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Monomer–dimer transition of the conserved N-terminal domain of the mammalian peroxisomal matrix protein import receptor, Pex14p

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ABSTRACT

Pex14p is a central component of the peroxisomal matrix protein import machinery. In the recently determined crystal structure, a characteristic face consisting of conserved residues was found on a side of the conserved N-terminal domain of the protein. The face is highly hydrophobic, and is also the binding site for the WXXXF/Y motif of Pex5p. We report herein the dimerization of the domain in the isolated state. The homo-dimers are in equilibrium with the monomers. The homo-dimers are completely dissociated into monomers by complex formation with the WXXXF/Y motif peptide of Pex5p. A putative dimer model shows the interaction between the conserved face and the PXXP motif of another protomer. The model allows us to discuss the mechanism of the oligomeric transition of the full-length Pex14p modulated by the binding of other peroxins.

1. Introduction

Peroxisome is an organelle in eukaryotic cells that functions in various metabolic pathways, including the β-oxidation of very long fatty acids [1]. Peroxisomal matrix proteins are synthesized in the cytosol and are subsequently imported post-translationally into peroxisomes by a dynamic, complicated system consisting of Pex1p–Pex26p [2–7]. Pex14p with a molecular mass of 41 kDa is anchored in the peroxisomal membrane and is a central component of the peroxisomal matrix protein import machinery [8–16]. The protein contains multiple domains such as the conserved N-terminal, transmembrane and coiled-coil domains. The conserved N-terminal domain comprising residues 25–70 (in mammalian homologues) interacts with Pex5p and Pex19p. The PXXP motif near/within the conserved N-terminal domain interacts with Pex13p. It has been reported that Pex14p forms homo-dimers by the coiled-coil domain [14], or larger oligomers by the GXXXG and AXXXA motifs in the transmembrane domain [15]. In addition, the conserved N-terminal domain of Pex14p from protozoan parasite *Leishmania donovani* (LdPex14p) has been found to form a stable dimer [17]. Pex14p is formed in different oligomeric states by interactions with other peroxins in distinct manners [15]. Pex14p assembles into oligomers with molecular weights of 300–450 kDa in the absence of other peroxins. Large oligomeric states of 200–700 kDa are observed in the interaction with Pex13p, while smaller oligomers (~100 kDa) are formed in the presence of Pex5p or Pex19p.

We have recently determined the crystal structure of the conserved N-terminal domain of mammalian Pex14p [18]. The domain consists of three helices with a right-handed twist that is stabilized by the rigid hydrophobic core. Two concave pockets are formed at the side of the molecule. The residues of the side are conserved to a high degree. Two phenylalanine residues (Phe35 and Phe52), which are highly hydrophobic, are exposed to the solvent. The pockets are surrounded by several positively charged residues such as Arg25, Lys34, Arg40, Lys55 and Lys56. Consequently, the two pockets on the surface are suitable for recognizing the helical WXXXF/Y motif of Pex5p [16,19]. The two conserved aromatic residues of the WXXXF/Y motif can be stabilized by aromatic–aromatic and cation–π interactions with the conserved N-terminal domain. In vitro and in vivo assays confirmed that the Phe35/Phe52 residues are essential to the Pex14p functions including the interaction between Pex14p and Pex5p. It is noteworthy that two molecules assemble into a dimer in the crystal lattice.

The molecular basis for recognition of the WXXXF/Y motif of Pex5p by the domain has also been investigated with NMR

Abbreviations: BS^3, bis[sulfosuccinimidyl] suberate; CD, circular dichroism; DPC, decylphosphocholine; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; LDAO, lauryldimethylamine-*N*-oxide; MES, 2-(*N*-morpholino)ethanesulfonic acid; NCS, non-crystallographic symmetry; PAGE, polyacryl-amide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography.

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spectroscopy by Neufeld et al. [20]. Actually, a peptide of the motif is bound into the pockets. The root mean square deviation between NMR and the crystal structures is calculated to be 1.1 Å for C_α atoms, indicating that the two structures are essentially identical. They further indicated that the region is shared as a common binding site by the F/YFXXXF motif of Pex19p. However, the peptide-free structure was not revealed, while clear signals and drastic shifts in response to complex formation with the motifs have been reported. Consequently, the oligomeric architecture of the domain in the solution remains to be elucidated, although it is expected to play an important role in the dynamics of the peroxisomal matrix protein import machinery.

We report herein that the conserved N-terminal domain in the isolated state exhibits a monomer–dimer transition. A putative homo-dimer model of the conserved N-terminal domain is proposed. The model allows us to discuss the mechanism of the oligomeric transition of the full-length Pex14p modulated by the binding of other peroxins.

2. Materials and methods

2.1. Construction, expression, and purification

Pex14p(25–70) was prepared as reported [18]. It contains eight vector-derived residues at the N-terminus. The cDNA coding for amino acid residues at 21–70 of *Rattus norvegicus* (rat) Pex14p was amplified from rat full-length *PEX14* by polymerase chain reaction (PCR) with a pair of primers, 5′-GTGCCGAATTCATT GAGGGACGCAATGTGGTGCCTCGAGAGCC-3′ (underlined, EcoRI site) and 5′-TCGAGTCGACTCACGACTGCTGGAAAGCCAGGTC-3′ (underlined, SalI site), and ligated into the EcoRI/SalI site of pGEX-6P-1 (GE Healthcare). The cDNA construct encoding the glutathione *S*-transferase (GST)-fused Pex14p(21–70) [GST-Pex14p(21–70)] was transformed into *Escherichia coli* XL1-Blue cells. The transformed cells were grown to an OD_{600} of 0.4 at 18 °C. Expression of the fusion protein was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were lysed by sonication on ice. The centrifuged supernatant was applied to a GSTrap FF column (GE Healthcare). GST was removed by PreScission protease (GE Healthcare) in the column. The variant still contains eight amino acid residues derived from the expressing vector. Pex14p(21–70) without any vector-derived residues was prepared from PreScission protease-treated Pex14p(21–70) using 40 U/ml Factor Xa protease (Novagen) in 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$ at 20 °C in 16 h, then purified as Pex14p(25–70), as reported previously [18].

2.2. Peptide synthesis

Peptides of the WXXXF/Y motif of Pex5p, 5p1–5p7 and the variants of 5p1 at the conserved aromatic residue(s) 5p1AF, 5p1WA, 5p1AA, and 5p1FW, were synthesized by the Fmoc solid phase method and purified to >95% purity by a custom peptide synthesis service (Invitrogen). The sequences are as follows: 5p1, 114-LSENWAQEFLAAG-126; 5p2, 136-NETDWSQEFIAEV-148; 5p3, 155-SPARWAEEYLEQS-167; 5p4, 181-TTDRWYDDYHPEE-193; 5p5, 240-QAEQWAAEFIQQQ-252; 5p6, 254-TSEAWVDQFTRSG-266; 5p7, 297-EAHPWLSDYDDLT-309; 5p1AF, 114-LSEN**A**AQEFLAAG-126; 5p1WA, 114-LSENWAQE**A**LAAG-126; 5p1AA, 114-LSEN**A**A-QE**A**LAAG-126; 5p1FW, 114-LSEN**F**AQE**W**LAAG-126, where the mutated residues are shown in bold letters.

2.3. Native-PAGE for in vitro binding assay

The complex formation of Pex14p variant with Pex5p peptide was investigated by Native-PAGE analysis using a 15% gel and a running buffer containing 30 mM histidine and 30 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.1. The mixtures of protein and peptide were prepared with protein/peptide 1:2 (w/w) and incubated for 30 min at room temperature before Native-PAGE analysis. The gel was stained with Coomassie brilliant blue.

2.4. Circular dichroism (CD)

A quarts cuvette with a pass length of 1 mm was filled with a solution containing 0.1 mg/ml Pex14p(21–70) with or without 0.025 mg/ml 5p1 in 20 mM Tris–HCl buffer, pH 7.4. CD spectra were measured using a J-805 CD spectropolarimeter (JASCO) in a range from 190 to 250 nm at 20 °C. The scan speed and scan step were set to be 10 nm/min and 0.1 nm, respectively. Five scans were averaged.

2.5. Chemical crosslinking

The mixture of the Pex14p variant and Pex5p peptide was prepared with protein/peptide 1:1 (w/w). All samples were incubated for 30 min at room temperature before crosslinking. The molar ratio of protein/bis[sulfosuccinimidyl] suberate (BS^3) was 1:50. Samples (0.6 mg/ml protein in 20 mM Tris–HCl pH 7.4) were incubated with BS^3 for 30 min at room temperature. The quenching of crosslinking was completed by adding 500 mM Tris–HCl pH 7.5 to the crosslinking solution to give a 50 mM final concentration of Tris–HCl in the crosslinking solution, then incubating the solution for 15 min at room temperature. Finally crosslinked samples were analyzed by 5–20% gradient SDS–PAGE stained with Coomassie brilliant blue.

2.6. Size-exclusion chromatography (SEC)

Superdex 75 10/300 GL (GE Healthcare) was equilibrated with 150 mM NaCl and 20 mM Tris–HCl pH 7.4. Samples were prepared

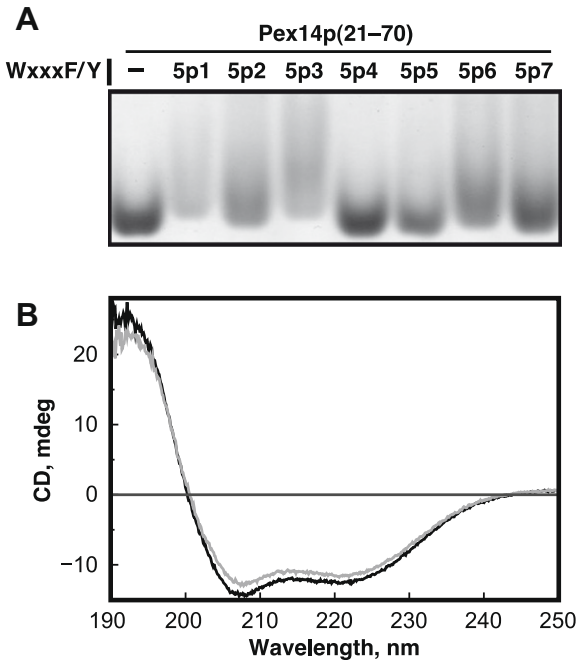

Fig. 1. Properties of Pex14p(21–70). (A) Native-PAGE analysis of the complex formation between Pex14p(21–70) and the WXXXF/Y motif peptides of Pex5p (5p1–5p7). (B) CD spectra for 0.1 mg/ml Pex14p(21–70) in 20 mM Tris–HCl pH 7.4 buffer, and the mixture of 0.1 mg/ml Pex14p(21–70) and 0.025 mg/ml 5p1 (1:1 in molar ratio) in 20 mM Tris–HCl pH 7.4 buffer are represented as gray and black lines, respectively. Both exhibit a helix-rich conformation.

in equilibrium buffer, and 50 μl of each (0.85–1 mg/ml) was applied to the column. The mixtures of Pex14p variant and Pex5p peptide were prepared with protein/peptide 1:1 (w/w). The crosslinked samples were prepared as described in Section 2.5.

3. Results and discussion

Native-PAGE analysis indicates that the truncated variant of mammalian Pex14p, Pex14p(21–70) has the ability to interact with synthetic WXXXF/Y motif peptides (5p1–5p7) (Fig. 1A). The 5p4 and 5p7 peptides display low affinities as reported [18,19]. The circular dichroism (CD) spectrum (Fig. 1B) for Pex14p(21–70) indicates that the variant takes on a helix-rich conformation, like the crystal structure [18]. The spectrum for the Pex14p(21–70)–5p1 complex also exhibits similar features that are consistent with the NMR structure [20]. This result implies that there is no significant difference in conformation for Pex14p(21–70) between peptide-free and peptide-bound states.

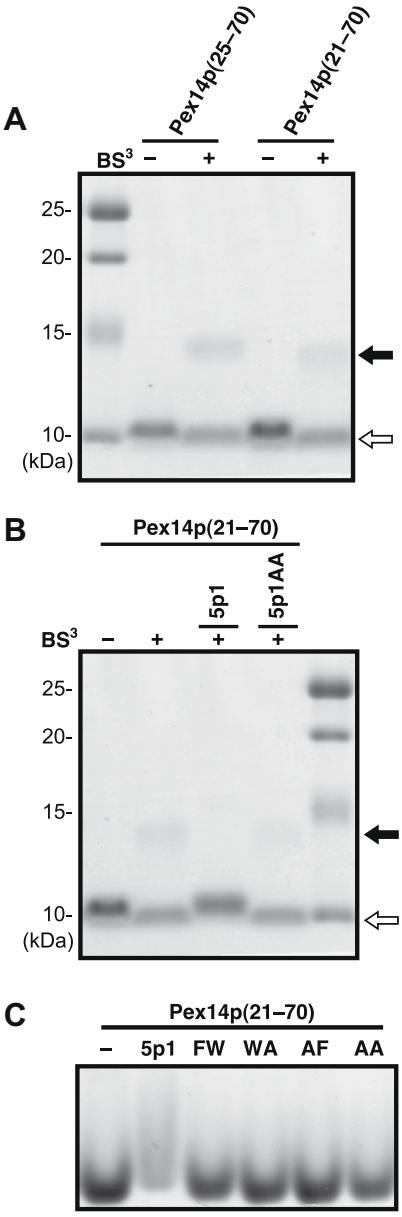

Fig. 2. Dimer formation of the conserved N-terminal domain. (A) Chemical crosslinking of Pex14p(21–70) and Pex14p(25–70) analyzed by SDS–PAGE. The discrepancy between the real and apparent molecular weights was also reported in the SDS–PAGE analysis of other truncation variants. (B) Effects of the addition of a WXXXF/Y motif peptide of Pex5p, 5p1, and its variant 5p1AA, in which the conserved aromatic residues are replaced by alanine. (C) Native-PAGE analysis for the complex formation between Pex14p(21–70) and 5p1, and its variants at the conserved aromatic residue(s), 5p1FW, 5p1WA, 5p1AF, and 5p1AA.

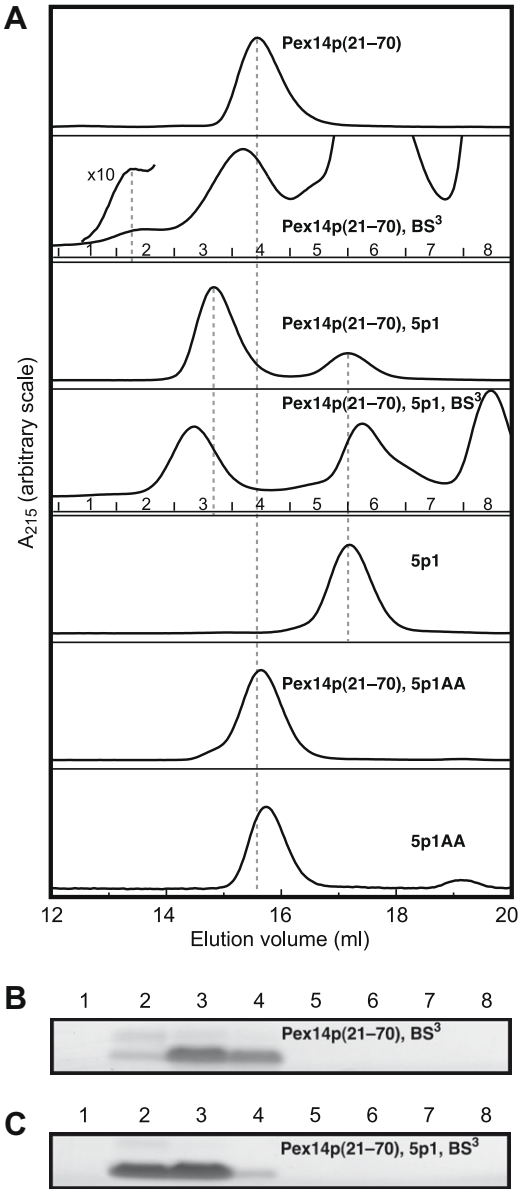

Fig. 3. Size-exclusion chromatography (SEC) of Pex14p(21–70). Elution profiles for Pex14p(21–70) in the presence or absence of the crosslinker BS^3 (A). Isolated Pex14p(21–70) only showed one peak at 15.6 ml. Crosslinked Pex14p(21–70) showed four peaks at 13.4, 15.3, 17.4, and 19.6 ml, of which the two higher peaks at 17.4 and 19.6 ml corresponded to crosslinkers. According to the SDS–PAGE analysis (lane numbers are the fraction numbers given in the elution profile) (B), the peaks at 15.3 and 15.6 ml correspond to monomeric Pex14p(21–70), and the peaks at 13.4 ml to dimeric Pex14p(21–70). The effects of the WXXXF/Y motif peptides (5p1 and 5p1AA) were also investigated. 5p1 and 5p1AA exhibit different elution volumes of 17.2 and 15.7 ml, respectively. The mixture of Pex14p(21–70) and 5p1 showed two peaks at 14.8 and 17.2 ml. The crosslinked mixture showed three peaks at 14.5, 17.4, and 19.6 ml, of which 14.5 ml peak correspond to the Pex14p(21–70)–5p1 complex, as confirmed by SDS–PAGE analysis (C). Therefore, the peaks at 14.8 and 14.5 ml correspond to the Pex14p(21–70)–5p1 complex. The peak at 17.2 ml and the partial peak at 17.4 ml correspond to unbound 5p1. The partial peak at 17.4 ml and the peak at 19.6 ml correspond to crosslinkers. The crosslinked samples were slightly shifted in SEC due to the modification. The mixture of Pex14p(21–70) and 5p1AA showed only one peak at 15.6 ml. This peak corresponds to the mixture of non-interacted Pex14p(21–70) and 5p1AA according to the isolated SEC analysis of each.

In order to investigate the oligomeric state of the conserved N-terminal domain of Pex14p in the solution, chemical crosslinking was performed for Pex14p(21–70) with a linker reagent bis[sulfosuccinimidyl] suberate (BS^3) (Fig. 2A). A band for dimers as well as a band for monomers was observed in the SDS–PAGE analysis with the addition of BS^3. Similar results were also observed by using Pex14p(25–70) in the crystallographic study (Fig. 2A). The addition of detergents such as lauryldimethylamine-*N*-oxide (LDAO) or decylphosphocholine (DPC) also provided the same results (data not shown), indicating that the dimerization is not due to non-specific hydrophobic interactions. On the other hand, the dimer formation was inhibited in the presence of a WXXXF/Y motif peptide of Pex5p (5p1) (Fig. 2B). Substitutions of the conserved aromatic residues of the WXXXF/Y peptide (5p1AA) led to a defect in inhibition of the dimer formation (Fig. 2B). Aromatic residues of the WXXXF/Y motif create the aromatic–aromatic interactions with the two phenylalanine residues (Phe35 and Phe52) of Pex14p. In fact, binding assay with Native-PAGE indicated that mutated peptides at the conserved aromatic residues (5p1FW, 5p1WA, 5p1AF, and 5p1AA) could not form complexes with Pex14p(21–70) (Fig. 2C). These results indicate that the dimeric interface is identical to the binding site for the WXXXF/Y motif of Pex5p.

The dimer formation was further investigated by size-exclusion chromatography (SEC) (Fig. 3A). However, isolated Pex14p(21–70) only showed one peak at 15.6 ml. In contrast, crosslinked Pex14p(21–70) showed four peaks at 13.4, 15.3, 17.4 and 19.6 ml, of which the two higher peaks at 17.4 and 19.6 ml corresponded to crosslinkers. According to the SDS–PAGE analysis (Fig. 3B), the peaks at 15.6 and 15.3 ml correspond to monomeric Pex14p(21–70), and the peak at 13.4 ml to dimeric Pex14p(21–70). The effects of the WXXXF/Y motif peptides (5p1 and 5p1AA) were also investigated. 5p1 and 5p1AA appear to have been oligomerized to different degrees in aqueous solution, exhibiting different elution volumes of 17.2 and 15.7 ml, respectively (Fig. 3A). The mixture of Pex14p(21–70) and 5p1 showed two peaks at 14.8 and 17.2 ml. The crosslinked mixture showed three peaks at 14.5, 17.4 and 19.6 ml, of which the 14.5-ml peak corresponds to the Pex14p(21–70)–5p1 complex, as confirmed by SDS–PAGE analysis (Fig. 3C). Therefore, the peaks at 14.8 and 14.5 ml correspond to the Pex14p(21–70)–5p1 complex. The peak at 17.2 ml and the partial peak at 17.4 ml correspond to unbound 5p1. The partial peak at 17.4 ml and the peak at 19.6 ml correspond to crosslinkers. The crosslinked samples were slightly shifted in SEC due to the modification. The mixture of Pex14p(21–70) and 5p1AA showed only one peak at 15.6 ml. This peak corresponds to a mixture of non-interacted Pex14p(21–70) and 5p1AA, according to the isolated SEC analysis of each. These results suggest that Pex14p(21–70) homo-dimers are completely dissociated into monomers when they pass through the column. That is, the transition between the monomer and dimer is too fast to detect by SEC. As for the mixture of Pex14p(21–70) and the WXXXF/Y motif peptide 5p1, both the non-treated and crosslinked samples provided shifted peaks at an elution volume of ~14.5 ml, indicating a tight interaction in the Pex14p(21–70)–5p1 complex. 5p1AA, a variant of 5p1, showed no interaction with Pex14p(21–70), as indicated by SEC analysis (Fig. 3A). This finding is consistent with chemical crosslinking and Native-PAGE analyses (Fig. 2B and C).

The asymmetric unit of the Pex14p(25–70) crystal contains two protein molecules that are related by a non-crystallographic two-fold axis. A putative dimer model was constructed based on the crystal structure with some modifications (Fig. 4A). Four residues of 21-NVVP-24 were added to the reference structure by replacing the eight vector-derived residues at the N-terminus of the crystallized variant. The conserved surfaces of each monomer in the dimer model were in contact with each other and not exposed to the solvent.

Interestingly, the N-terminal PXXP motif is predicted to form hydrophobic interactions with a pocket consisting of Phe35 and Phe52 residues in the partner molecule (Fig. 4B). The PXXP motif is identified as a binding site for Pex13p, since the truncation of residues 21–25 leads to a loss of binding ability with Pex13p [15]. It has been reported that some mutations at the Pex5p/Pex19p binding region of Pex14p impair the complex formation with Pex13p but not with Pex5p or Pex19p [20,23]. This result ap-

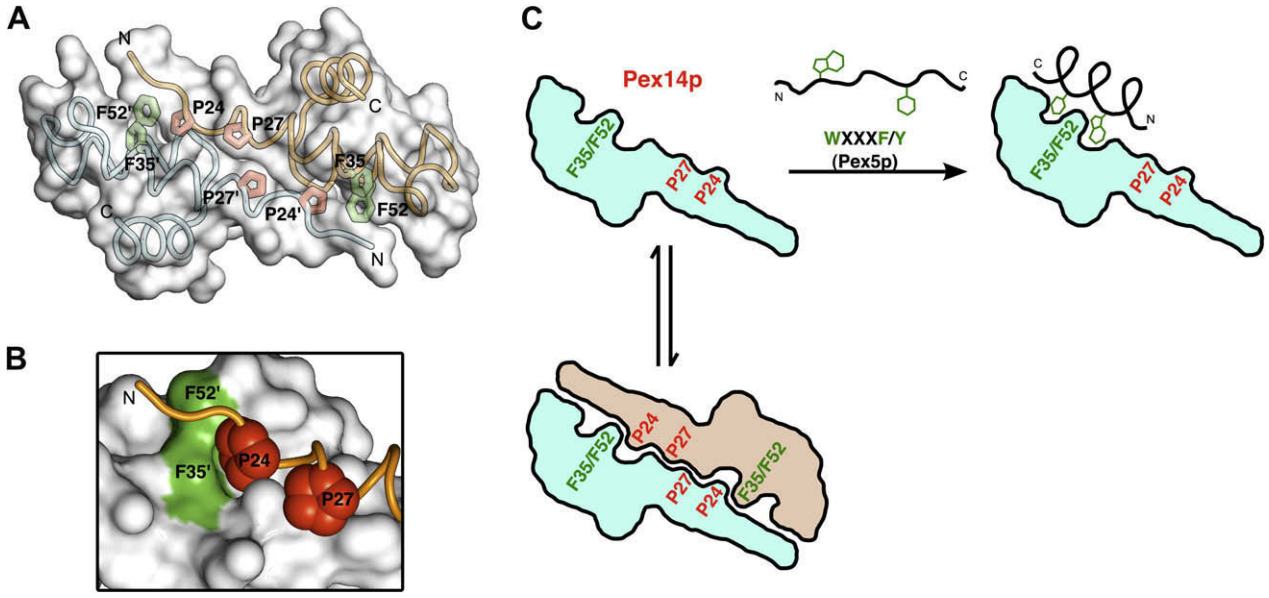

Fig. 4. Monomer–dimer transition of the conserved N-terminal domain. (A) A dimer model of the conserved N-terminal domain. Two molecules of Pex14p(21–70) are represented as tube models in sky blue and orange. Pro24, Pro27, Phe35, and Phe52 are represented as stick models. The molecular surface for each chain is drawn transparently. The model was constructed based on the non-crystallographic symmetry (NCS) dimer in the crystal structure of Pex14p(25–70) (PDB ID: 3FF5) [18] using the Coot [21] and XtalView [22] programs. (B) Close-up view of the interaction between proline residues (Pro24 and Pro27 in the PXXP motif) and phenylalanine residues (Phe35 and Phe52). The partner molecule is represented as surface. (C) Schematic representation of the monomer–dimer transition of the conserved N-terminal domain. The WXXXF/Y motif of Pex5p forms a helix when binding to the conserved N-terminal domain of Pex14p. The molecule figures were prepared by the PyMOL program [25].

pears strange, although the dimer structure can provide a reasonable interpretation, with the mutation leading to a tighter dimer. The binding of Pex5p/Pex19p is strong enough to lead to interactions with Pex14p even in such cases. Actually, LdPex14p as a stable dimer can also interact with Pex5p [17,24]. In the crystallographic dimer of Pex14p(25–70) lacking Pro24, the side chain of the artificially attached phenylalanine residue creates the interaction as a substitute for Pro24 [18].

The stable dimer of LdPex14p may be due to electrostatic interactions between protomers because the charge distribution on the domain of LdPex14p is different from that of mammalian Pex14p despite the high homology. In particular, a negatively charged residue (aspartic acid) is expected to be located at the position corresponding to Pro24 of the mammalian Pex14p [22]. The aspartic acid plausibly forms salt-bridges with positively charged residues on the dimeric interface.

Previous experiments by ultracentrifugation using full-length Pex14p indicated that Pex14p is in various oligomeric states according to the distinct interactions with other peroxins [15]. The oligomerization of Pex14p is mainly mediated by the coiled-coil domain and the GXXXG and AXXXA motifs in the transmembrane domain. However, we show in this paper that the conserved N-terminal domain mediates a transient dimerization by interaction between the conserved face and the PXXP motif of another protomer, which are the binding sites for Pex5p/Pex19p and Pex13p, respectively (Fig. 4C). The dimeric mode can explain why the oligomeric state of Pex14p is modulated by the complex formation with other peroxins. The collective inactivation by the dimer formation of the conserved N-terminal domain of Pex14p is quite elaborate as a central controlling mechanism for diverse peroxins as well as a preventive mechanism for low-affinity–non-physiological interactions. Such a systematically regulated transition between different oligomeric states of Pex14p may be indispensable for rapid formation and collapse of the protein translocation channel of the peroxisomal matrix protein import machinery.

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