Characterization of the Interaction between Recombinant Human Peroxin Pex3p and Pex19p

IDENTIFICATION OF TRP-104 IN Pex3p AS A CRITICAL RESIDUE FOR THE INTERACTION**

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Proteins required for peroxisome biogenesis are termed peroxins. The peroxin Pex3p is a peroxisomal membrane protein (PMP), involved in peroxisomal membrane biogenesis. It acts as a docking receptor for another peroxin Pex19p, which is a specific carrier protein for newly synthesized PMPs. Here we have determined the physicochemical properties and binding manners of Pex3p-Pex19p interaction, in terms of the affinity, the stoichiometry, and the binding site in Pex3p. The cytosolic domain of human Pex3p was overproduced, using an Escherichia coli expression system and was highly purified by two chromatography steps. Gel filtration chromatography analyses and intrinsic tryptophan fluorescence titrations revealed that a one-to-one complex is formed between monomeric Pex3p and monomeric Pex19p. The tryptophan fluorescence spectrum of Pex3p showed a large 18-nm blue shift of the maximum emission wavelength by the binding of Pex19p. This result indicates that either one or two tryptophan residues of Pex3p (Trp-104 and Trp-224) are directly involved in binding to Pex19p. We investigated the binding activities of the wild-type and tryptophan mutants of Pex3p by pull-down assays and surface plasmon resonance analyses. As a result, the wild-type and the W104A and W104F mutants showed Kd values of 3.4 nM, 1080 nM, and 66.2 nM, respectively. The affinity differences with mutation affected their peroxisomal redoing activities in pex3 ZPG208 cells. These findings suggest that the indole ring of Trp-104 directly interacts with Pex19p to facilitate the specific peroxisomal translocation of the Pex19p-PMP complexes.

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3 The abbreviations used are: PMP, peroxisomal membrane protein; SPR, surface plasmon resonance; GST, glutathione S-transferase; λmax, the maximum emission wavelength; β-DDM, n-dodecyl-β-D-maltoside; PTS, peroxisome targeting signal; NTA, nitritoltriacetic acid.

Peroxisomes are single membrane-bound organelles in a wide variety of eukaryotic cells. They play essential roles in several metabolic processes, including β-oxidation of fatty acids and hydrogen oxide-based respiration (1). The importance of this organelle for human survival is highlighted by lethal peroxisomal biogenesis disorders, such as Zellweger syndrome (2, 3). Peroxisome biogenesis requires multiple “peroxin” proteins encoded by PEX genes. Most of the peroxin defects cause mislocalization of peroxisomal matrix proteins, and generate peroxisomal membrane ghosts, lacking their internal contents (4). In contrast, a defect in Pex3p, Pex16p, or Pex19p results in no detectable peroxisome structures, with mislocalization of peroxisomal membrane proteins (PMPs) (5–9). Thus, these peroxins are thought to be involved in the synthesis and assembly of peroxisomal membranes and/or the correct translocation of PMPs. A recent study demonstrated that in Saccharomyces cerevisiae preperoxisomal compartments containing Pex3p budded from the endoplasmic reticulum membrane in a Pex19p-dependent manner, and then matured into functional peroxisomes (10). This result highlights the special roles of Pex3p and Pex19p in the early stages of peroxisome biogenesis.

Pex3p has been proposed to act as a docking receptor on the peroxisomal membrane and to accommodate translocating complexes of PMPs (11). Human Pex3p is a 42-kDa protein in which the N-terminal 33-amino acid region contains the membrane-anchoring region, with the C-terminal region exposed to the cytosol to display receptor function (11, 12). Pex3p can form a ternary complex with Pex19p and PMPs (13, 14). Because Pex19p acts as a chaperone-like carrier protein for newly synthesized PMPs (13–19), the binding of Pex3p and Pex19p is an essential event in the targeting of PMPs to the peroxisomal membrane. In previous studies, the physiological roles of Pex3p and Pex19p have been investigated using immunofluorescence assays, pull-down assays, and yeast two-hybrid systems (7, 9, 11, 13, 20, 21). These studies revealed that the interaction between...
Trp-104 in Human Pex3p Is Involved in Pex19p Binding

Pex3p and Pex19p is important for the biogenesis of peroxisomal membranes, and suggested the functional regions of these proteins. However, no common recognition sites of Pex3p for Pex19p have been found. Most of the experiments were performed by the induction of a short fragment of the objective Pex3p gene, although it is uncertain whether the truncated gene product maintains the authentic three-dimensional structure. Actually, it was suggested that the Pex3p-Pex19p interaction requires nearly full-length Pex3p (7). To evaluate the important residues of Pex3p for Pex19p binding, highly purified protein samples are preferable, and the physicochemical properties of the interaction should be quantitatively analyzed while considering protein structures. However, no such experimental data have been reported for Pex3p.

Here, we have characterized the binding properties of the Pex3p-Pex19p interaction, using highly purified human Pex3p. As for the molecular mechanisms of Pex3p-Pex19p interactions, an important question is how Pex3p specifically chooses Pex19p as a binding partner from the many different proteins in the cytosol. To address this question, we have analyzed the physicochemical properties and the binding of the Pex3p-Pex19p interaction with regard to affinity, stoichiometry, and binding site in Pex3p using fluorescence measurements and surface plasmon resonance (SPR) analyses. We have concluded that Trp-104 in Pex3p is a critical amino acid residue for binding.

EXPERIMENTAL PROCEDURES

Materials—Synthetic oligonucleotides were purchased from Hokkaido System Sciences Co. Restriction enzymes and DNA modifying enzymes were obtained from Takara Bio, Inc., Toyobo Co., Ltd., and New England Biolabs, Inc. The pT7Blue T-Vector, and pET-16b plasmids, and E. coli strain BL21(DE3) were purchased from Novagen Inc. Plasmid pGEX-6P-1 and pcDNA3.1+ were obtained from GE Healthcare and Invitrogen, respectively. Human PEX3 and PEX19 cDNAs (GenBankTM accession numbers, human PEX3: NM_003630 and human PEX19: NM_002857), were obtained as described(13). We prepared a mutant form of Pex19p in which all Cys residues are replaced with Ala to avoid nonproductive disulfide molecules. The mutagenesis was carried out by a modification of the overlap extension PCR method (22), using pET-16b/PEX19(1–296, C8A, C128A, C226A, C229A, C296A) and pGEX6-P-1/PEX3(aa, 34–373), and pCDNA3.1+ /PEX3-HA template: pET-16b/PEX19(aa, 1–296, C8A, C128A, C226A, C229A, C296A) was also constructed, using the forward primer 5′-GCATGACACCATGCTGAGTTCAGATATATTAGTATGAAAC-3′ and the reverse primer 5′-CTTTATTATTGATCTAGGAGTTGGTAT-3′, to introduce an NdeI site at the N terminal and a BglII site at the C terminal, respectively. The PCR product was ligated with the pT7Blue T-Vector, and the DNA sequence of the insert was confirmed, using an ABI PRISM 310 sequencer (Applied Biosystems). The plasmid was then digested with NdeI and BglII, and the fragment containing the PEX3(amin acids 34–373) gene was inserted into pET-16b, at the NdeI/BglII site.

Expression and Purification of Recombinant Proteins—His10-Pex3p-(34–373) was overproduced, using E. coli BL21(DE3) strain as the host. The transformants were grown in Luria-Bertani broth containing 50 μg/ml ampicillin at 37 °C. Cells were grown to an A600 of 0.7–1.0, and then were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 18–20 h at 18 °C. Cells were harvested by centrifugation. The cell pellets from 1-liter cultures were resuspended in 20 ml of buffer A, containing 50 mM Tris–Cl, pH 7.5, 0.3 M NaCl, 20 mM imidazole, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride, and were disrupted by sonication (7 min, 30 s on and 30 s off, output 5 and 50% duty cycle) on ice with a Branson Sonifier 250 (Branson Ultrasonomics). The cell lysate was centrifuged at 30,000 × g for 30

| Mutation | Primer | Sequence (from 5′ to 3′) |
|----------|--------|-------------------------|
| W202F | W202FF | ATGATCCATTATTCTCCAGGTTGCT |
| W202F | W202FR | ATGATCCATTATTCTCCAGGTTGCT |
| W104A | W104AF | AGGCGCGCTATTTCTGCTGCTGACT |
| W104F | W104FF | AGGCGCGCTATTTCTGCTGCTGACT |
| W224A | W224AF | ATGATCCATTATTCTCCAGGTTGCT |
| K100A | K100AF | ATGATCCATTATTCTCCAGGTTGCT |
| K100A | K100AR | ATGATCCATTATTCTCCAGGTTGCT |
| I103A | I103AF | AGGCGCGCTATTTCTGCTGCTGACT |
| I103A | I103AR | AGGCGCGCTATTTCTGCTGCTGACT |
| L107A | L107AF | AGGCGCGCTATTTCTGCTGCTGACT |
| L107A | L107AR | AGGCGCGCTATTTCTGCTGCTGACT |
| K108A | K108AF | AGGCGCGCTATTTCTGCTGCTGACT |
| K108A | K108AR | AGGCGCGCTATTTCTGCTGCTGACT |
| F112A | F112AF | AGGCGCGCTATTTCTGCTGCTGACT |
| F112A | F112AR | AGGCGCGCTATTTCTGCTGCTGACT |

GAGG-3′, to introduce an NdeI site at the N terminal and a BglII site at the C terminal, respectively. The PCR product was ligated with the pT7Blue T-Vector, and the DNA sequence of the insert was confirmed, using an ABI PRISM 310 sequencer (Applied Biosystems). The plasmid was then digested with NdeI and BglII, and the fragment containing the PEX3(amin acids 34–373) gene was inserted into pET-16b, at the NdeI/BglII restriction sites. The expression plasmid for glutathione S-transferase (GST)-Pex3p-(34–373) was constructed using essentially similar procedures to those described above.

The expression plasmid pcDNA3.1+ /PEX3-HA was also similarly constructed, using the forward primer 5′-GCATGACACCATGCTGAGTTCAGATATATTAGTATGAAAC-3′ and the reverse primer 5′-GCATGACACCATGCTGAGTTCAGATATATTAGTATGAAAC-3′. The PCR-generated fragment with Nhel and KpnI restriction sites was subcloned in-frame into the pcDNA3.1+ expression vector.

Site-directed Mutagenesis—To prepare expression plasmids for the mutant proteins, a QuikChangeTM site-directed mutagenesis kit (Stratagene) was used according to the manufacturer’s instructions. The template plasmids, the primers used in this study, and the generated plasmids are listed in Table 1. Specific mutations were confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins—His10-Pex3p-(34–373) was purified by Ni-NTA agarose chromatography and batch elution, using 20 mM imidazole in buffer A. The purified protein was fractionated by SDS-PAGE. The purity and yield of the purified protein were determined by SDS-PAGE and UV absorbance at 280 nm, respectively.

TABLE 1

| Mutation | Primer | Sequence (from 5′ to 3′) |
|---------|--------|-------------------------|
| W202F | W202FF | ATGATCCATTATTCTCCAGGTTGCT |
| W202F | W202FR | ATGATCCATTATTCTCCAGGTTGCT |
| W104A | W104AF | AGGCGCGCTATTTCTGCTGCTGACT |
| W104F | W104FF | AGGCGCGCTATTTCTGCTGCTGACT |
| W224A | W224AF | ATGATCCATTATTCTCCAGGTTGCT |
| K100A | K100AF | ATGATCCATTATTCTCCAGGTTGCT |
| K100A | K100AR | ATGATCCATTATTCTCCAGGTTGCT |
| I103A | I103AF | AGGCGCGCTATTTCTGCTGCTGACT |
| I103A | I103AR | AGGCGCGCTATTTCTGCTGCTGACT |
| L107A | L107AF | AGGCGCGCTATTTCTGCTGCTGACT |
| L107A | L107AR | AGGCGCGCTATTTCTGCTGCTGACT |
| K108A | K108AF | AGGCGCGCTATTTCTGCTGCTGACT |
| K108A | K108AR | AGGCGCGCTATTTCTGCTGCTGACT |
| F112A | F112AF | AGGCGCGCTATTTCTGCTGCTGACT |
| F112A | F112AR | AGGCGCGCTATTTCTGCTGCTGACT |
min at 4 °C. The supernatant was applied to a 10-ml column of Ni\(^{2+}\)-NTA-agarose equilibrated with buffer A. The column was washed with 100 ml of 50 mM Tris-Cl, pH 7.5, 0.3 mM NaCl, 50 mM imidazole, and 10% glycerol. His\(_{10}\)-Pex3p-(34–373) was eluted stepwise with 0.25 mM imidazole. The fractions containing His\(_{10}\)-Pex3p-(34–373) were concentrated with a Vivaspin concentrator (cut off: 10 kDa, Sartorius) and were further purified by gel filtration chromatography, using a Superdex200 16/60 column (GE Healthcare) with a running buffer of 20 mM HEPES-Na, pH 7.5, 0.5 mM NaCl, and 10% glycerol. All purification steps were carried out at 4 °C. For the Pex3p-(34–373) mutants, W104A, W224A, and W104F were expressed and purified by the same method. K100A, I103A, L107A, K108A, and F112A were expressed and purified by one-step affinity chromatography with the Ni\(^{2+}\)-NTA agarose, as described above. Hereafter, His\(_{10}\)-Pex3p-(34–373) is referred to as Pex3p.

His\(_{10}\)-Pex19p and GST-Pex19p were prepared as described (13), with some modifications. In the purification of His\(_{10}\)-Pex19p, we used buffers without dithiothreitol. His\(_{10}\)-Pex19p W202F was also purified by the same method. To obtain Pex19p without tag, the glutathione-Sepharose 4B (GE Healthcare) eluate of GST-Pex19p was digested with PreScission Protease (GE Healthcare) according to the manufacturer’s instructions. After removing glutathione in the eluate by dialysis, the protease and GST tag were removed by passing through the glutathione-Sepharose 4B column. The flow-through fractions were further purified by an anion exchange chromatography as described (13).

**Measurement of Protein Concentrations**—Protein concentrations were measured by UV absorbance at 280 nm. The A\(_{280}\) of 280-nm values we used were calculated from the amino acid composition (23). The A\(_{280}\) at 280-nm values are as follows: 0.56 for the wild-type Pex3p and the Pex3p mutants without tryptophan replacement, 0.43 for the Pex3p W104A and W224A mutants, and 0.28 for His\(_{10}\)-Pex19p and 0.89 for GST-Pex19p. The concentration of the tryptophan-less Pex19p mutant, which has little absorbance at 280 nm, was measured by the Bradford method (24). The standard curve was calibrated with the wild-type Pex19p, for which the concentration was determined by UV absorbance at 280 nm.

**SDS-PAGE and Western Blotting Analysis**—SDS-PAGE was performed by the method of Laemmli (25). Samples containing Pex3p were loaded without heating. Proteins separated in the polyacrylamide gel were stained with Coomassie Brilliant Blue R-350 (GE Healthcare). For the Western blotting analyses, rabbit anti-human His\(_{10}\)-Pex3p polyclonal antibodies and rabbit anti-human His\(_{10}\)-Pex19p polyclonal antibodies were obtained from the antibody production service by Qiagen. The goat anti-rabbit IgG (H+L)-AP conjugate (Bio-Rad) was used as the secondary antibody. The color development was carried out by 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride.

**Gel Filtration Chromatography Analysis of Pex3p, Pex19p, and Their Complex**—Gel filtration chromatography analyses were performed on a Superdex200 10/300 GL column (GE Healthcare) at 4 °C. The running buffer consisted of 50 mM Tris-Cl, pH 7.5, 0.3 mM NaCl, and 10% glycerol, and the flow rate was 0.6 ml/min. Each sample (200 µl), containing Pex3p at 10 µM, Pex19p at 10 µM, or a mixture of them at 10 µM, was loaded, and the elution profiles were monitored by the absorbance at 280 nm. The column was calibrated with using a gel filtration standard (Bio-Rad) containing thyroglobulin, 670 kDa; γ-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa and vitamin B12, 1.35 kDa.

**Intrinsic Tryptophan Fluorescence Measurement**—Steady-state fluorescence measurements were performed using a Shimadzu RF-5300PC spectrophotofluorometer (Shimadzu). Samples containing 0.5 µM Pex3p and 0.125–2.0 µM Pex19p (20 mM HEPES-Na, pH 7.5, 0.2 mM NaCl, and 10% glycerol) were prepared. Intrinsic tryptophan fluorescence spectra derived from Pex3p (Trp-104 and Trp-224) were measured at 25 °C. In this experiment, the Pex19p W202F was used to avoid the influences induced by the original tryptophan residue of Pex19p. The excitation wavelength was set at 295 nm, and the emission spectra were measured between 300 and 450 nm. The excitation slit width was 3 nm and the emission slit width was 10 nm. The emission spectra were corrected by subtracting the spectra of each concentration of the Pex19p W202F in the same buffer.

**Pull-down Assay**—Pull-down assays for the binding between Pex3p and Pex19p were performed as described below. 10 µl of glutathione-Sepharose 4B beads were incubated with 125 µl of GST-Pex19p at 0.3 mg/ml in binding buffer (20 mM HEPES-Na pH 7.5, 0.5 mM NaCl and 10% glycerol) for 10 min at 4 °C. After the incubation, the beads were washed twice with 125 µl of the binding buffer, and then a 100-µl aliquot of Pex3p or its mutants at 0.2 mg/ml was added. 10 µl of the suspension were collected as input samples. The remaining suspensions were diluted with 1 ml of the binding buffer and incubated at 4 °C for 10 min. The beads were washed twice with 1 ml of the binding buffer and were resuspended in 100 µl of the binding buffer (samples: bound). Proteins in the samples “Input” and “Bound” were eluted in SDS-sample buffer and analyzed by SDS-PAGE.

As for the binding between Pex3p and Pex19p-PMP22 complex, the binding assays were performed essentially in the same procedures as described (13). Briefly, His\(_{10}\)PMP22 mRNA was translated with the cell-free protein synthesis system in the presence of 24 µg of Pex19p, which did not have a tag region. After the 20-h translation at 26 °C, the reaction mixture was centrifuged for 10 min at 18,000 x g. Then, 32 µg of the wild-type GST-Pex3p or the GST-Pex3p W104A mutant was added to the supernatant and incubated for 5 min on ice. The reaction mixtures were purified by chromatography on 100-µl Ni\(^{2+}\)-NTA-agarose columns, using the buffer containing 20 mM Tris-Cl, pH 8.0, 0.5 mM NaCl, 10% glycerol, and 20 mM imidazole for washing, or 200 mM for elution. Each eluate was precipitated with trichloroacetic acid, and the precipitates were analyzed by the Western blotting. In this study, each 1:2000 dilution mixture of anti-Pex3p, anti-Pex19p, and anti-PMP22 antibodies was used as the primary antibody. We confirmed that these three antibodies did not cross-react with each other in advance.

**Circular Dichroism (CD) Analysis**—CD spectra were measured using a JASCO J-725 spectrometer (Jasco International Co., Ltd.). Samples were dialyzed against 20 mM sodium phosphate buffer, pH 7.5. A quartz cell with a 1-mm light pathlength was used. All measurements were performed at 20 °C. Data were collected as the average of 16 time measurements.
**SPR Analysis**—Equilibrium binding analyses of the interaction between Pex3p and Pex19p were performed with a Biacore2000 instrument (GE Healthcare). Goat anti-GST antibodies (GE Healthcare) were immobilized on CM5 sensor chips (GE Healthcare) in two flow cells by standard amine coupling, following the manufacturer’s instructions. Then, purified GST-Pex19p was captured on flow cell 2 via the GST antibody, while flow cell 1 was used as a reference. All experiments were performed at 25 °C, using buffer containing 10 mM HEPES-Na, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) Tween 20 (running buffer). Various concentrations of Pex3p and its mutants were injected. All samples to be injected were dialyzed against the running buffer to reduce the bulk refractive index changes caused by buffer variations. Association and dissociation phases were both monitored for 3–5 min. After each cycle, the surfaces were regenerated by injections of 10 mM glycine-Cl, pH 2.2, for 1 min and 50 mM NaOH for 1 min. The equilibrium dissociation constant ($K_D$) was obtained by non-linear fitting to a one-site model of the Langmuir binding isotherm (see Equation 1). Curve fitting was performed using KaleidaGraph software (Synergy Software Co.).

$$\text{RU}_{\text{bound}} = \frac{[\text{Pex3p}] \cdot [\text{RU}_{\text{max}}]}{K_D + [\text{Pex3p}]}$$

(Eq. 1)

**Morphological Analysis**—Peroxisome-deficient Chinese hamster ovary cell mutant, pex3 ZPG208 (7) was cultured as described (26). DNA transfection to Chinese hamster ovary cells was performed by lipofection (8). At 60-h post-transfection, peroxisomes in cells were detected by indirect immunofluorescence light microscopy as described (20, 27).

**RESULTS**

**Expression and Purification of His$_{10}$-Pex3p-(34–373)**—To investigate the molecular properties of the human Pex3p protein, we prepared highly purified Pex3p lacking the N-terminal 33-residue hydrophobic segment (Pex3p-(34–373)). The purification of His$_{10}$-Pex3p-(34–373) is summarized in Fig. 1. Even though the hydrophobic segment was removed, Pex3p was prone to aggregate formation. We found that a low temperature and a high salt concentration were required to improve its solubility. For example, during the dialysis of His$_{10}$-Pex3p-(34–373) at 0.6 mg/ml in a high salt buffer (10 mM HEPES-Na, pH 7.5, 0.5 M NaCl, 10% glycerol) against a buffer without salt (10 mM HEPES-Na, pH 7.5, 10% glycerol), the protein formed precipitates and finally achieved a 0.1 mg/ml concentration in a 20,000 g supernatant of the dialyzed solution.

**Determination of the Molecular Weights of Pex3p and the Pex3p-Pex19p Complex**—We analyzed the molecular weights of Pex3p and the Pex3p-Pex19p complex by gel filtration chromatography analyses. Purified Pex3p (theoretical molecular mass: 40.9 kDa) was eluted in a sharp peak at the retention volume of 15.5 ml (Fig. 2). This location corresponds to an apparent molecular mass of 39.6 kDa, which is close to the monomeric size. Purified Pex19p (theoretical molecular mass: 36.1 kDa) and the mixture of equivalent molar amounts of Pex3p and Pex19p were each eluted, respectively, in sharp peaks at the retention volumes of 13.8 ml and 13.2 ml (Fig. 2).

These locations correspond to apparent molecular masses of 97.2 and 135.2 kDa. Pex19p is suggested to exist as a monomer, as determined by an analytical ultracentrifugation experiment (supplemental Fig. S2). In gel filtration chromatography,
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![Graph showing tryptophan fluorescence spectra of Pex3p with increasing Pex19p concentrations.](image)

**FIGURE 3.** Tryptophan fluorescence spectra of Pex3p with increasing Pex19p concentrations. Intrinsic tryptophan fluorescence spectra of 0.5 µM Pex3p (open circles) and those with Pex19 at 0.125 µM (open diamonds), 0.25 µM (open triangles), 0.5 µM (open squares), and 2.0 µM (closed circles) were measured as described under “Experimental Procedures.” The experiments were performed three times, and representative data are shown. *Inset,* relative fluorescence intensities (F/F₀) at 322 nm were plotted. The error bar represents the S.D. (*n* = 3). The initial fluorescence intensity of 0.5 µM Pex3p is F₀.

![Diagram showing binding assay results.](image)

**FIGURE 4.** Binding assays. A, pull-down assays for tryptophan mutants of Pex3p and Pex19p or Pex19p-PMP22 complex. His₁₀-PMP22 was synthesized by the cell-free protein synthesis system in the presence of GST-Pex19p. After protein synthesis, the wild-type GST-Pex3p or the GST-Pex3p W104A mutant was added to the soluble fraction containing the Pex19p-His₁₀-PMP22 complex. Input, corresponds to 1% amount of the total proteins. The mixtures were purified by Ni²⁺-NTA-agarose and the imidazole eluates (Bound) were analyzed by Western blotting. Glutathione-Sepharose 4B beads (Input). After washing away the unbound proteins, the beads were collected (Bound). Proteins in the Input and Bound samples were eluted in SDS-sample buffer and analyzed by SDS-PAGE, using a 12.5% gel. The proteins separated in the gel were stained with Coomassie Brilliant Blue R-250. The experiments were performed three times, and representative data are shown. B, binding assays for the Pex3p W104A mutant and the Pex19p-PMP22 complex. His₁₀-PMP22 was synthesized by the cell-free protein synthesis system in the presence of GST-Pex19p. After protein synthesis, the wild-type GST-Pex3p or the GST-Pex3p W104A mutant was added to the soluble fraction containing the Pex19p-His₁₀-PMP22 complex. Input, corresponds to 1% amount of the total proteins. The mixtures were purified by Ni²⁺-NTA-agarose and the imidazole eluates (Bound) were analyzed by Western blotting. The blotted GST-Pex3p, Pex19p, and His₁₀-PMP22 were detected using a mixture of antibodies. The experiments were performed twice, and representative data are shown.

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Retention volume of Pex19p corresponds to a larger molecular weight than its theoretical one because of its extended conformation. The apparent molecular mass of 135.2 kDa is nearly equal to 136.8 kDa, the sum of the molecular masses of Pex3p and Pex19p, suggesting that they form a complex in a 1:1 molar ratio.

**Intrinsic Tryptophan Fluorescence Titration of Pex3p with Pex19p**—To investigate the binding manner between Pex3p and Pex19p, we measured the tryptophan fluorescence changes of Pex3p induced by the binding of Pex19p. Pex3p has two tryptophan residues (Trp-104 and Trp-224) and Pex19p has one tryptophan residue (Trp-202). In this experiment, the Pex19p W202F mutant was used as an alternative to that of the wild-type, to avoid the influences induced by the original tryptophan residue of Pex19p. This mutation seems not to have essential effects on the binding to Pex3p because the Trp-202 locates in the region, which was suggested not to participate in the binding to Pex3p (13). In addition, we confirmed that the binding affinity of the Pex19p W202F mutant toward Pex3p was the same level as that of the wild-type Pex19p, by a pull-down assay. As shown in Fig. 3, the maximum emission wavelength (λmax) for Pex3p was 340 nm, and with an increase in the Pex19p concentration, the λmax blue-shifted to 322 nm. The emission intensities at 322 nm were increased in a Pex19p-dependent, saturable manner (Fig. 3, inset). This result indicates that the binding of Pex19p with Pex3p induced these fluorescence changes. Because a large (18-nm) blue shift in λmax was observed, we speculated that either one or both of the tryptophan residues could be directly involved in the binding to Pex19p. In addition, in the same experiment in buffers containing or not 0.5 M NaCl, the fluorescence spectra shifted in the same manner, and the binding curves were also the same. However, the shift was suppressed, depending on the increase in n-dodecyl-β-D-maltoside (β-DDM: critical micelle concentra-
the same assay with a Pex3p W104F mutant to elucidate whether the aromatic ring in Trp-104 played a role in binding. As a result, the W104F mutant showed slightly less affinity for Pex19p than the wild-type, but more than the W104A mutant. We also investigated whether the Pex3p W104A mutant showed decreased affinity to the Pex19p harboring PMP. His10-PMP22 was synthesized in vitro in the presence of Pex19p, and the soluble fraction was prepared by centrifugation. The wild-type GST-Pex3p or the GST-Pex3p W104A mutant was added in the soluble fraction, and the complex of His10-PMP22, Pex19p and GST-Pex3p was isolated with Ni2+-NTA-agarose. As a result, the binding of the GST-Pex3p W104A mutant to the Pex19p-His10-PMP22 complex was much weaker than that of the wild-type (Fig. 4B). This result suggests that the replacement of Trp-104 with Ala diminishes binding activity toward Pex19p harboring PMPs, as well as toward Pex19p by itself.

**SPR Analysis**—To quantitatively clarify the involvement of the side chain of Trp-104 in the binding, we measured the dissociation constant (K_D) for the binding of the wild-type Pex3p, and the W104A and W104F mutants by SPR analyses. Because the Pex3p W104A showed decreased affinity to the Pex19p-PMP22 complex as well as Pex19p, it seemed that the replacement of the Trp-104 had essentially a similar effect on affinity to Pex19p either with or without PMPs. Thus, to assess the K_D of the Trp mutants, we evaluated the K_D of the Pex3p-Pex19p interaction. Fig. 5A shows overlaid SPR sensorgrams of the wild-type Pex3p. The values of equilibrium responses over the maximum response derived from non-linear fitting of Equation 1 (B/B_max) were plotted against the corresponding concentrations of the wild-type Pex3p. The same procedure was applied to the Pex3p W104A and W104F mutants (Fig. 5B). The SPR analyses revealed that the wild-type Pex3p has a high affinity for Pex19p, with a K_D of 3.4 ± 0.7 nM, whereas the W104A mutant has about 300-fold lower affinity (K_D of 1080 ± 125 nM), than the wild-type. The W104F mutant, in which the indole ring of the original tryptophan was replaced with a benzene ring, has about 20-fold lower affinity than the wild-type (K_D of 66.2 ± 17.9 nM). These results are consistent with the results of the pull-down assays. Therefore, the side chain aromatic ring of Trp-104 seems to play a key role in the specific binding activity of Pex3p to Pex19p.

**CD Analysis**—To investigate whether the replacement of Trp-104 with Ala induced large structural changes, we measured and compared the CD spectra of the wild-type Pex3p and the W104A mutant. As shown in Fig. 6, their CD spectra were quite similar. This result indicates that the mutation did not induce large structural changes. Therefore the vastly lower affinity of the W104A mutant to Pex19p is not caused by structural disorder in Pex3p, but by the lack of the side chain structure of Trp-104.

**Pull-down Assay using Mutants of Conserved Amino Acids Neighboring Trp-104**—We investigated whether the amino acids in the vicinity of Trp-104, are also involved in binding. The protein sequence of human Pex3p was aligned with that of several mammalian and yeast species using the ClustalW program. Fig. 7A shows the alignment of the region surrounding Trp-104 in human Pex3p. As shown in this figure, Lys-100 and Leu-107, as well as Trp-104, are conserved among species. In addition, Ile-103 and Phe-112 are conserved in terms of their hydrophobic characteristics, and Lys-108 is conserved in terms of its basic character, with the exception of *Saccharomyces cerevisiae*. To determine whether these residues are involved in binding, we prepared single amino acid mutants of Pex3p, K100A, I103A, L107A, K108A, and F112A, respectively, and
carried out pull-down assays with GST-Pex19p by the same method described above. As a result, none of these mutants showed decreased affinity comparable to that of the W104A mutant (Fig. 7B). This result indicates that these conserved residues have fewer contributions to binding than Trp-104. Therefore, Trp-104 is one of the most important residues, among conserved amino acid residues in this region, for binding to Pex19p.

**Peroxisome Restoring Activity of the Pex3p W104A Mutant**—

We investigated the effect of the decreased affinity of Pex3p W104A to Pex19p on the peroxisome assembly. Wild-type full-length Pex3p-HA or its mutants were expressed in the pex3 ZPG208 cells, and peroxisome assembly was verified by immunofluorescent staining with antibodies to the peroxisome targeting signal (PTS)1 and Pex14p, under immunofluorescence light microscopy. In the cells transfected with the plasmid encoding the wild-type Pex3p, both PTS1 proteins and Pex14p were detected in a punctate staining pattern, in a superimposable manner (Fig. 8, panels c and d), whereas, in those transfected with the plasmid coding for the Pex3p W104A mutant, no punctate structures were detectable (Fig. 8, panels e and f). These results suggest that the Pex3p W104A mutant is defective in peroxisome restoring activity. In contrast, the Pex3p W104F mutant apparently retained the restoring activity (Fig. 8, panels g and h). In addition, the W224A mutant, which had essentially the same affinity to Pex19p as that of the wild-type, restored peroxisome assembly (Fig. 8, panels i and j).

Based on the findings in our SPR analyses that the side chain aromatic ring of Trp-104 exerts strong effects on the affinity of Pex3p to Pex19p, we conclude that tight binding between Pex3p and Pex19p via the side chain of Trp-104 is crucial for peroxisome biogenesis.

**DISCUSSION**

The present work demonstrates the physicochemical properties of Pex3p with respect to Pex19p binding, which reflects the intracellular traffic mechanism of PMPs. This is the first time that human Pex3p has been highly purified in a soluble form, although its membrane-anchoring segment (Met-1–Gly-33) is truncated, and the Pex3p displays the biological binding function. Pex3p is susceptible to precipitation, even though the membrane-anchoring region was removed. We found that a
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low temperature and a high concentration of salts were required to prevent the precipitation. In addition, in SDS-PAGE, Pex3p samples that were heated at 95 °C showed bands derived from non-productive self-aggregation, and thus we had to avoid heating the samples. These characteristic properties of Pex3p may have caused some inconsistencies in previous results. Here, we have generated experimental data taking into consideration its molecular properties.

Using the purified preparations of Pex3p and Pex19p, Trp-104 of Pex3p was identified as a critical residue for binding to Pex19p. In the fluorescence titrations of Pex3p with tryptophan-less Pex19p, the original λ_max of Pex3p, which possesses two tryptophan residues (Trp-104 and Trp-224), was 340 nm and with an increase in the Pex19p concentration, it shifted to a shorter wavelength and reached 322 nm (Fig. 3). In general, for tryptophan, its fluorescence emission intensity and λ_max are highly sensitive to the polarity of the microenvironment in which the indole side chain is localized. Thus, the shift of λ_max observed in this experiment indicates that at least one of the tryptophan residues in Pex3p becomes more hydrophobic by the binding of Pex19p. From this result, we surmised that the tryptophan residues in Pex3p directly participate in the binding to Pex19p. This assumption was confirmed by the following findings. (i) The pull-down assay showed that the affinity of the Pex3p W104A mutant for Pex19p was significantly decreased, while on the other hand, the Pex3p W224A had essentially the same affinity as that of the wild-type (Fig. 4A). (ii) In the SPR analysis, whereas the wild-type Pex3p showed a K_D value of 3.4 nM, the Pex3p W104A mutant showed about 300-fold decreased affinity (K_D, 1080 nM), and in the case of the W104F mutant, in which the indole ring of the original tryptophan is replaced with a benzene ring, a moderate 20-fold decrease of the affinity was observed (K_D, 66 nM) (Fig. 5B). (iii) The CD analysis suggested that the replacement of Trp-104 with Ala induces minimal structural perturbation (Fig. 6). (iv) Several conserved amino acids in the proximity of Trp-104 in Pex3p have only minor contributions to the Pex19p binding (see below, Fig. 7, A and B). Taken together, these findings indicated that not only does Trp-104 directly participate in Pex19p-Pex3p binding, but also that the aromatic ring of Trp-104 is a critical component of the binding.

The physiological importance of the Trp-104 residue was highlighted by studies of the deficient peroxisome restoring activity in pex3 ZPG208 cells (Fig. 8). The wild-type Pex3p restored the peroxisome assembly, but Pex3p W104A did not, suggesting that a substantial decrease of the binding affinity precludes peroxisome assembly.

Present studies suggest that several conserved amino acids in proximity to Trp-104 in Pex3p have little influence in the binding to Pex19p (Fig. 7, A and B). The sequence alignment analysis showed that a region from Lys-100 to Arg-114 is highly conserved in Pex3p sequences. To find assisting residues for Trp-104 in Pex3p-Pex19p interaction, we tried to alter the conserved residues in this region. Ala substitutions indicated that the residues, Lys-100, Ile-103, Leu-107, Lys-108, and Phe-112 appeared to contribute little to the binding. However, the tight binding between Pex3p and Pex19p is unlikely to occur solely by this tryptophan residue. In fact, the Pex3p W104A mutant still has a K_D value for Pex19p binding of 1080 nM; that is, the W104A mutant retains a certain level of affinity. Thus, the other residues of another region in Pex3p could be involved in Pex19p binding. There is a computationally predicted hydrophobic region, from Ile-109—Val-131 in Pex3p and this hydrophobic region is conserved among the Pex3p proteins from yeast species (28). This region could contain candidates for the other binding sites.

Our stoichiometric analyses demonstrated that the Pex3p monomer is sufficient for the binding of a single molecule of Pex19p. In the gel filtration chromatography analyses, the mixture of equimolar amounts of Pex3p and Pex19p showed a single peak in the elution profile (Fig. 2). In the fluorescence titrations, the changes in the fluorescence intensities from Pex3p were saturated when Pex3p and Pex19p were mixed in a 1:1 molar ratio (Fig. 3, inset). These findings suggest that Pex3p and Pex19p form a complex in a 1:1 molar ratio. This indication was supported by a native-PAGE gel shift assay (supplemental Fig. S3). With regard to the assembly states of these two proteins, Pex3p is judged to exist as a monomer by gel filtration chromatography analysis. Pex19p is also suggested to exist as a monomer, based on our analytical ultracentrifugation analysis (supplemental Fig. S2). Thus, our experimental data suggest that a molecule of monomeric Pex3p is complexed with a molecule of monomeric Pex19p. In addition, Pex19p reportedly forms a complex with PMP70 in a 1:1 molar ratio (29). Consequently, each single molecule of Pex3p, Pex19p, and PMPs is likely to form a ternary complex on the peroxisomal membrane, although it is still unclear whether the N-terminal membrane-anchoring region of Pex3p has some influence on the interaction among the three proteins.

Pex3p was found to bind to Pex19p with high affinity (K_D, 3.4 nM), which is comparable to that observed in other intracellular translocation systems such as the specific binding between Pex14p and Pex5p. Recently, a titration analysis of the Pex3p-Pex19p interaction by native-PAGE demonstrated that the K_D value was 3.4 μM, which is higher than our suggested value (30). It is likely that the K_D value of Pex3p for Pex19p varies with different assay conditions, because its solubility and stability are strongly influenced by solution conditions. However, our SPR assay was performed in a buffer that is similar to physiological conditions, we suggest that the Pex3p-Pex19p interaction has a high affinity, with a K_D value on the nanomolar level. This proposed high affinity of the Pex3p-Pex19p interaction is similar to the properties of the interaction between the cytosolic peroxisomal protein carrier, Pex5p, and its docking receptor, Pex14p. Pex5p is a specific carrier of peroxisomal matrix proteins containing PTS1 (31, 32), and Pex14p is a docking receptor for Pex5p (33). SPR analyses demonstrated that Pex5p bound to Pex14p(1—78) with high affinity (K_D, 0.9 nM, long form human Pex5p and 3.3 nM, short form human Pex5p) (34). These K_D values are similar to that of the interaction between Pex3p and Pex19p. Because the specific interactions of Pex3p with Pex19p and Pex14p with Pex5p are essential events for the translocation of PMPs and PTS-containing proteins to the peroxisome, respectively, it is believed that such a high affinity is necessary for Pex3p and Pex14p in order to choose their specific binding partners from the many proteins of the cytosol. On
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the other hand, the fact that Pex3p is tightly bound to Pex19p raises the question of how Pex19p is released from Pex3p. Indeed, it has been demonstrated that Pex19p seems to be released from the peroxisome to the cytosol after the translocation of PMPs (14). Our experimental data showed that Pex19p could not bind to Pex3p in the presence of gentle detergents such as β-DDM. This suggests that a hydrophobic interaction is very important for binding, and its perturbation could trigger the release of Pex19p.

In conclusion, our results revealed some physicochemical characteristics of the interaction between Pex3p and Pex19p. A molecule of monomeric Pex3p is complexed with a molecule of monomeric Pex19p, with a $K_D$ at a low nanomolar level. Trp-104 of Pex3p plays a key role in this interaction.

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