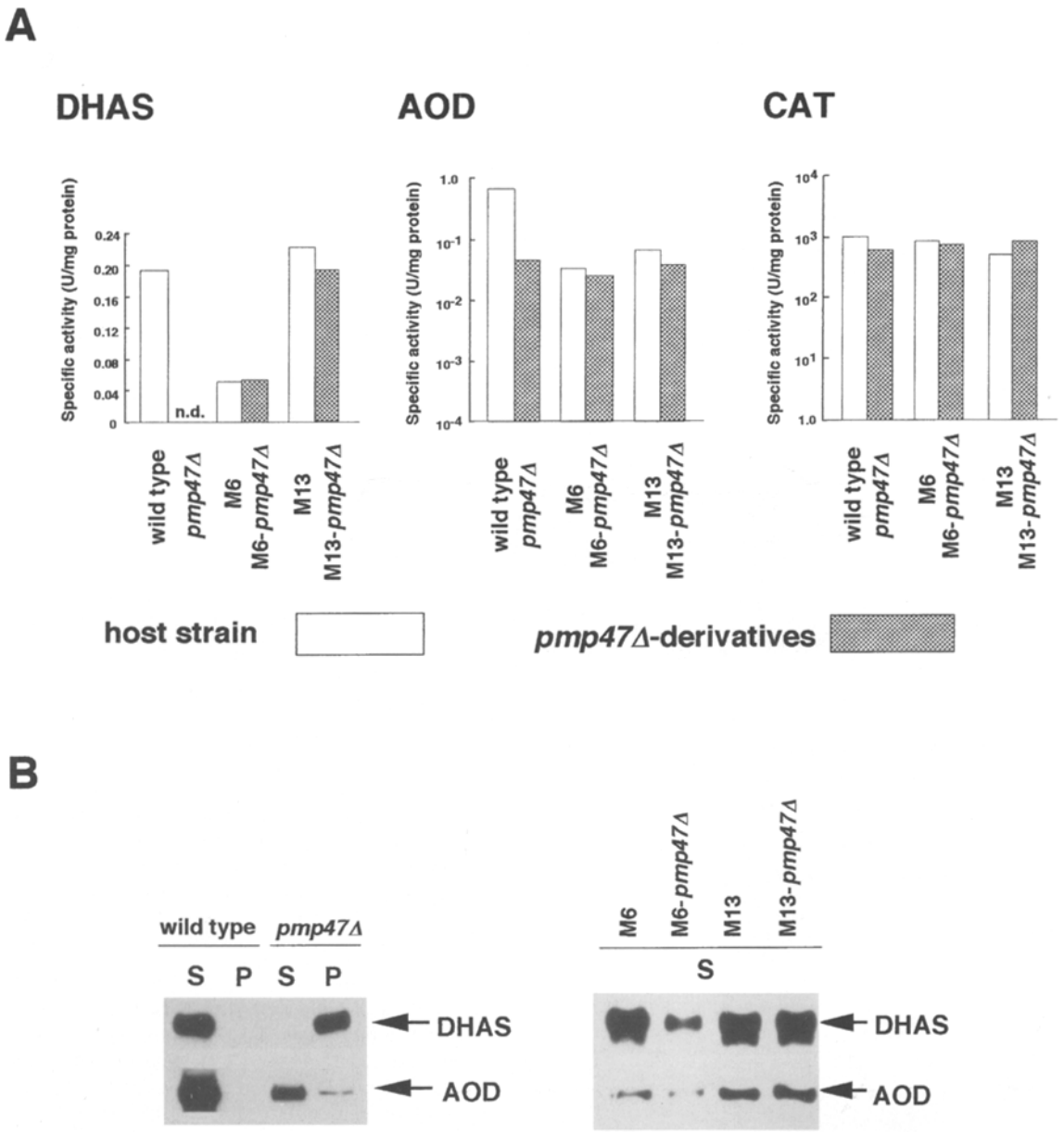


Figure 4. DHAS was inactive and was present as insoluble material in *pmp47Δ* but was in a soluble and active form in *pmp47Δ* derivatives from two peroxisome-deficient strains M6 and M13. (*A*) Methanol-induced enzyme activities after induction on methanol plus glycerol for 16 h. *n.d.*, not detected. (*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.

tion contained ~5–10% of DHAS aggregates from lysed cells, and this could not be removed by differential centrifugation. Similar to the wild-type cells, cytochrome *c* oxidase activity was found in fractions 5–8 of sucrose gradient. Also, CAT activity and the peak of AOD were found in fractions 13 and 14. However, DHAS behaved differently. In addition to peroxisomal fractions 12 and 14, DHAS protein was detected in fractions 4 and 6, overlapping with the mitochondrial marker. Thus, a fraction of the DHAS protein had not been imported into peroxisomes. The main peroxisomal peak fraction 14 was subjected to 30 mM Tris-Cl, pH 8.0. While AOD was released to the supernatant, similar to wild-type cells, DHAS was not releasable with this treatment (Fig. 5 *C*). From these experiments, we assume that AOD and CAT had translocated into peroxisomes as well as the wild-type strain, and that most parts of aggregated DHAS were not transported to peroxisomes.

EM Observations

The subcellular morphology of the wild-type and *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-

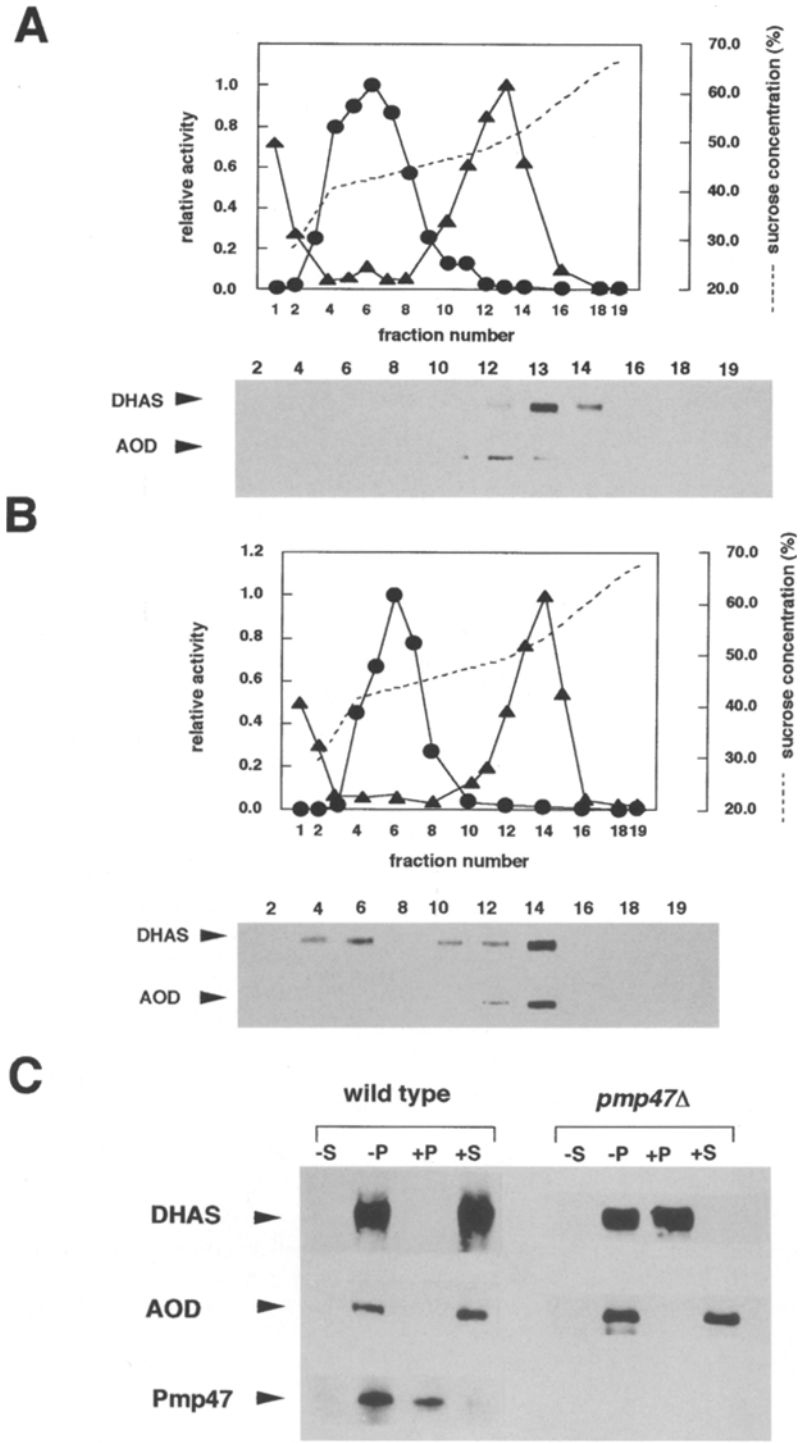


Figure 5. Semicontinuous sucrose gradient fractionation of organelle pellets from (*A*) wild-type and (*B*) *pmp47Δ* strains. Cells were grown on methanol plus glycerol medium for 16 h, and then organellar pellets were prepared and fractionated on a sucrose gradient as described in Materials and Methods. 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-Cl, pH 8.0, from peroxisomal fractions of *pmp47Δ*. The peak of peroxisomal fraction for wild type (fraction 13 in *A*) and that for *pmp47*Δ (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-Cl, 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, *pmp47*Δ 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 *pmp47*Δ 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., 1995*b*). 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. Comigration 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 nonutilizers 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 (*PSR1Δ*) 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 were osmotically ruptured, and the cell debris was removed by low speed centrifugation at 500 *g*, as described above. The organellar 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 organellar pellet fraction (Fig. 7 *A*). These pellettable 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*). Immuno-EM (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

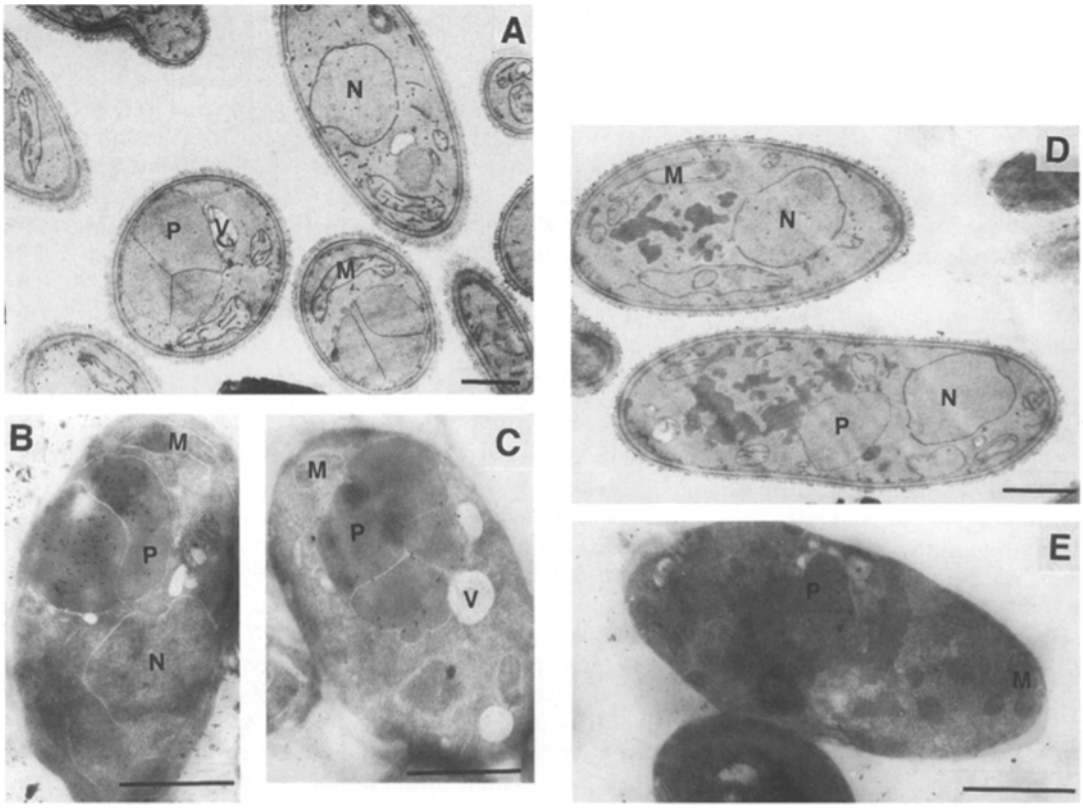


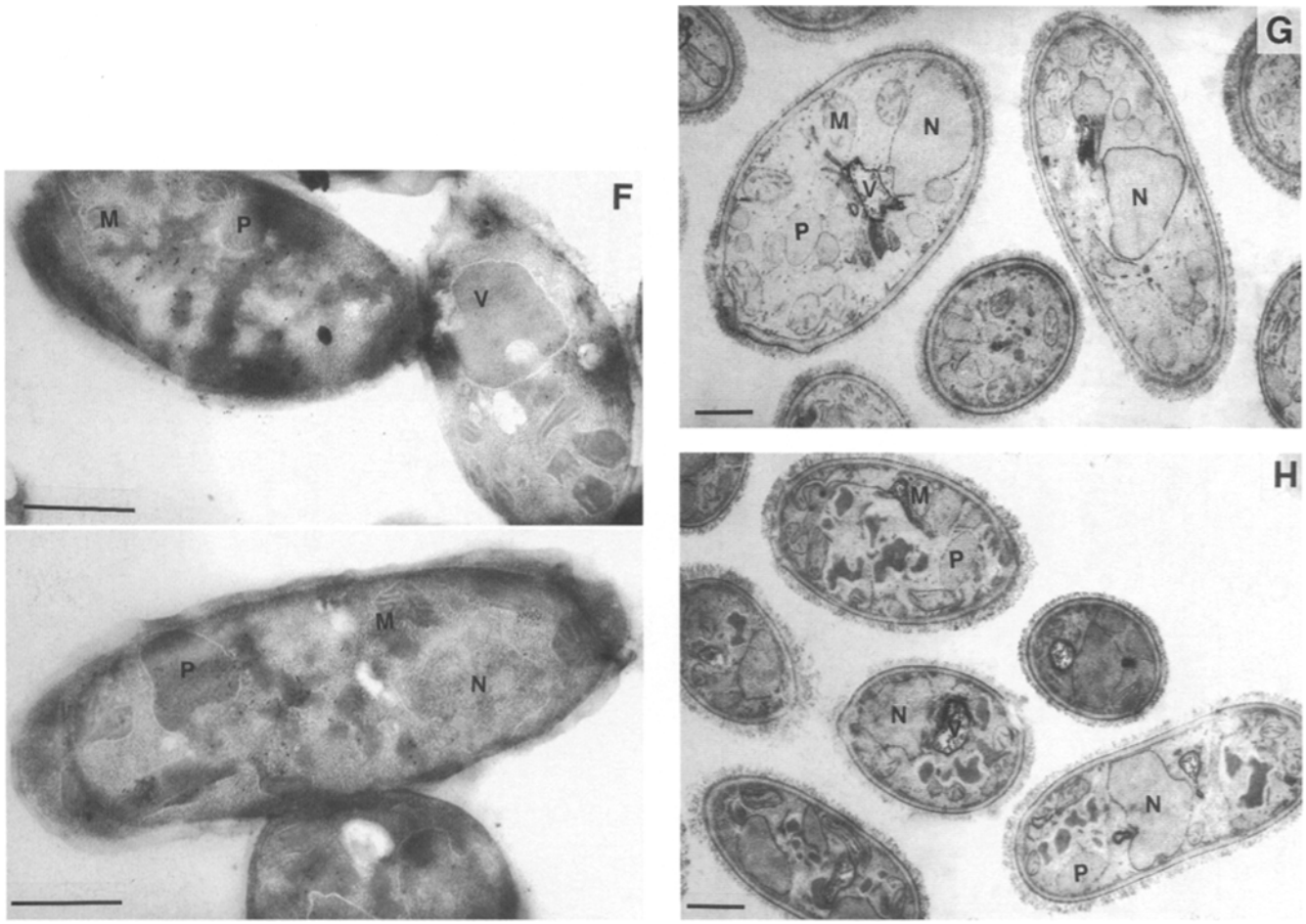
Figure 6. Subcellular morphology and immunocytochemical experiments of (*A–C* and *G*) the wild-type and (*D–F* and *H*) *pmp47Δ* cells grown on (*A–F*) methanol plus glycerol for 10 h, and (*G* and *H*) oleate for 16 h. Overall morphology with (*A*, *D*, *G*, and *H*) $KMnO_4$-fixed cells and immunocytochemical 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 *pmp47Δ*. (*E*) Anti–AOD-antibodies reacted with the peroxisome matrix in *pmp47Δ*. (*F*) On the other hand, anti-DHAS antibodies did not react with the peroxisome matrix, but with aggregates in the cytoplasm in *pmp47Δ*. *P*, peroxisome; *N*, nucleus; *V*, vacuole; *M*, mitochondrion. Bar, 1 μm.

each time point, proteins and mRNA were extracted for immunoblot and Northern analysis, respectively. As shown in Fig. 8 *A*, Pmp47 protein concentrations reached 40% of the maximum as early as 2 h after induction. DHAS and AOD were detected only after 4 h of induction. To compare these observations at mRNA levels, mRNA was extracted and hybridized with radiolabeled *PMP47–*, *AOD-*, *DHAS-*, and *C. boidinii* actin-DNA (Fig. 8 *B*). The band representing Pmp47-mRNA was detectable in glucose-grown cells. Induction of this species was very rapid, reaching 50% of maximal value after 1 h and obtaining a plateau after 2 h. The mRNAs for DHAS and AOD were detected only after 2 h. In contrast, the concentration of actin mRNA (as a control) was almost constant with the induction time (Fig. 8 *B*). From these results, we conclude that the induction of Pmp47 occurs before the matrix enzymes and that this is regulated mainly at the mRNA level.

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-

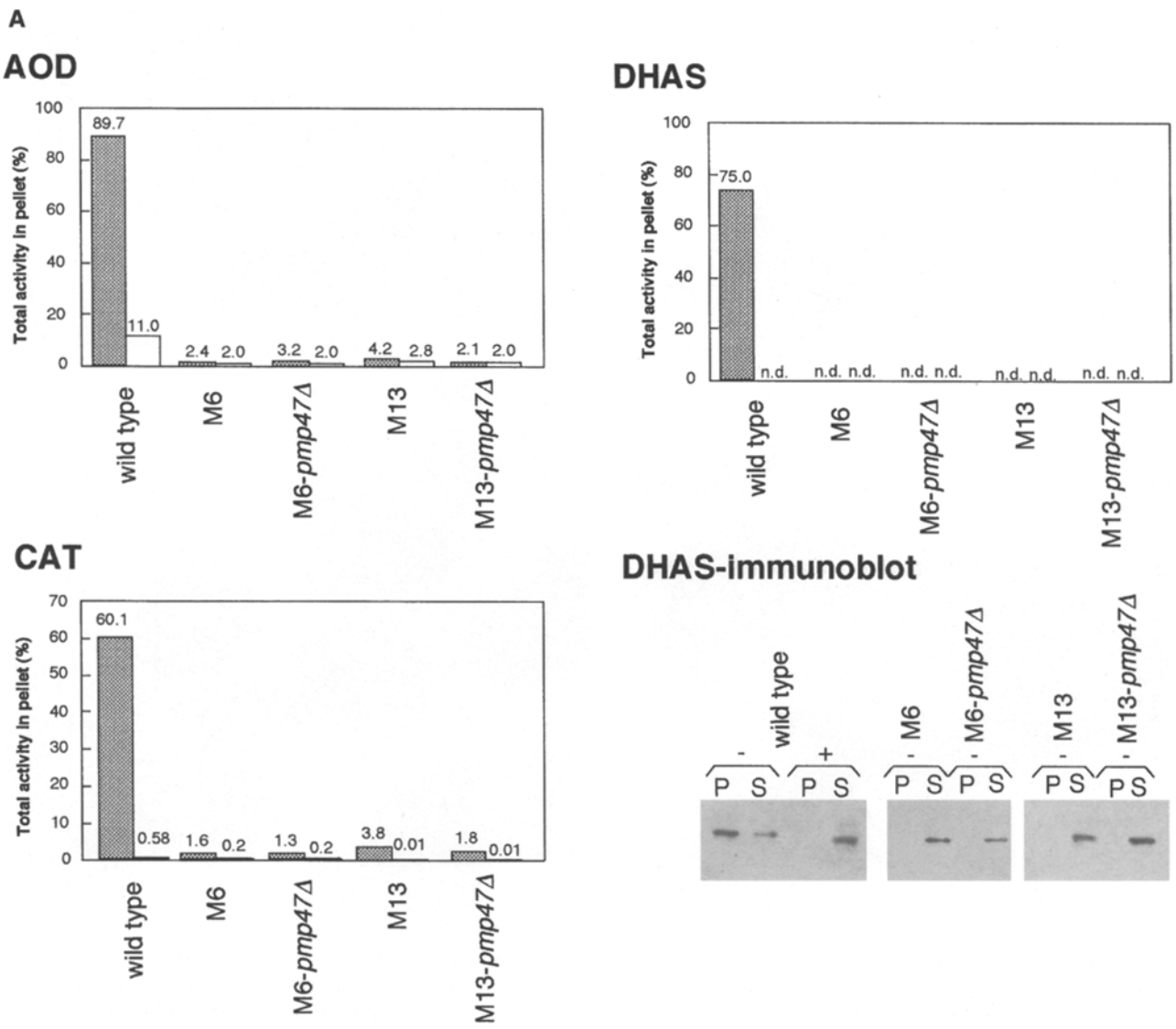
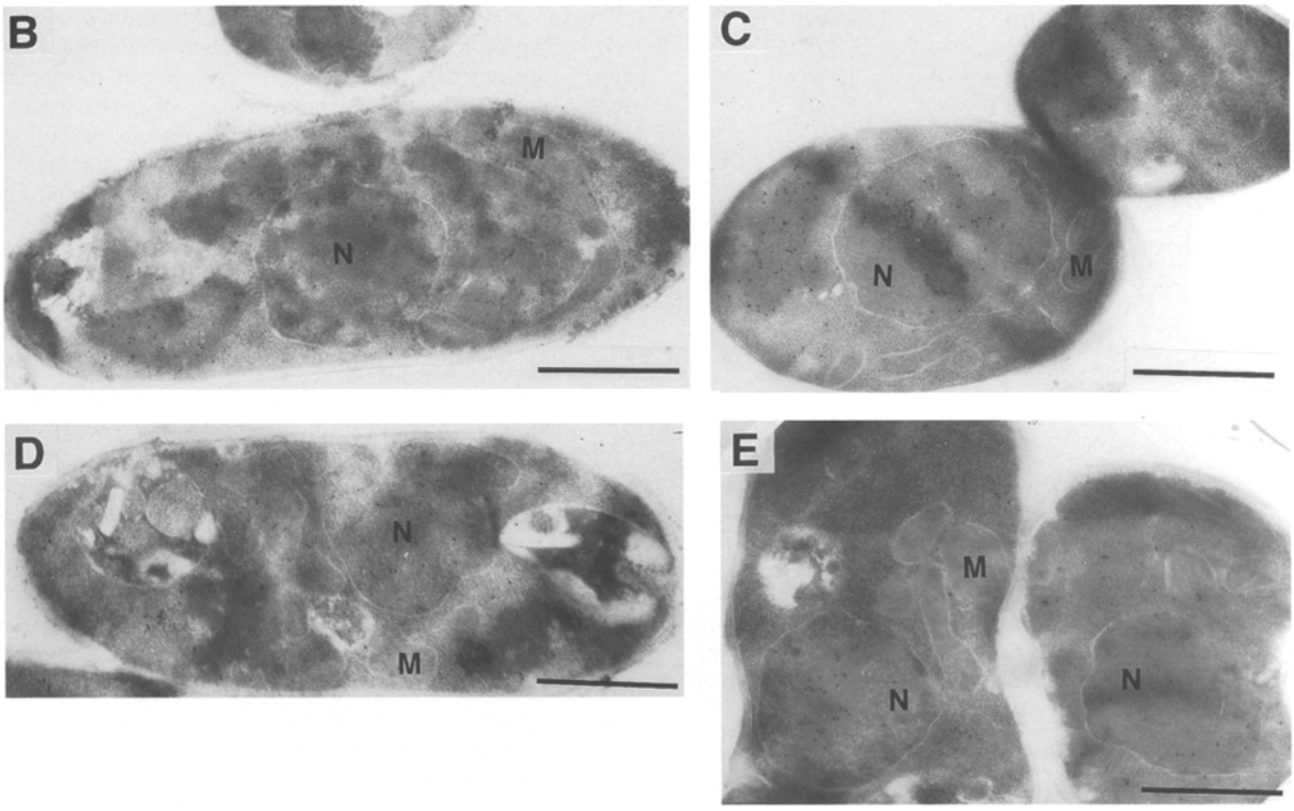


Figure 7. DHAS is active but mislocalized in peroxisome-deficient mutant strains (M6 and M13) and their *pmp47Δ* derivatives (M6-*pmp47Δ* and M13-*pmp47Δ*). (*A*) Activities of peroxisomal enzymes in the organellar pellet after differential centrifugation in the (*open box*) presence and (*shadow box*) 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 (− Triton X100) (−T), 0.208 for wild-type strain (+ Triton X100) (+ 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.

tent with the present and previous observations. (*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 *pmp47*Δ will have to exist free from the translocation ma-

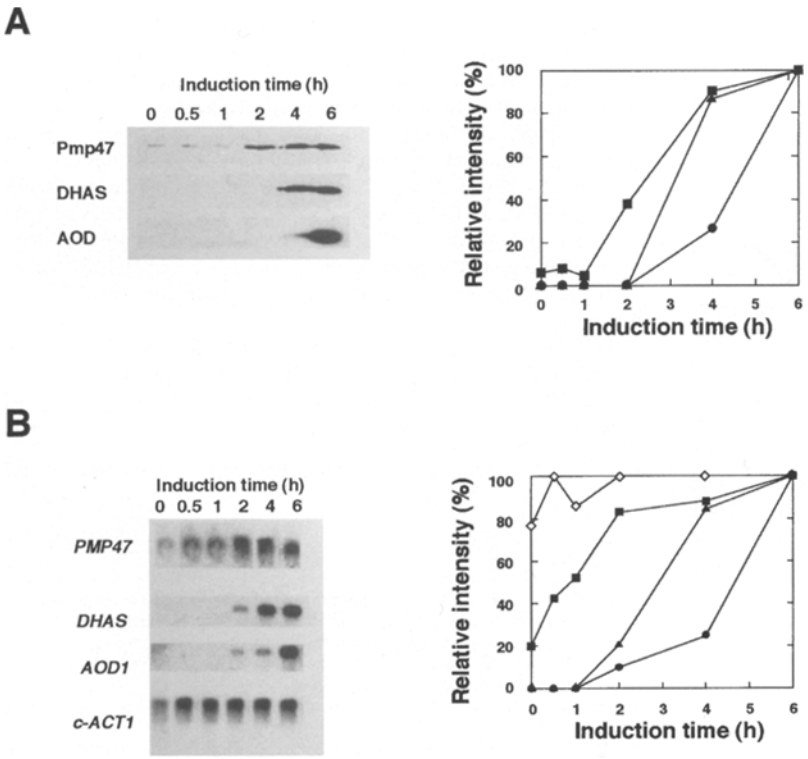


Figure 8. Induction of Pmp47, AOD, and DHAS, and their mRNAs during the early stage of peroxisomal induction. (*A*) Immunoblot analysis. (*B*) Northern analysis. Proteins (3.0 μg) and mRNAs (1.0 μg) extracted from each time point after transfer to methanol medium were loaded on to each lane. The bands 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 BglII-Sal I fragment of pMOX33) (Sakai and Tani, 1992), *PMP47* (1.6-kb HincII-HindIII fragment from pMP471) (this study), *DASI* (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).

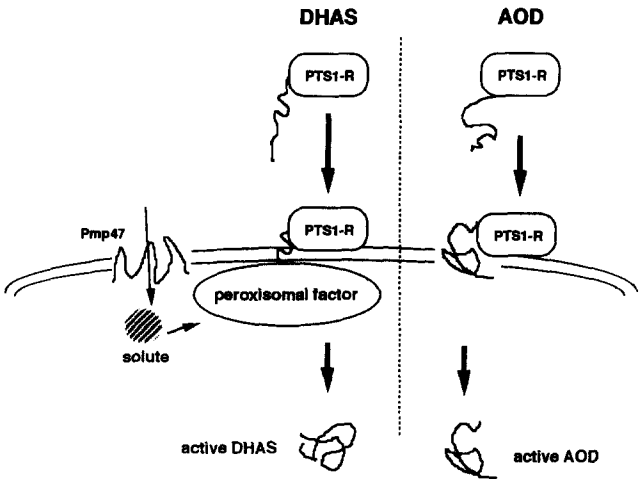


Figure 9. The diversity of peroxisomal protein import pathway (the PTS1 pathway) and a working hypothesis for the mechanism of DHAS import and folding. Both DHAS and AOD have a PTS1 motif sequence, NHL and ARY, respectively. However, only DHAS seems to demand a solute transported by Pmp47 for showing 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.

chinery or the membrane. However, in the experiment, all DHAS protein existed as aggregates in *pmp47Δ* (Fig. 4 *B*). (*c*) If the folding of DHAS was inhibited by the translocation machinery or peroxisomal membranes, the aggregates should contain a considerable amount of AOD, since AOD is one of the major component of purified peroxisomal membranes. However, anti-AOD labeled gold particles did not react with cytoplasmic aggregates.

What does Pmp47 directly transport? Since the protein family to which Pmp47 belongs is known to transport small solute compounds such as ADP/ATP, phosphate, tricarboxylate, 2-oxoglutarate/malate, etc., we favor the hypothesis that Pmp47 is an ATP/ADP exchanger, based on the following observations: (*a*) The highest sequence similarity of Pmp47 among the nearly 40 transporters in the family is with the mitochondrial ATP/ADP exchanger (Jank et al., 1993; Kuan and Saier, 1993). (*b*) The inverted topology of Pmp47 with respect to the mitochondrial ATP/ADP exchanger (McCammon et al., 1994) suggests an opposite direction of the exchange of substrates that is consistent with the function of the two organelles. ATP within mitochondria is required for translocation (Cyr et al., 1993), while there is a cytoplasmic ATP requirement for peroxisomal import (Imanaka et al., 1987; Wendland and Subramani, 1993). (*c*) Peroxisome proliferation may require energy in the form of ATP. (*d*) ATP is present in 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. boidinii*. 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 organellar 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.

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