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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

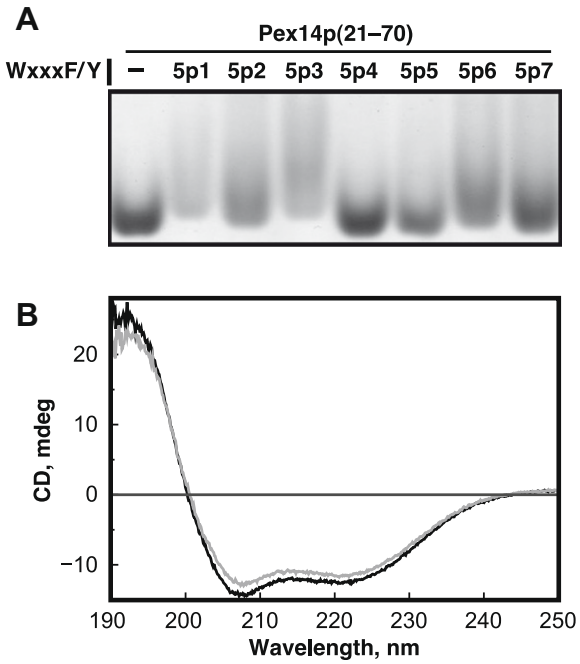

Fig. 1. Properties of Pex14p(21–70). (A) Native-PAGE analysis of the complex formation between Pex14p(21–70) and the WXXXF/Y motif peptides of Pex5p (5p1–5p7). (B) CD spectra for 0.1 mg/ml Pex14p(21–70) in 20 mM Tris–HCl pH 7.4 buffer, and the mixture of 0.1 mg/ml Pex14p(21–70) and 0.025 mg/ml 5p1 (1:1 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.