Peroxisome is a ubiquitous organelle present in nearly all eukaryotic cells and involved in various oxidative enzymatic reactions such as the α- and β-oxidation of fatty acids. Enzymes catalyzing these reactions are well characterized and found in the matrix of the organelle. These matrix proteins are synthesized in the cytoplasm and imported across its membrane. Proteins localized in the peroxisomal membranes are also known, and some of them are involved in the transport of small metabolites and peroxisomal proteins. Biogenesis of the organelle proceeds via a cellular machinery consisting of more than 20 proteins (peroxins) that effect the recognition, targeting, and import of peroxisomal proteins (1). In human, dysfunction of the machinery results in inherited metabolic disorders and peroxisome biogenesis disorders such as Zellweger syndrome. Some of the genes responsible for the disease have yet to be identified. To characterize the protein composition of the organelle, therefore, is important not only in studying the proteins involved in the functions of the organelle, but also in elucidating the molecules responsible for the organelle's biogenesis.

Because of the rapid expansion of protein sequence databases, mass spectrometry (MS)-based identification of proteins, which relies on the sequence data base, is becoming more realistic and more reliable (2–4). One important field of application of this emerging technique is to analyze proteins in whole cells and tissues, and thousands of proteins expressed can be detected and identified (5, 6). The same technique can be used to analyze subcellular fractions ranging from organelles to large macromolecular complexes such as ribosomes. Analyzing subcellular fractions reduces the complexity of the samples to be analyzed and should yield more information on the minor protein components that would otherwise escape detection (7–10). A classical way of cell fractionation, i.e. differential centrifugation after homogenization, is clearly not adequate for such analyses, since the analytical methods employed may detect minor contaminants. Methods are necessary that allow purification of such macromolecular complexes without losing the structural or functional integrity of the complex. Peroxisomes are usually purified by combining the classical differential centrifugation and density gradient centrifugation. These fractions still contain other contaminating organelles such as mitochondria.

In the present study, we combined the Nycodenz density gradient centrifugation with immunosolation using anti-PMP70 (70-kDa peroxisomal membrane protein) antibody. The fractions obtained were practically free from contaminating mitochondria. MS-based proteomic analyses (11) gave an image of the “proteome” of the liver peroxisomes with the composition...
and the relative abundance of the protein components. In addition to the known peroxisomal proteins, several endoplasmic reticulum (ER) and mitochondrial proteins, which seem to exist in the organelle, and a few new proteins of unknown function were identified. One of them is a peroxisome-specific isozyme of Lon protease, which acts as molecular chaperone. The protein may play an important role in the biogenesis of the peroxisome.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibody against rat PMP70 was raised using a synthetic peptide corresponding to the C-terminal 15 amino acids (12). Specific antibody was purified from rabbit antiserum by affinity chromatography on an antigen-bound goat IgG-Sepharose column (SulfoLink, Amersham, UK). Goat anti-rabbit IgG (Fc fragment-specific) was obtained from the same source. Nycodenz was obtained from Daichi Pure Chemicals Co., Ltd. (Tokyo, Japan), while Dynabeads M-500 Subcellular were from DYNAL (Oslo, Norway). Protease inhibitor mixture tablets were purchased from Roche Diagnostics (Mannheim, Germany). Other chemicals and biochemicals were from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Rat Liver Peroxisomes—The peroxisomal fraction from rat liver was prepared by conventional differential centrifugation in sucrose followed by isopycnic centrifugation in Nycodenz (13) with some modifications. About 0.6 ml of a light mitochondrial fraction-containing peroxisomes was layered onto 10 ml of linear Nycodenz gradient (1.15 to 1.25 g/ml) in a P40ST rotor (Hitachi, Tokyo, Japan). The gradient rested on a 0.9 ml cushion of 1.30 g/ml Nycodenz. All Nycodenz solutions contained 0.25 mM sucrose, 1 mM EDTA, 0.1% (v/v) ethanol in 5 mM Hepes-KOH buffer (pH 7.4). Centrifugation was carried out at 77,000 × g for 180 min at 4 °C. Fractions of ~1.0 ml were collected, and the peroxisome-containing fraction was determined by the distribution of catalase activity (13).

Immunopurification of Peroxisomes—Anti-PMP70 specific antibody was bound to magnetic beads (1×108), using an anti-rabbit Fc fragment antibody as a linker antibody according to the manufacturer’s instruction. The peroxisome-enriched fractions from the Nycodenz density gradient (about 500 μg of protein) were incubated with the magnetic beads, which were subsequently washed in phosphate-buffered saline containing 2 mM dithiothreitol (pH 6.8). Gel pieces were washed twice with 0.25 M sucrose, 2 mM EDTA, and 0.1% ethanol, and eluted with 100 mM Tris-HCl buffer as above.

In-gel Digestion with Trypsin—The purified peroxisomal fraction was subjected to SDS-PAGE and stained with MS-compatible silver staining (1% of the sample obtained) (14) or with Coomassie Brilliant Blue (CBB) (10% of the sample). For the proteomic analysis, a single lane from CBB-stained gel was divided equally into 64 slices of 2-mm width. Each gel slice was cut into small pieces and put into the wells of a 96-well PCR plate (BM Bio, Tokyo, Japan). The gel pieces were destained by rinsing in 50% acetonitrile containing 25 mM NH4HCO3. Disulfide bonds of cysteines were reduced by incubating with 10 mM dithiothreitol in 25 mM NH4HCO3 at 56 °C for 1 h and alkylated with 55 mM iodoacetamide in 25 mM NH4HCO3 at room temperature for 45 min in the dark. Gel pieces were washed with 50% acetonitrile and dried in a vacuum concentrator (SpeedVac, Thermo Savant, NY). Gel pieces were re-hydrated with 15 μl of trypsin solution (10 ng/μl in 50 mM NH4HCO3 containing 5 mM CaCl2). In-gel digestion was performed at 37 °C overnight. Resulting peptides were extracted with 50% acetonitrile containing 5% trifluoroacetic acid. Extracts were dried in Speed-Vac and re-dissolved in 0.1% (v/v) formic acid. The peptide mixtures thus obtained were subjected to LC/MS and data-dependent tandem mass (LC/MS/MS) analyses.

Mass Spectrometric Analysis—LC/MS/MS analysis was carried out in a Q-Tof-type hybrid mass spectrometer (Micromass, Manchester, UK) interfaced on-line with a capillary HPLC (Waters-Micromass modular CapLC, Micromass) (15, 16). A linear gradient of acetonitrile containing 0.1% formic acid was split at a 1:10 ratio, and the flow slow was 40 μl/min. A 5 μl sample was injected into a nanoLC column (PepMap C18, 75 μm × 150 mm, LC Packings, Amsterdam, Holland). The column eluate was directly injected into a self-constructed nano-spray ion source with a tapered stainless capillary. The LC/MS/MS analysis was carried out in a data-dependent mode; peptide peaks above a certain threshold were subjected automatically to the collision-induced dissociation. Peak lists obtained from the MS/MS spectra were used to identify proteins using Mascot search engine (Matrixscience, London, UK).

Immunological Analyses—Antibody against the C-terminal 16 amino acids of RIKEN cDNA 1300002A08 clone, GGPVFYKTRPGLD5, was raised in rabbits (MBL, Nagoya, Japan). The distribution of the protein among subcellular fractions was determined by immunoblotting. Each 50 μg of protein of differential centrifugation fractions; nuclear (N), cytoplasmic (S), and light mitochondrial (ML) fractions, were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The blot membranes were incubated with the antibody and visualized by ECL Plus (Amersham Biosciences). Immunofluorescence microscopy of rat liver cells with anti-peroxisomal Lon protease antibody was performed as described previously (17). Immunofluorescence microscopy of human fibroblasts was previously described (18). Human fibroblasts used in this study were from a normal, peroxisomal D-bifunctional protein deficiency patient (19) and a Zellweger syndrome patient (20).

RESULTS

Purification of Peroxisomes by Antibody-conjugated Magnetic Beads—Peroxisomal fractions obtained from the conventional differential centrifugation were further enriched by Nycodenz gradient centrifugation. Fractions containing peroxisomes were determined based on the enrichment of a marker enzyme, catalase. Peroxisome was further purified by immunoisolation with magnetic beads coated with monospecific antibodies against PMP70 as described under “Experimental Procedures.” When two samples obtained before and after the immunopurification were compared, a few changes were noticed (Fig. 1A). Several bands disappeared during the purification, while some bands were clearly concentrated. Some of the protein bands that disappeared were cut out and subjected to the in-gel digestion/MS analysis. One protein was found of mitochondrial origin, carboamyl phosphate synthase (Fig. 1A). A few proteins of small molecular weight were from the cytoplasm (data not shown). It should be noted that major mitochondrial and ER proteins such as cytochrome c oxidase and cytochromes P-450 were not detected. Therefore, contaminating mitochondrial and cytoplasmic proteins seem to be effectively removed by the immunopurification. On the other hand, one of the typical peroxisomal membrane proteins, PMP22, was clearly concentrated during the purification. Interestingly, peroxisomal matrix proteins were not uniformly concentrated. The amount of the most abundant matrix protein, catalase, seemed to decrease, while the bands corresponding to another major matrix protein, uricase, clearly increased in its intensity (Fig. 1A). These results suggest that there seems to be some leakage of soluble matrix proteins during the purification process, although no drastic change of the composition was noted.

Proteins Identified in the Purified Peroxisomes—Each lane in the SDS-PAGE obtained from the purified peroxisomal fractions was evenly cut into 64 slices, regardless of the presence of stained bands, and subjected to the in-gel digestion. The resulting peptide mixtures were analyzed by LC-MS/MS as described under “Experimental Procedures.” Mascot search engine was used to identify proteins in the MS/MS ion search mode. Up to 10 proteins were identified in a single gel slice. Proteins thus identified are summarized in Table I.

We could identify 34 known peroxisomal proteins, and five proteins were those of unknown function. Furthermore, two mitochondrial proteins and six ER proteins were identified in addition to the four proteins from other subcellular fractions. Of the known peroxisomal proteins, seven proteins were peroxisomal membrane proteins. In addition to the two major matrix proteins, catalase, and uricase, as mentioned above, 25 proteins of the peroxisomal matrix were found in the list. Most of them are enzymes involved in lipid metabolism. Five proteins were peroxins (Pex1, -2, -3, -13, -14) involved in the
various steps of the peroxisome biogenesis. Several mitochondrial and ER proteins identified in the present study can be considered to be authentic peroxisomal proteins rather than contaminating proteins from other organelles. Reasoning for this conclusion will be discussed below in detail.

Since we could identify only 5 of 13 peroxins, and three peroxisomal membrane proteins, we have isolated peroxisomal membranes by treating the beads-bound peroxisome with mild alkaline treatment (Na₂CO₃). The washed membranes were eluted from the magnetic beads, and subjected to the same SDS gel electrophoresis/mass spectrometry-based analysis. The peroxins and peroxisomal membrane proteins thus identified are listed in Table II. We could identify 12 peroxins, and only two peroxins, Pex7 and Pex19, are missing in the list. This is not surprising, since these two proteins are predominantly cytoplasmic. It is interesting to compare the relative amounts of these proteins found in the whole and membrane fractions of the organelle. Pex1 is the most abundant peroxins in the whole fraction, but the amount of the protein relative to other peroxins decreased drastically. Pex1 has been shown to be cytoplasmic when examined by immunofluorescence microscopy. Our results suggest that part of the protein is associated with the peroxisome and can be washed away with alkaline treatment. On the other hand, Pex5, which have been predominantly cytoplasmic with a small amount associated with the peroxisome, were found in the washed membrane fractions. The association of the protein, therefore, is stronger to that of Pex1 proteins. All the peroxins that are membrane-bound (Pex2, Pex3, Pex6, Pex10, Pex11, Pex12, Pex13, Pex14, and Pex16) were concentrated in the membrane fraction, demonstrating the efficiency of the isolation method. Similarly, other membrane proteins of the organelle, which were not detected in the whole peroxisomal fraction, were found in the fraction. Judging from the normalized score, the relative abundance of ALDP and ALDPR seems very low compared with that of the abundant peroxisomal membrane proteins such as PMP70 and PMP22.

Proteome of Rat Liver Peroxisome—The positions of protein in SDS-PAGE are shown together with the image of a silver-stained gel (Fig. 1B). On the left side of the gel image, abundant proteins whose stained bands can be easily recognized are listed. Less abundant proteins whose bands are either faint or invisible are listed on the right side of the gel. In some cases, more than one protein of similar amounts were identified in a single gel slice, making the relationship between the stained bands and proteins obscure. Many of the abundant proteins were found not only in a single gel slice but also in neighboring slices. Furthermore, proteins were detected in positions where the apparent molecular mass differs appreciably from the theoretical mass calculated from the known sequence. Major proteins like catalase were, in fact, detected throughout many gel slices well above or below the theoretical mass range. They originated either from complexes that remained undissoctated even in the presence of SDS or from proteolytic fragments. In these cases, only the gel slices in which most of the proteins were detected were marked.

For the analysis of proteomes, regardless of whether it is a proteome from a cell or that of an organelle, it is important to know the abundance of each protein in that specific sample. Although this is not an easy task, we noticed that the probability score produced by the Mascot search engine is correlated with the amount of protein in that specific sample. The score presented for each protein identified is a sum of peptide scores detected in the LC/MS analysis. It should be noted that the more abundant a protein is, the more peptides that originated from the protein are likely to be observed. In other words, when one uses a large amount of protein for the analysis, the se-
Proteome of Rat Liver Peroxisome

| Protein name | NCBI accession number | M.W. | Top score | Total score | Normalized score | Taxonomy | Location | C-term. |
|--------------|-----------------------|------|-----------|-------------|------------------|----------|----------|---------|
| Peroxisomal membrane protein 2, 22 kDa (PMP22) | gi 133928796 | 22,562 | 186 | 585 | 1296.43 | rat | Px | Mem |
| ATP-binding cassette, subfamily D, member 3 (PMP70) | gi 6981450 | 75,780 | 312 | 908 | 599.10 | rat | Px | Mem |
| ATP-binding cassette, subfamily D, member 1 (ALDP) | gi 6671497 | 46,894 | 87 | 87 | 93.16 | mouse | Px | CRL |
| Peroxisomal acyl-CoA thioester hydrolyase 1 | gi 14548007 | 36,146 | 50 | 50 | 69.16 | mouse | Px | SKL |
| Peroxisome biogenesis factor 3 (Pex3) | gi 13786194 | 27,434 | 107 | 179 | 326.24 | rat | Mem | FIS |
| Peroxisomal acyl-CoA thioester hydrolyase 2 | gi 6754090 | 47,047 | 77 | 77 | 81.83 | rat | Px | AKL |
| Peroxisomal membrane protein 3, 35 kDa (Pex2) | gi 6978429 | 46,894 | 87 | 87 | 93.16 | mouse | Px | CRL |
| Peroxisomal membrane protein 2, 22 kDa (PMP22) | gi 12166980 | 45,269 | 164 | 508 | 561.09 | rat | Px | PGN |
| Peroxisomal membrane protein 3, 35 kDa (Pex2) | gi 6981450 | 75,780 | 312 | 908 | 599.10 | rat | Px | Mem |
| Peroxisomal membrane protein 2, 22 kDa (PMP22) | gi 133928796 | 22,562 | 186 | 585 | 1296.43 | rat | Px | Mem |
| ATP-binding cassette, subfamily D, member 3 (PMP70) | gi 6981450 | 75,780 | 312 | 908 | 599.10 | rat | Px | Mem |
| ATP-binding cassette, subfamily D, member 1 (ALDP) | gi 6671497 | 46,894 | 87 | 87 | 93.16 | mouse | Px | CRL |
| Peroxisomal acyl-CoA thioester hydrolyase 1 | gi 14548007 | 36,146 | 50 | 50 | 69.16 | mouse | Px | SKL |
| Peroxisomal acyl-CoA thioester hydrolyase 2 | gi 6754090 | 47,047 | 77 | 77 | 81.83 | rat | Px | AKL |
| Peroxisomal acyl-CoA thioester hydrolyase 1 | gi 14548007 | 36,146 | 50 | 50 | 69.16 | mouse | Px | SKL |

**Notes:**
- Proteins showing top scores above 100 (score for mitochondrial carboxyl phosphate synthetase) were listed. Proteins known to be localized in peroxisomes are listed even if they show scores less than 100. Top score refers to the highest score returned from the Mascot search engine for the individual protein in each gel slice. Scores from the neighboring gel slices were added to give total score for each protein.
- The Mascot score was normalized for molecular weight by dividing with the molecular weight and multiplying with 50,000, a typical molecular weight of proteins.

**Table I**

List of identified proteins

**Proteins showing top scores above 100:**
- Mitochondrial carboxyl phosphate synthetase (EC 1.3.3.20)
- Peroxisomal acyl-CoA thioester hydrolyase 1
- Peroxisomal acyl-CoA thioester hydrolyase 2
- Peroxisomal membrane protein 3, 35 kDa (Pex2)
- Peroxisomal membrane protein 2, 22 kDa (PMP22)
- ATP-binding cassette, subfamily D, member 3 (PMP70)
- ATP-binding cassette, subfamily D, member 1 (ALDP)

**Lipid metabolism**
- α-Oxidation
  - Hydroxyacyl-CoA 3 (medium-chain)
  - Hydroxyacyl-CoA 1, liver; glycolyzed oxidase 1
- β-Oxidation
  - Catalase
  - Peroxisomal acyl-CoA oxidase (EC 1.3.3.6) chain A, peroxisomal splice form II

**Oxidation of polyunsaturated fatty acid**
- Putative peroxisomal 2,4-dienoyl-CoA reductase
- Putative short-chain dehydrogenase/reductase
- Isozyme dehydrogenase I, soluble
- Peroxisomal acyl-CoA thioester hydrolyase 1

**Bile acid synthesis**
- Bile acid-CoA dehydrogenase: amino acid n-acyltransferase
- Glycolipid synthesis
  - Alkyl-dihydroxyacetonephosphate synthase 1 precursor
  - Dihydroxyacetone phosphate acetyltransferase

**Amino acid metabolism**
- Serine-pyruvate aminotransferase;
  - Alanine-glyoxylate aminotransferase
- Peroxisomal sarcosine oxidase
- Urease (urate oxidase)
- Antioxidant enzyme B166
- Peroxisomal bifunctional enzyme
- Peroxisomal multifunctional enzyme type II
- Long-chain acyl-CoA synthetase 2
- Trihydroxypropylsontanol-CoA oxidase
- Peroxisomal acyl-CoA thioester hydrolyase 2
- Peroxisomal acyl-CoA thioester hydrolyase 1

**Mitochondrial proteins**
- Glutathione S-transferase, mitochondrial (GST13-13)
- Carboxyl-phosphate synthetase 1
- ER proteins
  - Cytochrome b5
  - Aldehyde dehydrogenase 3A2
  - Microsomal glutathione S-transferase 1 (microsomal GST-1)
  - Cytochrome 55 reductase (EC 1.6.2.2)
  - Protein-disulfide isomerase A6 precursor, microsomal
  - UDP-glucuronosyltransferase 1-1 precursor, microsomal
  - Membrane proteins
    - RAB9, member RAS oncogene family
    - Visceral-associated membrane protein, associated protein A (VAP-A)
    - Visceral-associated membrane protein, associated protein B and C (VAP-B)

**Cytoplasmic proteins**
- β-Actin
- Putative proteins

**Proteins showing top scores above 100:**
- Mitochondrial carboxyl phosphate synthetase
- Peroxisomal acyl-CoA thioester hydrolyase 1
- Peroxisomal acyl-CoA thioester hydrolyase 2
- Peroxisomal membrane protein 3, 35 kDa (Pex2)
- Peroxisomal membrane protein 2, 22 kDa (PMP22)
- ATP-binding cassette, subfamily D, member 3 (PMP70)
- ATP-binding cassette, subfamily D, member 1 (ALDP)
Proteome of Rat Liver Peroxisome

List of proteins identified in peroxisomal membranes

| Protein name | NCBI accession number | M.W. | Top score | Total score | Normalized score | Taxonomy | Location | Comment |
|--------------|-----------------------|------|-----------|-------------|------------------|----------|----------|---------|
| PEX1 | gi 129555 | 34,745 | 186 | 345 | 496 | rat | Px | Pex2 |
| PEX2 | gi 2911135 | 104,250 | 327 | 608 | 292 | rat | Px | Pex6 |
| PEX3 | gi 27698157 | 45,097 | 168 | 350 | 388 | rat | Px | Pex13 |
| PEX4 | gi 26441964 | 24,121 | 122 | 195 | 404 | rat | Px | Pex24 |
| PEX7 | gi 13928796 | 22,562 | 334 | 870 | 1928 | rat | Px | Pex22 |
| PEX10 | gi 25453374 | 40,944 | 215 | 907 | 1108 | rat | Px | Pex10 |
| PEX11 | gi 16758202 | 40,655 | 274 | 469 | 577 | rat | Px | Pex12 |
| PEX12 | gi 27663382 | 35,122 | 207 | 598 | 851 | rat | Px | Pex16 |
| PEX13 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | PMP34 |
| PEX14 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | LACS |
| PEX15 | gi 15375324 | 83,315 | 191 | 382 | 497 | rat | Px, Mem | PMP70 |
| PEX16 | gi 21553069 | 38,625 | 235 | 626 | 810 | mouse | Pex16 |
| PEX17 | gi 19074911 | 24,502 | 122 | 245 | 457 | rat | Px, Mem | PMP3 |
| PEX18 | gi 13929034 | 70,648 | 417 | 417 | 295 | rat | Px, Mem | ALDRP |
| PEX19 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | VLACS |
| PEX20 | gi 129555 | 34,745 | 186 | 345 | 496 | rat | Px | Pex2 |
| PEX21 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | PMP22 |
| PEX22 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | PMP70 |
| PEX23 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | LACS |
| PEX24 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | PMP34 |
| PEX25 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | LACS |
| PEX26 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | PMP70 |
| PEX27 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | LACS |
| PEX28 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | PMP34 |
| PEX29 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | LACS |
| PEX30 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | PMP70 |
| PEX31 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | LACS |
| PEX32 | gi 25742739 | 78,128 | 1325 | 3616 | 2314 | rat | Px, Mem | PMP34 |

Scores of abundant proteins that are more sequence-specific fragment ions of high S/N are observed from peptides of abundant proteins.

To elucidate the relationship between the Mascot score and the relative abundance, we compared the Mascot score with the band intensity of the silver-stained gel calculated by image analysis (NIH Image, rsb.info.nih.gov/nih-image/). The Mascot score was normalized for molecular weight by dividing by the molecular weight and multiplying with 50,000, a typical molecular weight of proteins. Scores of abundant proteins that are found in successive slices were added. Only proteins whose bands are unambiguously identified were included in the comparison (Fig. 2). As is clear from the figure, there is a good correlation between the two parameters. Other less abundant proteins, which are not included in the figure and show low Mascot scores and band intensities, should be localized near the origin. It should be noted that although the silver staining is a clear indication of the protein amount, the linearity is very limited, and the staining varies appreciably depending on the protein. Therefore, both staining intensities and normalized Mascot score can be considered as semi-quantitative indicators of proteins. Judging from the correlation, whether a protein is highly or less abundant from the normalized Mascot score can be determined.

We have included these scores in the list of the peroxisomal proteins (Table I). The proteins are listed in decreasing order of the score. By comparing the list and the gel image, a rough image of the proteome of liver peroxisomes can be obtained. In addition to the two major matrix proteins, catalase, and uricase, several enzymes in the lipid metabolism are of high abundance. Some of the membrane proteins such as PMP22 and PMP70 are abundant proteins, comparable to the average matrix proteins. On the other hand, the peroxins are mostly minor components in the organelle.

In this context, proteins known to be localized in other subcellular fractions should be reexamined. Some of them have high scores compared with the abundant authentic peroxisomal proteins, suggesting that these proteins are intrinsic peroxisomal proteins rather than contaminating ones. Of these, aldheyde dehydrogenase and cytochrome b5 belong to the abundant proteins in the organelle. It may not be surprising to find such enzymes in peroxisomes. Proteins involved in the vesicle trafficking such as rab family proteins were also identified in the preparation.
fied has homology to Lon protease and has an AAA (ATPases associated with diverse cellular activities) domain (Fig. 3A). A close examination of sequences of Lon proteases of various origins indicated that at least two isoforms exist in mammalian cells. One isoform has been characterized as mitochondrial Lon protease, which is homologous to plant Lon2 sequences (21, 22). The present isoform is likely an orthologue of the plant Lon1. Both types share similar structural characteristics with an AAA domain and the C-terminal region containing a protease active site. The N-terminal parts are more diverse compared with the C-terminal half. It should be noted that the putative protease shows structural characteristics that are intermediate between the two mammalian and plant isoforms. This suggests that the bacterial protein is ancestral to the two eukaryotic isoforms.

To confirm the peroxisomal localization of the newly-identified Lon isozyme, we have studied the cellular localization of the protein in detail. For that purpose we have raised a specific antibody against a synthetic peptide based on the mouse C-terminal sequence. It differs from the human sequence at only two positions, and crossreacts with the human protein. The localization of the protease was studied by subcellular fractionation, immunocytochemistry, and immunoelectron microscopy (Fig. 4). In the immunoblotting, single band corresponding to expected size (about 95-kDa) was detected only in the ML fraction (Fig. 4A). When human fibroblasts were stained with the antibody, the immunofluorescence was found in a punctate pattern typical for peroxisome-associated proteins (Fig. 4B). A similar punctate pattern was observed when cells were stained with anti-catalase antibody (data not shown). These results established that the hypothetical proteins identified in the human and mouse genomic or cDNA sequences are the peroxisomal-specific isozyme of Lon protease, which is distinct from the known mitochondrial isozyme. The same antibody was used for immunoelectron microscopy. As shown in Fig. 4C, immunogold particles were associated mainly with the peroxisome core, an electron dense structure inside of the peroxisomal membranes. For a more quantitative analysis, labeling densities of several subcellular components were calculated. The particle density in the cytoplasmic area was 1.66 ± 0.44 particles/μm², while that in the peroxisomes was 28.57 ± 0.44 particles/μm². Within the peroxisome area, the labeling density in the peroxisomal core (123.90 ± 24.79 particles/μm²) was much higher than that in the matrix (6.84 ± 1.14 particles/μm²). This indicates that the protein has specific localization in the peroxiso-
nal core. Judging from the band intensity and the Mascot score, the present peroxisomal isozyme is an abundant peroxisomal proteins.

Presence of a Novel Bi-functional Enzyme Containing Acyl-CoA Dehydrogenase Domain—Another protein of unknown function identified in the present study contains a typical acyl-CoA dehydrogenase (ACDH) domain (Fig. 3B). Two sequences are reported for the protein; human FLJ128592 and a longer sequence annotated as similar to A1987948. The N-terminal half of the longer molecule shows homology to the aminoglycoside phosphotransferase (APH) family. In the present MS-based analysis, peptides derived from both N- and C-terminal regions of the longer sequence were identified. Therefore, the shorter sequence seems to be a truncated form. These proteins end with three amino acids, Ala-Arg-Met or Ala-Lys-Ile, a shorter sequence seems to be a truncated form. These proteins are reported for the protein; human FLJ12592 and a longer CoA dehydrogenase (ACDH) domain (Fig. 3).

Another protein of unknown function, called Leishmania tarentolae endozenepine-related protein precursor, has been observed in the peroxisomes. This is the first demonstration that an ACDH is present not only in the mitochondria but also in the peroxisomes. Only acyl-CoA oxidase activity has previously been thought to be associated with the organelle (25).

Other Proteins of Unknown Function—Three other proteins are listed under “putative proteins” in Table I. “Homolog to endozenepine-related protein precursor” (accession number in NCBI, gi 12836616) has a high similarity to bovine endozenepine-related protein. It has an acyl-CoA binding protein (ACBP) domain in the N-terminal that has been characterized as a cytoplasmic protein involved in the acyl-CoA transport. In the present analysis, this putative protein was detected in gel slices of low molecular weight under 20 kDa, and only peptides derived from the C-terminal region were detected. Therefore, only the C-terminal domain, which lacks the ACBP domain, seems to be localized in the peroxisome.

RIKEN cDNA 2010003O14 (gi 13384998) is homologous to CGI-135. CGI-135 proteins are proteins of unknown function, but are evolutionarily conserved between human and Caenorhabditis elegans. CGI-135 shows similarity to Fis1p, which is required for Dnm1p GTPase-mediated mitochondrial fission (26). Tumor-related protein (gi 14572526) is homologous to CGI-111, which shows some similarity to MAR1, a mitochondrial-associated ribonuclease (27). In Leishmania tarentolae in which the enzyme was characterized, it is an endoribonuclease and the 18 amino acid sequence of N-terminal shows characteristics of an uncleaved mitochondrial sequence. However, the C-terminal sequence of the tumor-related protein, found in this study, is Ser-Lys-Val and may function as a PTS-1. Because of the low abundance, the peroxisomal localization of these proteins should be determined by other techniques before conducting functional analysis.

DISCUSSION

Organelle proteomics, which is becoming a popular way to analyze cellular fractions, is advantageous in two respects; one is the decreased complexity of the samples to be analyzed, and the other is the information on the subcellular localization of protein components provided. Unfortunately, such analyses have been conducted often without much consideration of the methods and purity of samples subjected to analysis (7–9). On the other hand, the analytical methods employed are becoming more and more sensitive, enabling detection of less abundant components. In the present study, peroxisomal fractions obtained by differential centrifugation were further purified by density gradient and by immunopurification. Judging from the behavior of several mitochondrial and cytoplasmic proteins, the final preparation seems to be practically free from the contamination of the two subcellular fractions. Interestingly, several ER proteins, especially those present in the lumen of ER, have been detected. Since major ER proteins such as cytochrome P-450 isoforms were not detected, it is possible that some of these proteins are also localized in the peroxisomes. One example is aldehyde dehydrogenase, which is an endoplasmic reticulum protein. Judging from the abundance of the protein, this protein may well be an intrinsic peroxisomal protein involved in the peroxisomal oxidative pathways. An immunoreactive protein has, in fact, been observed in the peroxisomal membranes (28). Cytochrome b_5 and NADH-cytochrome b_5 reductase, which form part of the microsomal electron transfer pathways, have been shown to be localized both to the endoplasmic reticulum and the outer membrane of mitochondria. The present study suggests that these proteins may also be localized to the outer surface of the peroxisomal membranes (29).

Many peroxisome proteins were identified that are involved in important functions of peroxisome. However, the number of identified peroxins and that of peroxisomal membrane proteins was relatively small. Several proteins that are known to be localized to the peroxisomal matrix were not identified either, suggesting that their amounts in the sample analyzed might be small. The limitation of the present analytical methods comes mainly from the huge difference in the relative abundance of the component proteins in a sample. This is demonstrated in our study of the peroxisomal membranes. By treating the peroxisomal membranes with mild alkaline, we could remove most of the abundant matrix proteins and concentrate the membrane-bound proteins. In addition to all the membrane-bound peroxins, we could detect Pex1 and Pex5 that are loosely associated with the membrane. Pex5 seems to be more tightly bound to the membrane than Pex1. These results again demonstrate the importance of the sample purification in organelle proteomics.

Five putative proteins were identified in the present study. The most abundant one was identified as the peroxisomal isozyme of Lon protease. Lon protease is an ATP-dependent protease with an AAA domain. It is distributed widely among various species including Archaea, Eubacteria, and Eukaryotes (30). Together with other proteases such as Clps, Lon protease is considered to function as molecular chaperone in bacteria (31). In eukaryotes these functions are affected by more complex proteosome machineries, but a Lon protease homolog has been found in mitochondria of yeast and mammals (32, 33). Lon proteases in plants are now classified into three isoforms; Lon1, Lon2, and Lon3 (21). Sequence comparison revealed the relationship among various Lon isoforms (Fig. 3A). The overall structure is well conserved, especially in the C-terminal half of the molecule that contains the AAA domain. The main differences are found in the N-terminal half, where the Lon protease domain localizes. The plant Lon1 isoforms share high homology with the peroxisomal isoform, whereas Lon2 isoforms are homologous to the mammalian mitochondrial Lon protease. The plant Lon1 isoforms contain the C-terminal sequence, Ser-Lys-Leu, essential for the peroxisomal import. Although the Lon1 isoform has been annotated as plant mitochondrial Lon protease (21), we can conclude that the isoform is the peroxisome-specific isoform in plants. Interestingly, the Lon3 in plants is considered to be a chloroplast isoform (21). If this is the case, plants have three distinct Lon proteases in three organelles; mitochondria, chloroplast, and peroxisome. In peroxisomes, two other proteins containing the AAA domain are known (Pex1p and Pex6p). As their names suggest, they are involved in peroxisomal biogenesis and the import of peroxisomal matrix proteins. Lon protease in yeast is located in the mitochondrial matrix and is involved in maintenance of respiration and intramitochondrial proteolysis (34). Although physiological func-
tions of Lon protease in peroxisome has yet to be elucidated, it is possible that the protease is involved either in the folding process of the matrix proteins imported across the peroxisomal membranes as molecular chaperone, or in the degradation process of the misfolded proteins.

Another unknown protein of interest is a bi-functional enzyme containing an ACDH domain (Fig. 3B). This protein has been annotated as similar to A1H87948. The C-terminal sequence of the enzyme is Ala-Arg-Met, which can function as a PTS-1 (24). It is, therefore, highly likely that the protein having an ACDH domain is an intrinsic peroxisomal protein. ACDHs in mitochondria are involved in the first step of mitochondrial β-oxidation of fatty acids. Contrary to the mitochondrial ACDHs, the peroxisomal enzyme has a phosphotransferase domain (APH) in its N-terminal half. It is of interest how this DHs, the peroxisomal enzyme has a phosphotransferase do-

ors-activated receptor-

precursor protein and directly regulated by peroxisome proliferator response element (35). It is intriguing to assume a physiological role of this C-terminal domain produced from a larger precursor in the transport/metabolism of lipid metabolites.

In summary, we isolated a peroxisome fraction that is practically free from contamination of other cellular organelles, and we identified many known peroxisomal proteins. Semi-quantitative analysis produced a rough image of relative content of each protein; a proteome of rat liver peroxisome. A few novel proteins were added to the list, one of which is the newly identified peroxisome-specific isozyme of Lon protease. Judging from the relative content of the protein, the Lon protease is an abundant peroxisomal matrix protein. In rat liver, this protein is mainly associated with the electron-dense core structure in the peroxisomal matrix. Since the Lon proteases function as chaperones, the peroxisome-specific Lon isozyme is probably involved directly in peroxisome biogenesis. The peroxisomal isozyme distinct from the mitochondrial form is found not only in mammals but also in plants. An intriguing hypothesis is that there is a distinct isoform for each of the three plant organelles: mitochondria, chloroplast, and peroxisome. This hypothesis has yet to be substantiated, although the roles played by Lon proteases in organelle biosynthesis are clearly important.

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