

## Characterization of Peroxisomal Pex5p from Rat Liver

Pex5p IN THE Pex5p-Pex14p MEMBRANE COMPLEX IS A TRANSMEMBRANE PROTEIN\*

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**Pex5p is the receptor for the vast majority of peroxisomal matrix proteins. Here, we show that about 15% of rat liver Pex5p is found in the peroxisomal fraction representing 0.06% of total peroxisomal protein. This population of Pex5p displays all the characteristics of an intrinsic membrane protein. Protease protection assays indicate that this pool of Pex5p has domains exposed on both sides of the peroxisomal membrane. The strong interaction of Pex5p with the membrane of the organelle is not affected by mild protease treatment of intact organelles, conditions that result in the partial degradation of Pex13p. Cytosolic Pex5p is a monomeric protein. In contrast, virtually all peroxisomal Pex5p was found to be part of a stable 250-kDa protein assembly. This complex was isolated and shown to comprise just two subunits, Pex5p and Pex14p.**

In the last years, great advances have been made in the characterization of the biogenesis mechanism of peroxisomes (reviewed in Refs. 1 and 2). Peroxisomal matrix proteins are made on free ribosomes and post-translationally imported into the organelle (3). At least two types of peroxisome-targeting sequences (PTSs)<sup>†</sup> have been defined for this class of proteins. The majority of peroxisomal matrix proteins contain the so-called PTS1, a C-terminal tri-peptide with the sequence SKL (and variants) (2, 4); a minor fraction of matrix proteins are targeted to the organelle via PTS2, a N-terminal nona-peptide with the sequence (R/K)(L/V/I)X<sub>5</sub>(H/Q)(L/A) (2, 5). Proteins capable of recognizing these two signal sequences have been identified and characterized in several organisms. These are Pex5p (6–9) and Pex7p (10–12), the receptors for PTS1- and PTS2-containing proteins, respectively.

Subcellular fractionation studies revealed that although the majority of Pex5p behaves as a soluble cytosolic protein, a small fraction of this peroxin is consistently found in the peroxisomal fraction (13–15). In mammals and yeast, the peroxisomal pool of Pex5p is strongly attached to the membrane of

the organelle (8, 9, 16), an interaction that most likely is mediated by proteinaceous components. In support of this view, several peroxisomal intrinsic membrane proteins capable of interacting directly with the PTS1 receptor have been identified (see below). These observations provide the basis for the currently accepted model of peroxisomal matrix protein import (see Refs. 1 and 2). According to this model, Pex5p interacts with PTS1-containing proteins in the cytosol, binds to a docking factor(s) at the surface of the peroxisomal membrane, releases its cargo to some peroxisomal membrane component, and is shuttled back to the cytosol. An extended version of this model, in which the Pex5p-ligand complex is completely translocated across the peroxisomal membrane, has also been proposed (15).

Particular attention has been given to the identification and characterization of proteins that are capable of interacting with Pex5p. By using the two-hybrid system, *in vitro* binding assays and co-immunoprecipitation analysis, direct interactions of Pex5p with Pex8p (17), Pex12p (18), Pex13p (13, 14, 19), and Pex14p (20, 21) have been demonstrated. It was proposed by several authors that Pex13p and Pex14p represent the peroxisomal docking factors for Pex5p (13, 14, 19–21), although recently, some doubts were raised regarding the role of Pex14p in this process (Ref. 22 and see under “Discussion”). Despite all these data, not much is known about the architecture of the peroxisomal import machinery. For instance, it is still not clear whether some of these peroxins are subunits of stable protein complexes or if all the interactions that have been characterized so far reflect the existence of transient events *in vivo*.

If there are stable peroxin complexes on the peroxisomal membrane, then a strategy involving the isolation and characterization of these complexes could provide important structural data regarding the peroxisomal import machinery. It is obvious that some technical limitations may arise when applying such strategy; peroxins are low abundance proteins and some are intrinsic membrane proteins. Nevertheless, we have recently observed that chemical amounts of rat liver Pex3p can be easily obtained under denaturing conditions (23). In this work, we extend this observation to rat liver Pex5p. This time, a purification protocol using native conditions was used. We show that peroxisome-associated Pex5p is part of a stable protein complex comprising Pex14p. Other striking observations regarding the peroxisomal pool of Pex5p are presented.

### EXPERIMENTAL PROCEDURES

**Subcellular Fractionation and Protease Protection Assays**—Isolation of peroxisomes and preparation of a cytosolic fraction from rat liver by differential centrifugation were performed as described (24) with minor modifications (23). The peroxisomes used in this work were 92–94% pure having minor contaminations with endoplasmic reticulum (4%) and mitochondria (2%), as judged by the relative specific activities of marker enzymes (calculated according to Ref. 24). Alkaline carbonate

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<sup>1</sup> The abbreviations used are: PTS, peroxisomal targeting sequence; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione S-transferase; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

extraction of peroxisomes (25) and alkaline sucrose gradient floatation of membranes (23) were performed exactly as described. Extraction of peroxisomes using solutions of low or high ionic strength was performed as follows: freshly isolated rat liver peroxisomes at 1 mg/ml in SIE buffer (0.25 M sucrose, 5 mM imidazole HCl, pH 7.2, 1 mM EDTA-NaOH, pH 7.2) or SIE buffer containing 0.5 M NaCl were sonicated three times for 10 s (with a 30-s interval on ice) using a Heat Systems/Ultrasonics sonicator (model W-375) equipped with a microtip and set to 50% duty cycle, output 2. After sonication, the samples were halved. One-half was kept on ice as a control, and the other was separated into membrane and soluble fractions by centrifugation for 1 h at  $135,000 \times g$ . Protein in the samples was precipitated with 10% (w/v) trichloroacetic acid (added from a 100% (w/v) stock solution). After 30 min on ice, the precipitated protein was pelleted (15 min at  $15,000 \times g$ ), washed with acetone, solubilized in Laemmli sample buffer (26) (15 min at 80 °C with vigorous agitation), and separated by SDS-PAGE.

For the protease protection assays, freshly isolated rat liver peroxisomes (at 2 mg/ml, unless otherwise indicated) in SIE buffer were incubated on ice in the presence of the protease (freshly dissolved in SIE buffer) for 30 min. Where indicated, Triton X-100 was added to a final concentration of 0.1% (w/v) before adding the protease. Protease treatment of sonicated organelles was performed as follows: immediately after addition of the protease, organelles were sonicated as described above and incubated on ice for 30 min. Proteases were inactivated by incubation on ice for 2 min with 2 mM phenylmethanesulfonyl fluoride (added from a 0.2 M stock solution in ethanol). The proteins were precipitated immediately with trichloroacetic acid and processed for SDS-PAGE as described above.

**Partial Purification of Peroxisomal Pex5p**—In a typical experiment, the purification procedure was started with a pellet of 30 mg of purified peroxisomes. The organelles were solubilized in 4.8 ml of buffer A (20 mM Tris-HCl, pH 8.0, 0.5% (w/v) sodium deoxycholate, 1% (w/v) Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 50  $\mu$ g/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 5  $\mu$ g/ml pepstatin A, 2  $\mu$ g/ml *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane, 40  $\mu$ g/ml bestatin, 5  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml aprotinin) for 30 min at 4 °C and subjected to ultracentrifugation at  $135,000 \times g$  for 30 min at 4 °C in the 65.13 angular rotor (Sorvall). The insoluble material was discarded. To the supernatant, 50% (w/v) polyethylene glycol 4000 in buffer A was added to achieve a final concentration of 10% (w/v). After incubation on ice for 15 min, the sample was centrifuged at  $15,000 \times g$  for 15 min. To the resultant supernatant, 50% (w/v) polyethylene glycol 4000 in buffer A was added to give a final concentration of 15% (w/v). This sample was incubated on ice for 15 min and centrifuged at  $15,000 \times g$  for 15 min. The pellet obtained (enriched in Pex5p) was solubilized in 600  $\mu$ l of buffer B (containing 44 mM Bis-Tris-HCl, pH 7.0, 0.75 M 6-aminocaproic acid, 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 50  $\mu$ g/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 5  $\mu$ g/ml pepstatin A, 2  $\mu$ g/ml *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane, 40  $\mu$ g/ml bestatin, 5  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml aprotinin) and centrifuged at  $15,000 \times g$  for 15 min. The resultant supernatant was mixed with 106  $\mu$ l of saturated ammonium sulfate solution (pH 7.0 with  $\text{NH}_4\text{OH}$ ), incubated on ice for 15 min, and centrifuged at  $15,000 \times g$  for 15 min. To the supernatant obtained, 94  $\mu$ l of saturated ammonium sulfate solution was added. After incubation on ice for 15 min, the sample was centrifuged at  $15,000 \times g$  for 15 min. At this step, the precipitated protein was washed three times with 1 ml of 25% saturated ammonium sulfate solution, centrifuging ( $15,000 \times g$  for 5 min at 4 °C) between each wash. This pellet was then solubilized in 600  $\mu$ l of buffer A and applied to the top of a discontinuous sucrose gradient (2.7 ml of 7.5%, 2.5 ml of 10%, 2.1 ml of 15.5%, 1.7 ml of 21%, and 1.5 ml of 25% (w/v) sucrose in a buffer containing 50 mM Tris acetic acid, pH 8.0, 1 mM EDTA, and 0.1% Nonidet P-40). After centrifugation at  $165,000 \times g$  for 16 h at 4 °C in a TST 41.14 swing-out rotor (Sorvall), 13 fractions of 0.85 ml were collected from the bottom of the tube and analyzed by Western blotting using the anti-Pex5p antibody. The Pex5p-enriched fractions (fractions 9–12) were pooled. Fifty  $\mu$ l (bed volume) of DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech) pre-equilibrated in buffer C (20 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, and 0.5% (w/v) Nonidet P-40) containing 25 mM NaCl, were added to this fraction. After incubation of the suspension for 2 h at 4 °C with occasional shaking, the DEAE-Sepharose beads were washed with 600  $\mu$ l of buffer C containing 25 mM NaCl. The resin was sequentially washed with 200  $\mu$ l of 100, 150, 350, and 500 mM NaCl in buffer C. Pex5p was recovered in the 350 mM NaCl wash step as revealed by immunoblot analysis using the anti-Pex5p antibody. In a typical experiment, 10  $\mu$ g of this Pex5p-enriched material were obtained by this procedure.

**Antibodies**—To raise an antibody against human Pex12p, a cDNA

encoding amino acids 1–359 was amplified from human skin fibroblasts total RNA by reverse transcription-PCR using the primers 5'-gcggaat-tcagcaggaaactatggctgagc-3' and 5'-ggcctgcagagacatgattcccttcagttctcagg-3' designed according to the published sequence (27). The 1.1-kilobase pair cDNA fragment was digested with the *Eco*RI and *Pst*I restriction enzymes and cloned into the pBluescript II KS vector (Stratagene). After digestion of the recombinant plasmid with *Eco*RI and *Not*I, the insert was cloned into the pGEX-5X-1 (Amersham Pharmacia Biotech) expression vector. The human Pex12p fused to the glutathione *S*-transferase (GST) was expressed in the XL1 strain of *Escherichia coli* and obtained as inclusion bodies. These were isolated, and the fusion protein was purified by SDS-PAGE and used to immunize rabbits (28).

The human Pex5p cDNA was amplified by reverse transcription-PCR using the primers 5'-ccggtcgacatggcaatgcggagctgtgtga-3' and 5'-gcg-gtcgacctgtcactggggcaggccaaacatag-3' designed according to the published sequence (8). This molecule was cloned into the pGEM®-T easy vector according to the manufacturer's instructions (Promega). The recombinant plasmid was digested with *Eco*RI and *Sal*I, and the insert was cloned in pGEX-5X-1 and pMAL-c2 (New England Biolabs) expression vectors. The recombinant proteins containing amino acids 142–639 of Pex5p fused to GST or maltose-binding protein were expressed in the XL1 strain of *E. coli*. The GST-Pex5p fusion protein was purified by SDS-PAGE and used to immunize rabbits (28). Anti-Pex5p antiserum was immunopurified using the GST-Pex5p fusion protein blotted onto nitrocellulose membrane as described (28).

The preparation and characterization of the antibody directed to human Pex13p were described previously (23). The anti-catalase antibody was obtained from Research Diagnostics, Inc. A monoclonal antibody (clone 7H10-BD4) directed to the  $\alpha$  subunit of the mitochondrial ATPase, a peripheral membrane protein (29), was obtained from Molecular Probes. Rabbit and mouse antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse antibodies (Sigma), respectively.

**Miscellaneous**—Total RNA from human fibroblasts was isolated using the High Pure RNA Isolation Kit (Roche Molecular Biochemicals). The reverse transcription-PCR was done using the Titan™ One Tube Reverse Transcription-PCR System essentially as described by the manufacturer (Roche Molecular Biochemicals).

Proteins were measured by the Lowry method using bovine serum albumin as standard, as described (30).

SDS-PAGE was performed in 1.0-mm-thick 10 or 12% polyacrylamide gels using the Laemmli discontinuous buffer system (26). Silver staining of polyacrylamide gels was performed as described (31).

Blue native-PAGE was carried out according to the method of Schagger and von Jagow (32) with slight modifications. Protein samples (~200  $\mu$ g) were solubilized in 70  $\mu$ l of buffer B (see above). After a clarifying spin (15 min at  $15,000 \times g$ ), 5  $\mu$ l of 5% (w/v) Coomassie Blue G-250 in 0.5 M 6-aminocaproic acid was added to the sample before electrophoresis. Samples containing high concentrations of salt were first dialyzed against 50 ml of buffer B for 1 h, before addition of 5% (w/v) Coomassie Blue G-250 in 0.5 M 6-aminocaproic acid. Samples were resolved on 5–13% polyacrylamide gradient slab gels prepared according to Schagger and von Jagow (32) but containing 0.1% (w/v) Nonidet P-40 in all solutions. The cathode buffer contains 50 mM Tricine, 15 mM Bis-Tris-HCl, pH 7.0, 0.05% (w/v) sodium deoxycholate, and 0.02% (w/v) Coomassie Blue G-250. The anode buffer contains 50 mM Bis-Tris-HCl, pH 7.0. The gels were run for 16 h at 100 V (with constant voltage) at 4 °C. The second dimension was performed on 10% SDS-polyacrylamide gels exactly as described (32).

For the native sucrose gradient centrifugation analysis, protein samples (usually 2 mg) were solubilized in 600  $\mu$ l of buffer A (see above) and loaded on the top of a discontinuous sucrose density gradient as described above. Centrifugation was carried out at  $165,000 \times g$  for 16 h at 4 °C in a TST 41.14 rotor (Sorvall). Thirteen fractions of 0.85 ml were collected from the bottom of the gradient, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE.

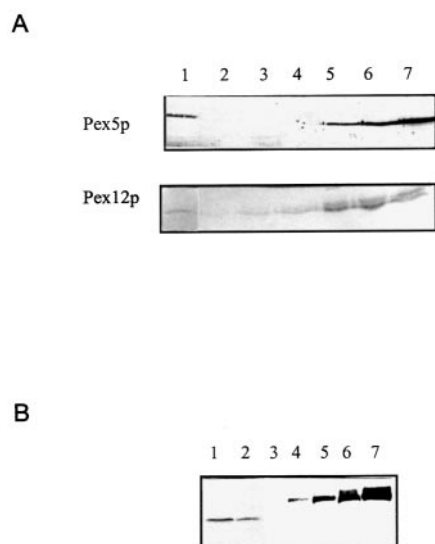
Western blotting onto nitrocellulose membranes (Schleicher & Schuell) was performed according to the manufacturer's instructions.

Densitometric analysis of immunoblots was carried out using the LKB BROMMA densitometer.

MALDI-MS of tryptic fragments of Coomassie Blue-stained protein bands in SDS-polyacrylamide gels was performed by HHMI/Keck Biotechnology Resource Laboratory (New Haven, CT).

## RESULTS

*A Considerable Fraction of Rat Liver Pex5p Is Peroxisome-associated and Behaves as an Intrinsic Membrane Protein*—As



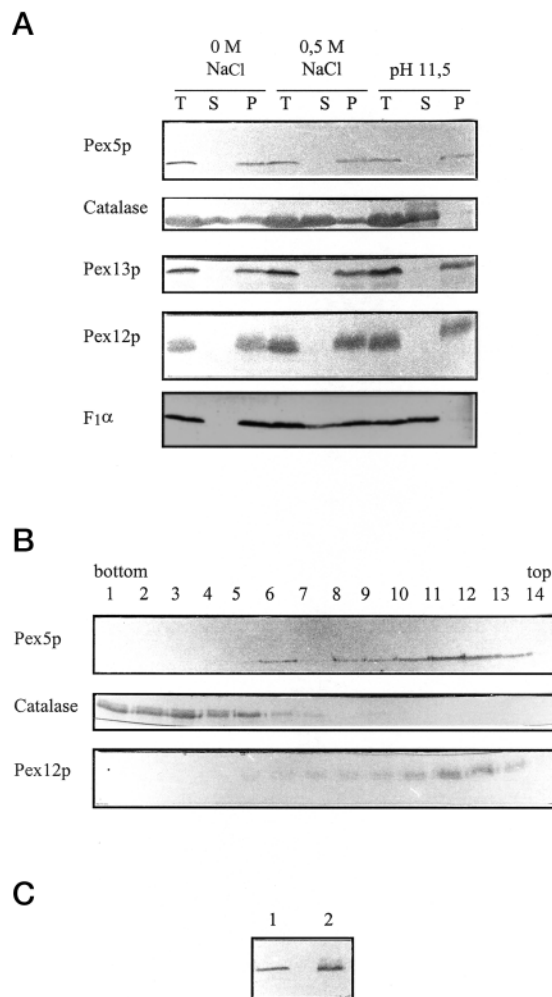
**FIG. 1. A considerable fraction of rat liver Pex5p is peroxisome-associated.** *A*, total rat liver homogenate (lane 1, 100  $\mu$ g of protein) and increasing amounts of highly pure rat liver peroxisomes (1, 3, 8, 16, 32, and 80  $\mu$ g of peroxisomal protein in lanes 2–7, respectively) were subjected to immunoblot analysis using an antibody directed to human Pex5p and Pex12p. *B*, highly pure rat liver peroxisomes (70 and 35  $\mu$ g of protein in lanes 1 and 2, respectively) and increasing amounts of a maltose-binding protein-Pex5p fusion protein (11, 33, 100, 300, and 900 ng of protein in lanes 3–7, respectively) were analyzed by immunoblotting using the immunopurified anti-Pex5p antibody. This antibody does not cross-react with the maltose-binding protein alone (data not shown).

a first step to characterize rat liver Pex5p we have determined the percentage of this protein that co-isolates with peroxisomes. For this purpose, highly pure peroxisomes and total homogenate from rat liver were subjected to Western blotting analysis using an antibody directed to human Pex5p (see Fig. 1*A*). Densitometric analysis of this blot (data not shown), together with the fact that peroxisomal protein corresponds to 2% of total rat liver protein (24), allowed us to estimate that 15% of Pex5p is peroxisome-associated. In a similar experiment, the amount of Pex5p in nanograms per microgram of total peroxisomal protein was also estimated (see Fig. 1*B*). A value of 0.6 ng of Pex5p per  $\mu$ g of total peroxisomal protein was obtained. This should be regarded as a minimal value since rat Pex5p may not be recognized by the anti-human Pex5p antibody as strongly as the human recombinant Pex5p protein used in this experiment as a standard.

Virtually all peroxisome-associated Pex5p behaves as an intrinsic membrane protein. Indeed, this pool of Pex5p cannot be extracted from the peroxisomal membrane either by sonication in low or high ionic strength buffers or by incubation of peroxisomal membranes at alkaline pH (see Fig. 2*A*). The possibility that Pex5p *per se* is not soluble under the conditions employed was still considered. However, this is clearly not the case. When alkali-treated peroxisomes are subjected to alkaline sucrose density floatation (23), both Pex5p and Pex12p (but not catalase) are found on the top of the sucrose gradient (Fig. 2*B*), a characteristic of peroxisomal intrinsic membrane proteins (23).

Finally, the behavior of peroxisome-associated Pex5p upon SDS-PAGE is not changed by reduction of the protein sample with dithiothreitol prior to electrophoresis (see Fig. 2*C*). Thus, the interaction of Pex5p with the peroxisomal membrane does not involve a thiol-cleavable covalent bound to some intrinsic membrane protein of this organelle.

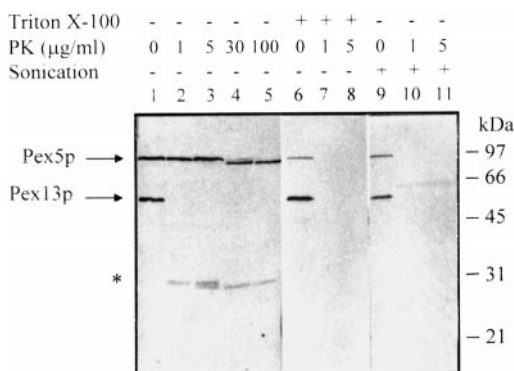
**Peroxisomal Pex5p Is a Transmembrane Protein**—As shown above, peroxisome-associated Pex5p displays all the character-



**FIG. 2. Peroxisomal Pex5p behaves as an intrinsic membrane protein.** *A*, 200  $\mu$ g of highly pure peroxisomes from rat liver were either sonicated in the presence of SIE buffer (0 M NaCl; see "Experimental Procedures") or 0.5 M NaCl in SIE buffer (0.5 M NaCl) or resuspended in 0.1 M  $\text{Na}_2\text{CO}_3$ , pH 11.5 (pH 11.5). The samples were halved. One-half was kept on ice for determination of recoveries (lanes T). The other half was separated into membrane (lanes P) and soluble fraction (lanes S) by centrifugation (1 h at  $135,000 \times g$ ). After trichloroacetic acid precipitation, the samples were analyzed by immunoblotting using antibodies directed to Pex5p, Pex12p, Pex13p, catalase, and the  $\alpha$  subunit of the mitochondrial ATPase complex (F1 $\alpha$ ). *B*, 2 mg of rat liver peroxisomes were resuspended in 2 ml of sucrose 52% (w/v) in 0.1 M  $\text{Na}_2\text{CO}_3$ , pH 11.5, and subjected to alkaline density gradient floatation exactly as described (23). The gradient was fractionated into 14 0.8-ml aliquots starting from the bottom of the tube (lane 1). After trichloroacetic acid precipitation, equivalent amounts of each fraction (derived from 150  $\mu$ g of peroxisomal protein) were analyzed by immunoblotting using antibodies directed to Pex5p, Pex12p, and catalase. *C*, 80  $\mu$ g of rat liver peroxisomes were solubilized in Laemmli sample buffer lacking  $\beta$ -mercaptoethanol with (lane 1) or without (lane 2) dithiothreitol (100 mM). Both samples were analyzed by immunoblotting using the antibody directed to Pex5p.

istics of a typical intrinsic membrane protein. What is the topology of this protein in the peroxisomal membrane? To address this question we have performed a protease protection assay. For this purpose, freshly isolated rat liver peroxisomes were treated with different amounts of proteinase K. After inactivation of the protease, the protein was precipitated with trichloroacetic acid and analyzed by Western blotting using antibodies directed to Pex13p and Pex5p. Mammalian Pex13p is an intrinsic membrane protein having at least its C-terminal SH3 domain exposed to the cytosol (13, 14). In agreement with this model, when rat peroxisomes are subjected to this proteolytic treatment, Pex13p is readily accessible to the added pro-



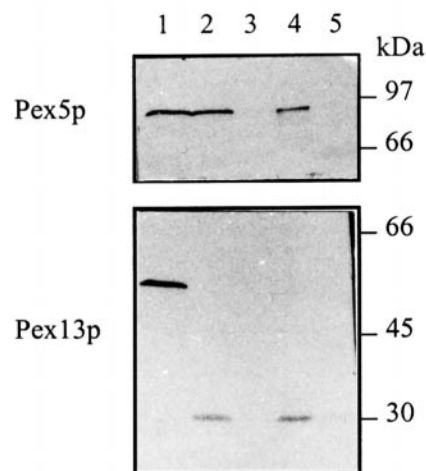


**FIG. 3. Peroxisomal Pex5p is a transmembrane protein.** 100  $\mu$ g of freshly isolated rat liver peroxisomes in SIE buffer (see "Experimental Procedures") were incubated on ice for 30 min in the presence of the indicated amounts of proteinase K (PK). Where indicated, Triton X-100 was added to a 0.1% (w/v) final concentration before addition of the protease. Some samples were sonicated immediately after the addition of the protease (as described under "Experimental Procedures"). After inactivation of the protease, the protein in the samples was precipitated with trichloroacetic acid and analyzed by immunoblotting using the antibody directed to Pex5p. The blots were reprobed with an antibody directed to Pex13p. The asterisk indicates the Pex13p fragments. The numbers at the right indicate the molecular masses of the applied standards in kDa.

tease (Fig. 3, lanes 2–5). However, a fragment of this protein (a polypeptide of 28 kDa) remains protease-protected even at the highest proteinase K concentration used in this experiment. This domain becomes accessible only when the peroxisomal membrane is disrupted by detergent (Fig. 3, lanes 7 and 8). It should be noted that under these conditions the 28-kDa fragment of Pex13p is extremely sensitive to proteinase K. Indeed, a protease concentration of only 0.1  $\mu$ g/ml is sufficient to degrade completely this peptide (data not shown) making it a good marker to monitor the integrity of the peroxisomal membrane.

When the behavior of rat Pex5p in this experiment is analyzed, a strikingly similar result is found. A domain of approximately 2 kDa is clipped from Pex5p when intact peroxisomes are subjected to the protease treatment. Although this cleavage requires relatively high proteinase K concentrations, it clearly occurs under conditions in which the 28-kDa fragment of Pex13p is fully protected (see Fig. 3, lanes 4 and 5). Thus, at least a short domain of peroxisomal Pex5p is exposed into the cytosol. Finally, when the peroxisomal matrix compartment is exposed to the protease by detergent treatment of the organelles, Pex5p is completely degraded (Fig. 3, lanes 7 and 8). Apparently, Pex5p is exposed on both sides of the peroxisomal membrane.

When performing a protease protection assay it is generally assumed that addition of a mild detergent (e.g. Triton X-100) just solubilizes the membrane of the organelle thus exposing the luminal domains of the protein under study to the action of the protease used in the assay. However, we have to consider that some protein complexes may not resist this solubilization procedure in an intact form. Thus, it would be possible that the partial protease resistance of Pex5p in intact organelles is just the result of a shielding effect performed by other protein(s) to which Pex5p binds on the surface of the peroxisome; after addition of the detergent this protein complex would be destroyed exposing Pex5p to the protease. In order to test this possibility peroxisomes were subjected to a mild sonication treatment in the presence of proteinase K. As shown above (see Fig. 2A) sonication of peroxisomes is not sufficient to disrupt the interaction of Pex5p with the peroxisomal membrane. When the peroxisomal matrix compartment is exposed to the



**FIG. 4. Peroxisomal Pex5p remains strongly attached to peroxisomal membrane after mild protease treatment of intact peroxisomes.** 200  $\mu$ g of freshly isolated rat liver peroxisomes in 40  $\mu$ l of SIE buffer were treated with trypsin (50  $\mu$ g/ml) for 30 min on ice. After inactivation of the protease, the organelles were diluted with 1.3 ml of 0.1 M  $\text{Na}_2\text{CO}_3$ , pH 11.5, and kept on ice for 30 min. Half of the sample was separated into membrane (lane 4) and soluble fraction (lane 3) by centrifugation (1 h at  $135,000 \times g$ ). The other half was kept on ice for determination of recoveries (lane 2). After trichloroacetic acid precipitation, the samples were analyzed by immunoblotting using antibodies directed to Pex5p and Pex13p. Lane 1, 100  $\mu$ g of untreated peroxisomes; lane 5, 100  $\mu$ g of Triton X-100-solubilized peroxisomes incubated with trypsin as described above. The numbers at the right indicate the molecular masses of the applied standards in kDa.

protease using this method, both Pex5p and Pex13p are digested even at low proteinase K concentrations (Fig. 3, lanes 10 and 11). Thus, the observed partial resistance to proteinase K of Pex5p in intact organelles is the result of shielding by the peroxisomal membrane itself. Taken together, these data indicate that rat peroxisomal Pex5p is a transmembrane protein.

**Pex5p Remains Attached to the Peroxisomal Membrane after Mild Protease Treatment of Intact Organelles**—As shown above, when intact peroxisomes are subjected to protease treatment only a small peptide of Pex5p is removed. This cleavage reaction is not very efficient, requiring a relatively high proteinase K concentration. Indeed, treatment of peroxisomes with low concentrations of this protease (e.g. 1–5  $\mu$ g/ml; see Fig. 3) results in no cleavage of Pex5p. A similar result is obtained when trypsin is used in these experiments; Pex5p remains uncleaved when intact organelles are incubated with trypsin concentrations up to 50  $\mu$ g/ml. Nevertheless, under these conditions Pex13p is degraded to a faster migrating species on SDS-PAGE (see below). Taking these data into consideration and the fact that Pex13p has been shown to interact with Pex5p (13, 14, 19), we have determined whether the cytosolic (protease-accessible) domains of rat Pex13p are essential for the interaction of Pex5p with the peroxisomal membrane. For this purpose, freshly isolated rat liver peroxisomes were incubated in the presence of trypsin (50  $\mu$ g/ml). After inactivation of the protease, the organelles were subjected to alkaline extraction in order to isolate intrinsic membrane proteins (see "Experimental Procedures"). Equivalent amounts of membrane (Fig. 4, lane 4) and soluble proteins (Fig. 4, lane 3) were analyzed by Western blotting using antibodies directed to Pex13p and Pex5p. As shown in Fig. 4, Pex13p was cleaved to a 30-kDa fragment by the action of trypsin. This fragment is still membrane-associated in agreement with its membrane topology model. As expected, Pex5p was not digested under these conditions. Most importantly, virtually all Pex5p remained resistant to the alkaline extraction. Thus, these results strongly suggest that the cytosolic (protease-accessible) do-

mains of Pex13p are not necessary to maintain Pex5p strongly attached to the peroxisomal membrane.

**Peroxisomal Pex5p Is Part of a Stable Protein Complex—**Besides recognizing PTS1-containing proteins *en route* to the peroxisome, Pex5p is able to interact with several other components of the import machinery of this organelle. Indeed, using the two-hybrid system, *in vitro* binding assays and co-immunoprecipitation analysis, Pex5p has been shown to interact directly with Pex8p (in *Saccharomyces cerevisiae* (17)), Pex12p (in human cells (18)), Pex13p (in fungi and human (13, 14, 19)), and Pex14p (in mammals and *S. cerevisiae* (20–22)). However, our knowledge about the architecture of this machinery is still limited. For instance, is still not clear whether all these peroxins are part of stable complexes or if they just interact in a transient way. We think that a strategy involving the isolation and polypeptide characterization of complex(es) containing Pex5p could provide some insights on this issue.

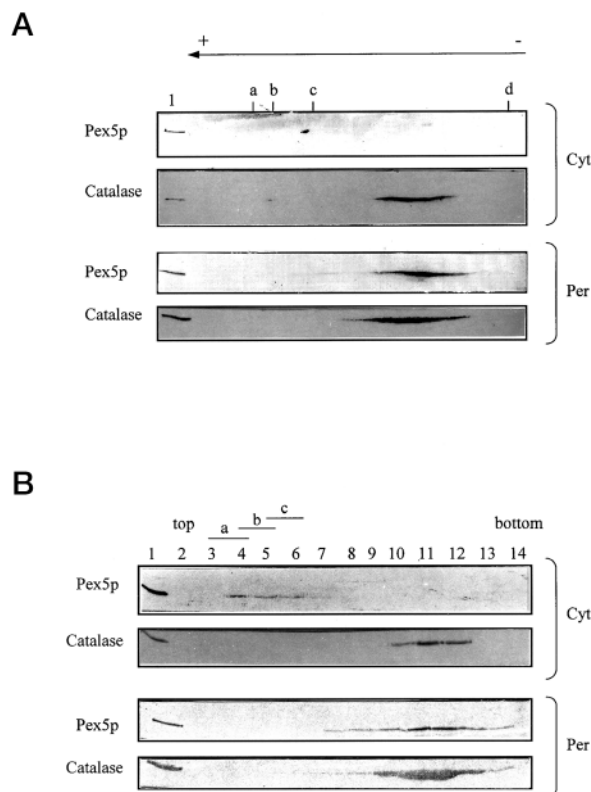
In order to test the feasibility of this approach, we have determined the molecular mass of peroxisomal Pex5p under native conditions. Considering that the interactions of Pex5p with all the other peroxins mentioned above are thought to occur at the peroxisomal membrane level (and not in the cytosol), we have included cytosolic Pex5p in this analysis. For this purpose, peroxisomal and cytosolic proteins from rat liver were resolved by blue-native gel electrophoresis (32) followed by a second dimension on SDS-PAGE and Western blot analysis.

As shown in Fig. 5A cytosolic Pex5p migrates with an apparent molecular mass of 70–90 kDa. The migration of catalase present in this fraction (presumably released into the cytosol during the isolation of peroxisomes) is also shown for comparison. Catalase is a tetrameric protein of 240 kDa (33). In sharp contrast with cytosolic Pex5p, peroxisomal Pex5p displays an apparent molecular mass of about 250 kDa. Similar results were obtained when the molecular masses of both the cytosolic and peroxisomal Pex5p were estimated by sucrose gradient sedimentation analysis (see Fig. 5B), although the (unknown) contribution of detergents and phospholipids to the size of the proteins under study was not considered.

Thus, the data presented suggest that cytosolic Pex5p is a monomeric protein under the experimental conditions used. In addition, we show that peroxisomal Pex5p is present in the membrane of the organelle as a high molecular mass complex. This complex, or at least a fraction of it, is stable and displays a molecular mass of about 250 kDa.

**Isolation of Peroxisomal Pex5p Under Native Conditions—**The fact that a stable protein complex containing Pex5p can be detected after detergent solubilization of peroxisomes encouraged us to isolate chemical amounts of this complex. A very simple procedure was developed for this purpose. It involves a polyethylene glycol precipitation, an ammonium sulfate precipitation, a sucrose density gradient centrifugation, and a DEAE-Sephacel adsorption step (see “Experimental Procedures” for details). Densitometric analysis of immunoblots containing equivalent amounts of these fractions using the anti-Pex5p antibody indicates that 12% of the initial amount of Pex5p was recovered in the last step (see Table I). The polypeptide compositions of the Pex5p-enriched fractions obtained in each step of the purification scheme are shown in Fig. 6. Three protein bands presenting apparent molecular masses of 90, 60, and 44 kDa are clearly enriched after the last step of the purification protocol (Fig. 6, lane 6).

In order to provide unambiguous proof that a Pex5p-containing complex was indeed isolated and also to identify the two proteins co-purifying with Pex5p, all the three major protein bands present in the Pex5p-enriched fraction (Fig. 6, lane 6)



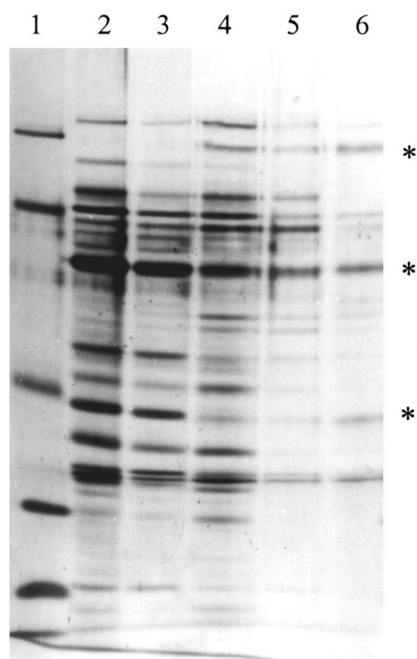
**FIG. 5. Peroxisomal Pex5p is part of a 250-kDa protein complex.** A, 200  $\mu$ g of rat liver cytosolic (Cyt) and peroxisomal protein (Per) were subjected to two-dimensional gel electrophoresis. Blue-native gel electrophoresis was used in the first dimension and SDS-PAGE in the second dimension. The direction of the first dimension is indicated by an arrow on the top of the figure. Letters a–d mark the position of molecular mass markers used to calibrate the first-dimension gel as follows: a, hen egg white ovalbumin (45 kDa); b, bovine serum albumin (66 kDa); c, rabbit muscle phosphorylase b (97 kDa); d, rabbit skeletal muscle myosin (470 kDa). The second-dimension gels were subjected to Western blotting. The blots were probed with anti-Pex5p and anti-catalase antibodies. Lane 1, 40  $\mu$ g of cytosolic protein (Cyt) and 40  $\mu$ g of peroxisomal protein (Per). B, 200  $\mu$ g of rat liver cytosolic (Cyt) and peroxisomal protein (Per) were subjected to sucrose density gradient centrifugation analysis (see “Experimental Procedures”). The gradients were fractionated into 13 0.85-ml aliquots starting from the bottom (lane 14). After trichloroacetic acid precipitation, the protein in the samples was analyzed by immunoblotting using antibodies directed to Pex5p and catalase. Lane 1, 40  $\mu$ g of cytosolic protein (Cyt) and 40  $\mu$ g of peroxisomal protein (Per). The position of the molecular mass standards (a–c, see above) are indicated. The 470-kDa marker (myosin) was found in the pellet of the gradient (data not shown).

**TABLE I**  
Partial purification of the Pex5p-Pex14p complex from rat liver peroxisomes

Procedure	Total Protein	Yield <sup>a</sup>	Purification
	mg	%	-fold
Peroxisomal extract	32.0	100.0	1.0
Polyethylene glycol fractionation	10.8	78.0	2.3
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation	0.445	32.5	23.3
Sucrose gradient centrifugation	0.135	29.5	70.0
DEAE-Sephacel adsorption	0.010	11.8	364.0

<sup>a</sup> Values were determined by densitometric analysis of immunoblots containing known amounts of each fraction using the anti-Pex5p antibody.

were subjected to MALDI-MS (see “Experimental Procedures”). The results of this analysis (see Table II) show that the 90-kDa polypeptide corresponds to Pex5p, as expected. Twelve peptides



**FIG. 6. Partial purification of a Pex5p-Pex14p complex.** Highly pure rat liver peroxisomes were solubilized with detergents and subjected to a clarifying centrifugation to remove insoluble material (see "Experimental Procedures" for details). An aliquot of this extract (2  $\mu$ g of protein) was loaded in lane 2. Solubilized proteins were sequentially subjected to polyethylene glycol precipitation (lane 3, 1.5  $\mu$ g of protein),  $(\text{NH}_4)_2\text{SO}_4$  fractionation (lane 4, 1.5  $\mu$ g of protein), sucrose gradient centrifugation (lane 5, 0.5  $\mu$ g of protein), and a DEAE-Sepharose adsorption step (lane 6, 0.5  $\mu$ g of protein). Only Pex5p-enriched fractions obtained at each step of the purification procedure are shown. Protein bands corresponding to Pex5p (90 kDa), Pex14p (60 kDa), and the Pex14p fragment (44 kDa) are marked with asterisks. Pex14p and the 44-kDa fragment of Pex14p co-migrate with two abundant peroxisomal proteins that display a brown color upon silver-staining. Co-enrichment of Pex14p and its 44-kDa fragment with Pex5p is easily observed by direct inspection of the silver-stained gel since both polypeptides display a dark-gray color after silver staining. Lane 1, molecular mass markers as follows: rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa).

comprising 27% of the mouse Pex5p sequence ((34) the rat Pex5p sequence is not yet available) and derived from the region encompassing amino acid residues 84–626 of this peroxin were detected by this analysis.

When the 60-kDa protein band was subjected to MALDI-MS, 16 peptides covering 45% of the rat Pex14p sequence (35) were detected (see Table II). These peptides are derived from the Pex14p region encompassing amino acid residues 26–376 (the last residue of this protein).

Unexpectedly, the 44-kDa protein band also corresponds to Pex14p. In this case, only 12 peptides comprising 36% of the rat Pex14p sequence and derived from the region encompassing amino acids residues 26 to 291 were detected upon MALDI-MS. It is possible that this polypeptide represents a truncated form of Pex14p probably lacking some (not more than 84) C-terminal amino acid residues.

These data clearly demonstrate that a Pex5p-Pex14p complex was isolated using the purification procedure described here. Is this protein assembly the same 250-kDa Pex5p-containing complex detected by immunoblot analysis after detergent solubilization of peroxisomes or were some subunits of this complex lost during the purification procedure? In order to answer this question, we have analyzed the Pex5p-Pex14p-enriched fraction described above (see Fig. 6, lane 6) by blue-native gel electrophoresis followed by a second dimension on

SDS-PAGE. As shown in Fig. 7, Pex5p displays exactly the behavior previously shown for peroxisome-associated Pex5p by Western blotting analysis (compare with Fig. 5A). (It is interesting to note that the high resolution provided by the blue-native gel electrophoresis system allows a partial separation of Pex5p-Pex14p complexes from Pex5p complexes containing the 44-kDa Pex14p fragment.) Thus, no components of the 250-kDa Pex5p-containing complex were lost during the purification procedure employed. This result also implies that there is more than one copy of at least one of the subunits in this Pex5p-Pex14p complex. Indeed, two-dimensional densitometric analysis of the gel shown in Fig. 7 indicates a Pex5p:Pex14p:Pex14p fragment molar ratio of 1:3.1:2.0 (conversion of the densitometric signals into molar equivalents was performed using the theoretical molecular masses of mouse Pex5p and rat Pex14p and considering that the 44-kDa protein corresponds to Pex14p lacking its 84 C-terminal amino acid residues). Assuming that these proteins do not exhibit an unusual staining behavior, then this stoichiometry would result in a complex with a predicted molecular mass of 255 kDa, a value in close agreement with the obtained experimentally.

#### DISCUSSION

In this work, a biochemical characterization of the peroxisome-associated pool of rat liver Pex5p is presented. As described previously (8), this form of the PTS1 receptor displays all the typical characteristics of an intrinsic membrane protein. Indeed, all attempts to extract Pex5p from the peroxisomal membrane using a variety of techniques intended to release peroxisomal soluble and peripheral membrane proteins have failed; solubilization of this form of Pex5p could be accomplished only when detergents were used. This observation could imply that 1) either Pex5p is in direct contact with the lipid bilayer of the peroxisomal membrane or 2) Pex5p is strongly associated with some intrinsic protein(s) of the peroxisomal membrane. (A combination of the two possibilities is also feasible.) Although there is no formal proof to support either of these models, it is generally believed that the strong interaction of the PTS1-receptor with the peroxisomal membrane is mediated by proteinaceous components. In support of this view, direct interactions of Pex5p with Pex12p (18), Pex13p (13, 14, 19), and Pex14p (20, 21), three peroxisomal intrinsic membrane proteins, have been shown. However, considering the nature of the techniques used in those studies, several aspects remain obscure; how strong are these interactions *in vivo*? Are they dynamic or are they involved in the formation of stable protein complexes? The data presented in this work provide some insights into these questions.

In order to gather some more information regarding the interaction of this peroxin with the peroxisomal membrane, we have tried to define the membrane topology of Pex5p. By using protease protection assays, peroxisomal Pex5p was found to be exposed on both sides of the membrane. Although this is the first time that such a membrane topology is shown for the PTS1 receptor, it is possible that this is not a characteristic unique to rat Pex5p. As noted by Terlecky *et al.* (9), peroxisome-associated (alkaline-resistant) Pex5p from *Pichia pastoris* is more susceptible to proteinase K when the peroxisomal membrane is first disrupted by addition of Triton X-100.

The identities of the Pex5p domains facing each side of the membrane are not known at the present. This will require the production of antibodies directed to different domains of this protein. Nevertheless, data concerning the topology of several Pex5p-binding peroxins and the domains involved in these interactions are already available. These data, together with the results presented here, can be used to infer some structural properties of the rat peroxisome-associated Pex5p.



TABLE II  
MALDI-MS analysis of tryptic fragments obtained from the three major protein bands present in the Pex5p-enriched fraction  
MALDI-MS was performed by HHMI/Keck Biotechnology Resource Laboratory (New Haven, CT).

Protein band	Sequence coverage	Peptides			
		Measured mass	Computed mass	Residues	
				Start	To
<i>Da</i>					
90 kDa	27% of mouse Pex5p sequence	2067.942	2067.923	84	100
		1539.682	1539.672	159	170
		2595.142	2595.118	185	205
		1174.662	1174.634	269	278
		1410.642	1410.630	281	292
		2554.122	2554.124	303	324
		2398.052	2398.023	304	324
		1380.662	1380.630	325	335
		2385.192	2385.170	434	457
		1158.662	1158.639	548	557
		1606.752	1606.740	594	608
		1808.902	1808.872	609	626
60 kDa and 44 kDa <sup>a</sup>	45% (36%) of rat Pex14p	940.532 (940.552)	940.559	26	34
		763.382 (763.382)	763.397	35	40
		771.412 (771.402)	771.423	43	49
		1113.692 (1113.692)	1113.690	130	139
		1278.712 (1278.722)	1278.693	184	195
		2061.112 (2061.142)	2061.037	196	213
		789.402 (789.392)	789.423	214	220
		1210.672 (1210.672)	1210.645	227	237
		1054.552 (1054.552)	1054.544	228	237
		1066.622 (1066.622)	1066.617	238	246
		3116.482 (3118.542)	3116.427	247	278
		1376.632 (1376.642)	1376.595	279	291
		1233.592	1233.565	311	320
		1428.742	1428.695	350	362
		1272.632	1272.594	351	362
		1345.622	1345.585	365	376

<sup>a</sup> Values for the 44-kDa fragment of rat Pex14p are given in parentheses.

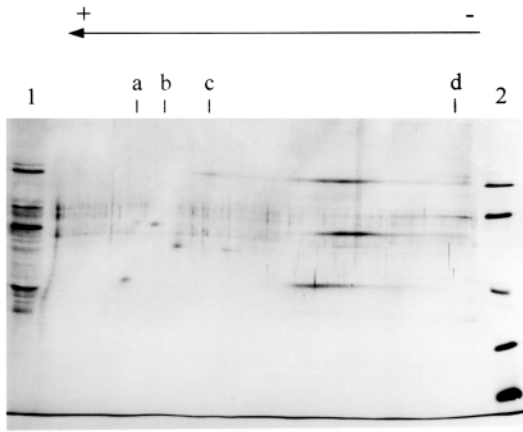


FIG. 7. **The Pex5p-Pex14p complex is a 250-kDa protein assembly.** 1.5  $\mu$ g of the partially purified Pex5p-Pex14p complex from rat liver peroxisomes (see Fig. 6, lane 6) was subjected to two-dimensional gel electrophoresis exactly as described in legend to Fig. 5. The direction of the first dimension is indicated by an arrow on the top of the figure. Letters a–d mark the position of molecular weight markers used to calibrate the first-dimension gel (see legend to Fig. 5). The second-dimension gel was stained with silver. Lane 1, 0.5  $\mu$ g of the protein sample loaded in the first-dimension gel; lane 2, molecular weight markers (see legend to Fig. 6).

The first peroxisomal integral membrane protein to be identified as a Pex5p-binding protein was Pex13p (13, 14, 19). This interaction involves the C-terminal SH3 domain of Pex13p (13, 14, 19) and an N-terminal domain of Pex5p (amino acid residues 100–213 in the *P. pastoris* Pex5p (36)). The finding that the SH3 domain of Pex13p faces the cytosol led to the proposal that this peroxin is the docking protein for the PTS1 receptor (13, 14, 19). However, recent studies in *S. cerevisiae* clearly

show that the steady-state levels of Pex5p present in a membrane-embedded complex comprising Pex14p, Pex17p, and a recombinant version of Pex7p remain unchanged after deletion of the gene encoding Pex13p (37). Furthermore, as shown here, rat liver Pex5p remains attached to the peroxisomal membrane in an alkaline-resistant manner when the cytosolic (protease accessible) domains of Pex13p are proteolytically removed. These data do not exclude the possibility that Pex13p is somehow involved in the recruitment of Pex5p from the cytosol into the peroxisomal membrane or, as has been suggested recently (36), in the release of Pex5p from the peroxisomal membrane into the cytosol. However, it seems plausible that once insertion into the peroxisomal membrane occurs, Pex5p no longer depends on this interaction to remain strongly associated with the membrane. Thus, the prediction that the N-terminal domain of the peroxisomal Pex5p described here is exposed into the cytosol based on its interaction with the SH3 domain of Pex13p is not necessarily correct.

Pex14p was the second peroxin to be identified as a Pex5p-binding protein (20–22, 38). In human, this interaction involves the N-terminal half of Pex5p and the first 78 N-terminal amino acid residues of Pex14p (39). The interaction of Pex14p with the peroxisomal membrane in yeast cells is still a matter of controversy (20, 38, 40). However, clear results regarding the mammalian Pex14p topology have been recently published (22). The data presented by Will *et al.* (22) strongly suggest that the N-terminal domain of human Pex14p is exposed into the lumen of the organelle. Based on this finding, the authors speculated that this would imply that the PTS1 receptor is in the lumen of the peroxisome at some stage of the protein import process, an hypothesis that would be in support of the “extended shuttle model of peroxisomal protein import” (15). Our results clearly indicate that there is no need to make this as-

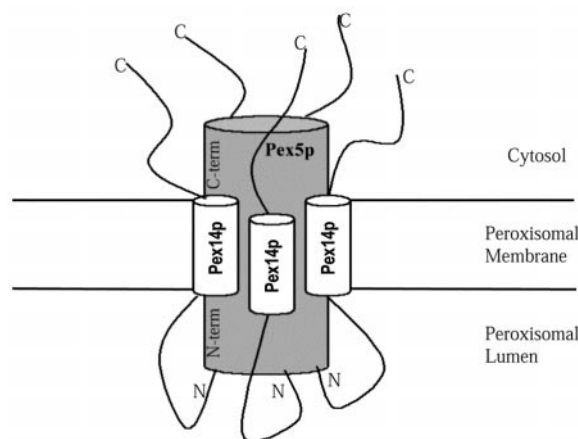


FIG. 8. Hypothetical model for the membrane topology of rat peroxisomal Pex5p. The C-terminal domain of Pex5p, containing the PTS1-binding domain, is facing the cytosol as proposed for the human (16) and *P. pastoris* (9) peroxisomal Pex5p. Pex14p, an intrinsic membrane protein, shields the PTS1 receptor from the hydrophobic environment of the membrane. The N-terminal domain of the PTS1 receptor is facing the lumen of peroxisomes based on the assumption that the N terminus of rat Pex14p is also exposed into this compartment.

sumption. The Pex5p-Pex14p interaction involves a Pex5p population that is still exposed on the cytosolic face of the peroxisomal membrane. The Pex14p membrane topology proposed by Will *et al.* (22) together with our results have another important implication; the Pex14p-binding domain of the Pex5p population described here is exposed into the lumen of the organelle.

We have shown that the rat liver cytosolic Pex5p behaves as a monomeric protein. In contrast, virtually all peroxisome-associated Pex5p was found to be part of a 250-kDa protein assembly. Chemical amounts of this complex were isolated and shown to consist of only two subunits, Pex5p and Pex14p. The ratio of Pex5p:Pex14p in the isolated complex was estimated to be 1:5. It is interesting to note that an almost similar Pex14p binding stoichiometry was described for recombinant human Pex5p (39). However, some doubts were raised by these authors concerning the possibility that all Pex14p-binding sites in Pex5p are occupied *in vivo*. Our data strongly suggest that, indeed, this is the case. Schliebs *et al.* (39) also noted that recombinant Pex5p is capable of forming homotetramers. In contrast, we found no evidence for homotetramerization of Pex5p. It remains to be shown whether this discrepancy results from the solubilization conditions used in our work or if it reflects the high protein concentrations used in the experiments performed with the recombinant protein.

One intriguing aspect of the data presented here concerns the biochemical homogeneity of the peroxisomal pool of rat Pex5p. As far as the detection limits of an immunoblot analysis allow us to infer, virtually all Pex5p present in rat liver peroxisomes behaves as an intrinsic membrane protein. This Pex5p population is inserted into the peroxisomal membrane having domains facing the two sides of the membrane. Finally, we provide data suggesting that all peroxisomal Pex5p is part of a stable protein complex comprising Pex14p (a hypothetical model regarding the Pex5p-Pex14p complex is presented in Fig. 8). These are quite unusual properties for a protein that presumably is in transit either to the cytosol (as proposed by the cycling receptor theory) or even to the peroxisomal matrix compartment (as proposed in the extended version of the cycling receptor model). If these models turn out to be true, then our results simply indicate that the disruption of the Pex5p-Pex14p interaction is a rate-limiting step in rat liver. However, considering the data presented here, the possibility that this

peroxisomal Pex5p pool may represent a resident population of molecules should still be considered. In this context, it is important to consider that peroxisomal (alkaline-resistant) Pex5p from *P. pastoris* is capable of interacting with a PTS1-containing peptide (9), suggesting that this membrane-embedded form of the PTS1-receptor may, indeed, function as such *in vivo*.

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