Insights into Peroxisome Function from the Structure of PEX3 in Complex with a Soluble Fragment of PEX19

Friederike Schmidt1,5, Nora Treiber2,5,6, Georg Zocher1, Sasa Bjelic3, Michel O. Steinmetz3, Hubert Kalbacher1, Thilo Stehle1,4* and Gabriele Dodt1*

From the 1Interfaculty Institute for Biochemistry, University of Tübingen, 72076 Tübingen, Germany, the 2Institute for Organic Chemistry and Biochemistry, University of Freiburg, 79106 Freiburg, Germany, the 3Biomolecular Research, Structural Biology, Paul Scherrer Institut, 5232 Villigen PSI, Switzerland, and the 4Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

5 These authors contributed equally to this work.
6 Current address: Max-Planck Institute of Immunobiology, Department of Cellular and Molecular Immunology, 79108 Freiburg, Germany

Running title: Structure of a sPEX3-PEX19Pep complex

* Correspondence should be addressed to G.D. (gabriele.dodt@uni-tuebingen.de, phone: +49 7071 2973349, fax: +49 7071 295191) or T.S. (thilo.stehle@uni-tuebingen.de, phone: +49 7071 2973043).

The human peroxins PEX3 and PEX19 play a central role in peroxisomal membrane biogenesis. The membrane-anchored PEX3 serves as the receptor for cytosolic PEX19, which in turn recognizes newly synthesized peroxisomal membrane proteins. After delivering these proteins to the peroxisomal membrane, PEX19 is recycled to the cytosol. The molecular mechanisms underlying these processes are not well understood. Here, we report the crystal structure of the cytosolic domain of PEX3 in complex with a PEX19-derived peptide. PEX3 adopts a novel fold that is best described as a large helical bundle. A hydrophobic groove at the membrane-distal end of PEX3 engages the PEX19 peptide with nanomolar affinity. Mutagenesis experiments identify phenylalanine 29 in PEX19 as critical for this interaction. Since key PEX3 residues involved in complex formation are highly conserved across species, the observed binding mechanism is of general biological relevance.

Peroxisomes are single membrane-bound organelles that carry out a variety of metabolic processes. In addition to the degradation of H2O2, the β-oxidation of very long chain or branched chain fatty acids and the synthesis of ether lipids are performed in these subcellular compartments (1, 2). The biogenesis of peroxisomes, including their formation and proliferation, as well as the degradation of peroxisomes, are highly dynamic processes that are adapted to metabolic needs (3). Defects in peroxisome biogenesis cause a number of severe inherited diseases, which are collectively referred to as peroxisome biogenesis disorders (4, 5). Studies in yeast and analysis of patients affected by these disorders have led to the identification of specific proteins involved in peroxisomal formation and maintenance (6). Fifteen such proteins, which are named peroxins, are currently known in humans and the corresponding genes (PEX genes) are highly conserved throughout the eukaryotic kingdom (7, 8).

All matrix proteins and most membrane proteins are imported post-translationally into peroxisomes. The machinery of peroxins that mediates the import of matrix proteins bearing a peroxisomal targeting signal is far better understood than the machinery that mediates the recognition and import of membrane proteins (9, 10). The peroxins PEX3, PEX16 and PEX19 are known to be essential for peroxisomal membrane biogenesis, as a loss of any of these proteins leads to the complete absence of detectable peroxisomal membrane structures (11). However, de novo formation of peroxisomes was observed in cells deficient for each of these peroxins upon complementation with the wild type gene, raising an intriguing question about the origin of the peroxisomal membrane (11-15). The ER membrane as the obvious source was disputed for a long time as several studies indicate that this process does not involve the classical COPI and COPII dependent
pathways (16-18). Recently, new evidence for an involvement of the ER as a peroxisomal precursor has been reported in yeast (19-21) and in mammalian cells (22-24), although the details of this process remain to be elucidated.

PEX19 is a farnesylated but hydrophilic protein that is predominantly found in the cytosol, with a smaller fraction transiently located at the peroxisomal membrane (12, 25). In the cytoplasm, PEX19 can act as a chaperone for newly synthesized peroxisomal membrane proteins (PMPs) by binding them during or after translation and keeping them in an import-competent form (26, 27). For the majority of PMPs, the PEX19-binding site matches the proposed membrane targeting signal (11, 28). Cargo-loaded PEX19 is directed to the peroxisomal membrane by docking to PEX3 (29). The predicted PEX3-binding domain of PEX19 is located within its first 56 amino acid residues, while the C-terminal part harbors the binding sites for other peroxisomal membrane proteins (11, 26, 30, 31). After insertion of the PMP, PEX19 is released into the cytosol to initiate another import cycle (32).

The peroxin PEX3 is anchored in the peroxisomal membrane via a short hydrophobic transmembrane segment within its N-terminal 33 residues, a region that is necessary and sufficient for targeting PEX3 to peroxisomes (33, 34). The cytosolic domain mediates the interaction with PEX19 (11). PEX3 is imported into peroxisomes in a PEX19-independent manner, and hence defines a separate import pathway (26). The role of PEX16 during the import of peroxisomal membrane proteins is less well defined, but it is thought to function as a docking site for PEX3 (35).

In order to define the parameters that underlie the interaction of PEX3 with PEX19, we solved the structure of a soluble domain of PEX3 in complex with a peptide corresponding to an N-terminal region of PEX19 (PEX19(26-373)). The soluble PEX3 domain comprises residues 41-373 and contains a cysteine to serine mutation at position 235 (sPEX3). In combination with affinity measurements and mutagenesis experiments, this structure provides insights into the determinants of recognition of the PEX3-PEX19 complex. As residues in the contact area are highly conserved among eukaryotes, our structure can serve as a general model for understanding the functions of PEX3 and PEX19 in peroxisomal biogenesis. Moreover, the structure presented here provides one of the first views of any interaction between two peroxins at high resolution.

**EXPERIMENTAL PROCEDURES**

**Protein expression**—Two human PEX3 fragments, comprising residues 26-373 and 41-373, were expressed in *E. coli*. The corresponding DNA regions including a preceding TEV protease cleavage site were cloned into the vector pET32a (Novagen), which includes an N-terminal His6-tag. Site-directed mutagenesis (QuikChange®, Stratagene) was used to generate Cys-Ser mutations at position 235 in both cases. These constructs were transformed into *E. coli* Rosetta2 (DE3) cells and grown at 37 °C to an OD 600 of 0.6, before protein expression was induced with 1 mM IPTG. Cells were grown for an additional 16 hours at 18 °C before harvesting. DNA coding for full-length human PEX19 with a preceding TEV protease cleavage site was cloned into the vector pColdI (Takara Bioscience), which includes an N-terminal His6-tag. The construct was transformed into *E. coli* BL21 (DE3) cells. Cells were grown at 37 °C to an OD 600 of 0.4, at which point the temperature was lowered to 15 °C before protein expression was additionally induced with 1 mM IPTG. Cells were then incubated for 16 hours at 15 °C.

**Protein purification**—All three proteins (PEX3(26-373)(C235S), PEX3(41-373)(C235S) and PEX19(1-299)) were purified using the same protocol. PEX3(41-373)(C235S) is referred to as sPEX3. In each case, 10 g of cells were resuspended in 50 mL buffer A (20 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 5 mM ß-mercaptoethanol, pH 8.0) and lysed with an EmulsiFlex®-C3 system (Avestin). After centrifugation (28,000 g, 30 min, 4 °C), the supernatant was loaded onto a 5 mL HisTrap HP column (GE Healthcare). Protein was eluted using a linear gradient of buffer B (20 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, 5 mM ß-mercaptoethanol, pH 8.0). Cleavage was carried out in a membrane tube (Spectra/Per®, Spectramulabs) overnight at 4 °C with 1 mg TEV / 40 mg of protein while dialyzing against 1 L buffer A. A second Ni2+-column removed residual uncleaved protein. Cleaved protein was then concentrated and
applied to a Superdex™ 200 10/300 (GE Healthcare) size exclusion column using buffer C (50 mM Tris, 200 mM NaCl, 0.5 mM Tris-(2-carboxyethyl)phosphine, pH 8.0). Purity and homogeneity of the proteins were confirmed by SDS-PAGE, native PAGE, and dynamic light scattering. Protein folding was analyzed with circular dichroism spectroscopy using a JASCO J-720 spectrophotometer. Protein concentrations were determined by measurements of absorption at 280 nm with a NanoDrop ND-1000 (PeqLab).

**Peptide synthesis**– PEX19-derived peptides were prepared using solid-phase synthesis based on the Fluoren-9-ylmethoxycarbonyl (Fmoc) strategy on a SyroII synthesizer (MultiSynTech, Witten, Germany) as described (36). Peptides were purified by HPLC using a C18 column, resulting in a purity of 95 %. Purity and identity of the products were confirmed by analytical HPLC, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS), and electrospray ionization mass spectrometry (ESI-MS).

**Affinity measurements using isothermal titration calorimetry (ITC)**– Affinity measurements for PEX326-373(C235S) with full-length PEX19 were carried out in buffer C at 25 °C with a VP-ITC system (Microcal). Purified PEX326-373(C235S) was present in 8 µM concentration, PEX19 was injected stepwise in 92 µM concentration. For binding studies between sPEX3 and different PEX19-derived peptides an ITC200 system (Microcal) was used. The protein was present in 17 µM concentration while the peptide fragments were injected stepwise (1 µL/s) in a 5-10 fold higher concentration. ITC experiments with PEX19-derived peptides were performed at 25 °C in buffer D (10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, 2.7 mM KCl, 0.5 mM Tris-(2-carboxyethyl)phosphine, pH 7.4). Binding isotherms were integrated and analyzed using Origin® 7 software supplied with the instrument according to a “one binding site” model.

**Crystallization and structure determination**– Purified sPEX3 at 2.5 mg/mL in buffer C was co-crystallized with PEX1995 (residues 14-33) in a molar 1:1 ratio using the hanging drop vapor diffusion method at 20 °C. The final crystallization condition was optimized to 10 mM Bis-Tris pH 5.6, 24 % (w/v) PEG 3350, 200 mM NaCl. Crystals appeared within 24 hours and were flash frozen in liquid nitrogen without adding cryoprotectant. X-ray diffraction data were collected at beamline BL14.1 (BESSY, Berlin, Germany). They belong to space group P2₁, and contain 1 complex in the asymmetric unit. Data were processed and scaled using the XDS package (37). Molecular replacement was carried out with PHASER (38, 39) using a highly twinned structure of PEX326-373(C235S) as the search model (40). This produced a clear solution, and unambiguous difference electron density for the bound PEX19 peptide (supplemental Fig. S3) and regions of sPEX3 that had not been included in the search model. Model building and refinement were carried out using COOT (41) and Refmac5 (38, 42), respectively. TLS (translation/libration/screw) groups suggested by the TLS-motion determination server (43) were employed in the refinement process. According to stereochemical analysis within COOT, 96.9 % of residues are located in favorable regions of the Ramachandran plot, whereas 3.1 % are located in allowed regions. Data collection and refinement statistics are given in Table 1. Buried surface areas were calculated using CCP4 programs (38). Electrostatic surface potentials were computed with APBS (44) in Pymol. The simulated annealing omit map was calculated in Phenix (45). The stereoview of the overall fold was displayed using Molscript (46). Coordinates and structure factors have been deposited with the RCSB Protein Data Bank (accession code 3MK4).

**RESULTS**

A PEX19-derived peptide binds sPEX3 with high affinity– The interaction of the two peroxins PEX3 and PEX19 is required for the import of all peroxisomal membrane proteins. In order to define the structure-function relationships that underlie this interaction, we expressed and purified a soluble version of human PEX3 that comprises most of its predicted cytosolic domain (sPEX3) as well as a slightly longer version starting at residue 26 (PEX326-373). A solvent-exposed cysteine at position 235 was mutated to serine in both proteins to prevent non-native oxidation. Earlier crystallization trials of the larger version of PEX3 in complex with full-length PEX19 failed. We therefore pursued co-crystallization experiments of sPEX3 with a PEX19-derived peptide that spans residues...
4-33 (PEX19\textsuperscript{op}). The design was based on a predicted α-helix in the proposed PEX3-binding region (30) that is located within the first 56 amino acid residues of PEX19 (29).

Affinity measurements using isothermal titration calorimetry (ITC) demonstrate that sPEX3 binds PEX19\textsuperscript{op} with high affinity ($K_d = 330$ nM, Fig. 1). The affinity of PEX3\textsuperscript{26-37}(C235S) for full-length PEX19 is still higher ($K_d = 10$ nM, Fig. 1). The molar ratio was calculated to be 1:1 for both complexes (supplemental Table S1). A comparison of the ITC data shows that the enthalpy for binding PEX19\textsuperscript{op} is 70% of the enthalpy released upon binding full-length PEX19 (supplemental Table S1). This indicates that most of the specificity of the PEX3-PEX19 interaction can be attributed to contacts with PEX19\textsuperscript{op}.

**Overall structure of sPEX3 in complex with PEX19\textsuperscript{op}**—The structure of the sPEX3-PEX19\textsuperscript{op} complex was determined by molecular replacement at 2.42 Å resolution (Table 1 and Experimental procedures). As search model, we used the core structure of the previously obtained, unrefined model of PEX3 comprising residues 26 to 373 (40). This unliganded structure could be solved to a resolution of 3.3 Å with phases obtained from multiple anomalous diffraction data using selenomethionine-derivatized crystals, but it could not be refined due to highly twinned X-ray data (twinning factor: 0.48). However, components of this model led to a unique solution for the structure presented here. Unambiguous difference electron density for the bound PEX19 peptide and regions of sPEX3 that had not been included in the search model provide confidence for the obtained solution. The cysteine residue at position 235 is located in a recessed, partially solvent-exposed area and would not be able to form intra- or intermolecular disulfide bridges in the wild type protein. Therefore, its mutation to serine is unlikely to alter the sPEX3 structure. The final structure has excellent geometry and agrees very well with the experimental data ($R_{	ext{free}} = 23.4\%$, Table 1).

sPEX3 adopts a new fold that is composed of ten α-helices and one short 3\textsubscript{10}-helix (Fig. 2 and supplemental Fig. S1). Searches for structural homologs with the programs DALI (47) and GANGSTA+ (48) revealed no significant matches. Although portions of several proteins that contain multiple parallel helices can be aligned with the sPEX3 structure, the overall folds of these proteins clearly differ from the sPEX3 fold, confirming that sPEX3 exhibits a new fold.

The long α3-helix forms the core of the structure, and the remaining helices are arranged circularly around it in five segments (Fig. 2A). As all helical axes are aligned in roughly the same direction, the structure can be described as a large helical bundle (Fig. 2B). The bundle is about 80 Å tall and 30 Å wide. Interfaces between the helices are mostly hydrophobic and devoid of water molecules, providing stability to the bundle. In contrast, the exterior of the bundle is predominantly hydrophilic. The C-terminal region of sPEX3 is oriented towards the N-terminus, and would thus face the peroxisomal membrane. Several of the helices in the bundle are kinked due to insertions or the presence of residues such as proline, which are not compatible with a regular helical structure. These kinks are probably needed in order to allow for tight packing between helices. Although the helical bundle is rigid and well defined by electron density, the positions of residues in several loop regions could not be determined due to their high mobility. Regions missing from the model are: Ala146-Gly151, Lys220-Pro232, Pro245-Gly252, Pro301-Ser317, and Gln369-Lys373 at the C-terminus. None of these regions are close to the site of interaction with PEX19\textsuperscript{op}.

Residues 14-30 of PEX19\textsuperscript{op} are well defined by electron density and permitted accurate model building. The C-terminal three amino acids (Lys31-Lys33) are not visible in the electron density maps and are not included in the model. The N-terminus of the peptide is involved in crystal contacts that are distant from the PEX3 binding groove. PEX19\textsuperscript{op} forms a single amphipathic α-helix that binds into a groove at the top of the helical bundle, opposite the N-terminus of sPEX3 (Figs. 2A and 2B). One side of the groove is shaped by helices α2 and α3, while the other side is formed by the loop that connects helices α4 and α5, and by a portion of helix α8.

The PEX19\textsuperscript{op}-binding site reveals three distinct interaction regions within sPEX3—The contact interface is formed by surfaces in sPEX3 and PEX19\textsuperscript{op} that are complementary in shape, producing an uninterrupted, contiguous interface that is devoid of water molecules and gaps and
buries a total area of 580 Å² from solvent. To facilitate the discussion of interactions, we have divided this interface into three contact areas along the sPEX3-binding groove (Fig. 3). The first and largest contact area (Fig. 3B and supplemental Fig. S3) is formed by sPEX3 residues located at the C-terminal end of helix α2 and the N-terminal region of helix α3. This helix-loop-helix motif of sPEX3 packs tightly against the C-terminal end of the PEX19pop helix. Contacts are predominantly hydrophobic and account for 42% of the total surface buried in the complex. A key interaction is centered at sPEX3 residue Trp104, which is strictly conserved in all eukaryotic organisms (Fig. 4 and supplemental Fig. S4A) and was previously shown to be crucial for the interaction with PEX19 (49). Trp104 inserts into a pocket formed by four PEX19pop residues: Leu22, Ala25, Leu26 and Phe29. Additional hydrophobic contacts involving sPEX3 residues Thr90, Leu93, Lys94, Lys100, Leu101 and Leu107 also contribute to the interaction.

The second contact region is formed at the loop that connects helices α4 and α5 of sPEX3 (Fig. 3A) and accounts for 21% of the total buried surface area. Contacts in this region involve the side chains of Lys197 and Leu196 of sPEX3, which protrude from this loop and engage residues close to the N-terminus of PEX19pop. Lys197 forms salt bridges with Asp15 and Glu17 of the peptide, as well as hydrophobic interactions with Leu18. Leu196 packs against Leu18, Leu21 and Leu22, which all project from the same face of the PEX19pop helix.

The third contact region, which buries 37% of the total contact area, is located at the N-terminus of helix α8 and the loop that precedes this helix (Fig. 3C). At the center of this region lies sPEX3 residue Lys324, which forms salt bridges and hydrogen bonds with Asp28 and Ser24, respectively, of PEX19pop. The remaining contacts in this region are exclusively hydrophobic and involve two proline residues of sPEX3. Pro321 faces towards peptide residues Leu21 and Leu22, whereas the ring of Pro327 packs tightly against the Phe29 side chain of the peptide. Located near the C-terminus of PEX19pop, Phe29 also interacts with the side chains of Ile326 and Asn330. Additional contacts are formed with the methyl group of Ala323, which faces towards Leu21, Leu22 and Ala25 of PEX19pop.

**Mutagenesis of contact residues**—In order to analyze the impact of mutations in PEX19pop on sPEX3 binding, structure-guided mutagenesis experiments were performed. Select PEX19pop amino acids that were judged to be critical for the interaction with sPEX3 were mutated and the interactions between the peptide and sPEX3 were analyzed with ITC. Amino acid Phe29, which contacts several sPEX3 residues via its hydrophobic side chain (Figs. 3B and 3C), was replaced with alanine. This F29A mutation completely abolished binding to sPEX3 (Fig. 1 and supplemental Table S1). The presence of a phenylalanine at position 29 is therefore critical for PEX3-PEX19 complex formation, consistent with the strict conservation of this residue among the PEX19 sequences of eukaryotic species (Fig. 4 and supplemental Fig. S4B). Secondly, PEX19pop residue Ala25 was mutated to leucine as well as tyrosine. The methyl group of Ala25 interacts with hydrophobic residues of sPEX3 (Figs. 3B and 3C). Inspection of the structure indicated that a leucine side chain might be able to form similar contacts, whereas a larger tyrosine side chain would not be tolerated. Consistent with this prediction, the $K_D$-value for the A25L mutation is 410 nM, which is only slightly lower than the affinity of wild type PEX19pop. We note that a leucine is present at this position in some yeast PEX19 proteins. Not surprisingly, replacement of Ala25 with tyrosine completely disrupted the interaction. The mutational data are summarized in supplemental table S1.

**Analysis of surface features and conservation**—In order to identify key surface features of sPEX3 and PEX19pop, we compared the sequences of both proteins from several organisms (supplemental Fig. S4) and displayed conserved surface residues as depicted in figure 4. This analysis reveals that all residues lining the PEX19pop-binding groove at the membrane-distal end of sPEX3 are well conserved (Fig. 4A). The structure of the complex identifies two key residues of sPEX3 that are involved in central contacts, Trp104 and Lys324. Not surprisingly, these two residues exhibit the highest degree of conservation. A similar analysis of PEX19pop residues shows that Ala25, Leu26, Asp28 and Phe29 are most highly conserved (Fig. 4B). Again, these residues all play central roles in complex formation (Fig. 3 and supplemental Table S1). An interesting
finding is that the C-terminal region of PEX19 \( ^{\text{PEP}} \) (residues 25-29) is more conserved than residues that lie closer to the N-terminus of the peptide (residues 18-24). This indicates that contacts formed by the C-terminal portion of PEX19 \( ^{\text{PEP}} \), and especially residues Leu26 and Phe29, are most important for a productive interaction and for conferring specificity.

While most of the remaining sPEX3 surface is quite variable, our analysis reveals a second cluster of highly conserved residues at the base of the molecule, near the N-terminal helix \( \alpha_1 \). This cluster includes an unusual number of surface-exposed large, hydrophobic residues (Ile49, Met67, Met72, Ile135, Ile140). In vivo, this region would be located close to the peroxisomal membrane. It is tempting to speculate that it forms the site of interaction with a second region of PEX19, or with other peroxisomal proteins.

Analysis of surface charges of sPEX3 (supplemental Fig. S2A) reveals a polar surface with many small positively and negatively charged patches, and two noticeably larger ones. The PEX19 \( ^{\text{PEP}} \)-binding groove exhibits a strong positive potential, consistent with several basic residues (Lys94, Lys100, Lys197 and Lys324) that line the groove and participate in PEX19 \( ^{\text{PEP}} \)-binding. In contrast, the PEX19 derived peptide exhibits a highly negative surface potential due to exposed aspartic and glutamic acids (supplemental Fig. S2B). A less conserved region at the base of sPEX3 exhibits a strong negative potential, which is due to an accumulation of acidic residues (Asp257, Glu266, Asp269, Glu272 and Asp275). Among these residues, Glu266, Glu272 and Asp275 are highly conserved in all eukaryotic species except yeasts. These features may indicate functional interactions of these residues with other proteins during peroxisomal biogenesis.

**DISCUSSION**

PEX3 has been reported to participate in different processes during peroxisomal biogenesis. In this study, we sought to define its structural features, as well as the specificity of its interaction with PEX19. Experiments performed in several laboratories have identified the N-terminal 56 amino acids of PEX19 as necessary and sufficient for the interaction with PEX3 and thus for docking PEX19 to the peroxisomal membrane (26, 29-31). Based on these studies, we have designed and synthesized a peptide that comprises the central part of this region of human PEX19, and determined the structure of the cytosolic domain of human PEX3 in complex with this peptide. sPEX3 folds into an elongated helical bundle that has no known structural homologs. Structural and functional analyses identified a single groove in sPEX3 as a high-affinity binding site for PEX19. This region is highly conserved across eukaryotic species. Additional regions of surface conservation of sPEX3 indicate contact points for other molecules involved in peroxisomal biogenesis.

Several observations support our finding that the described binding region identified within PEX19 is indeed the main interaction surface with PEX3. Although the sPEX3-PEX19 \( ^{\text{PEP}} \) interaction buries only a comparatively small surface area of 580 Å\(^2\), ITC measurements yield a high affinity of 330 nM for the sPEX3-PEX19 \( ^{\text{PEP}} \) complex. The \( K_d \)-value for the interaction between PEX3 \( ^{26-373} \) (C235S) and PEX19 is 10 nM, indicating a 33-fold higher affinity for full-length PEX19. This value is consistent with previous surface plasmon resonance studies resulting in a \( K_d \)-value in the low nanomolar range (3.4 nM) and a 1:1 molar ratio between PEX3 and PEX19 (49). The high affinity for PEX19 \( ^{\text{PEP}} \) can be attributed to an interacting surface that is complementary in shape, devoid of water molecules, and predominantly hydrophobic. The higher affinity for full-length PEX19 could be due to additional residues within the PEX19 N-terminus, which were not included in the synthesized peptide, but contribute to binding at the identified interaction site. However, since both termini of the PEX19 \( ^{\text{PEP}} \) helix point away from sPEX3 and since the sPEX3 structure lacks additional conserved residues at the top of the bundle, we consider this possibility unlikely. The observation that the enthalpy for PEX19 \( ^{\text{PEP}} \) binding is 70 % of the enthalpy for the full-length protein indicates that PEX19 \( ^{\text{PEP}} \) contributes significantly to the interaction. However, the difference in \( K_d \) also suggests that other residues of the full-length PEX19 protein are likely involved in contact formation. The conserved region at the base of sPEX3 is a possible candidate for additional interactions with full-length PEX19. A second binding site
within residues 124-140 of PEX19 has been proposed previously (30). However, this region is unlikely to interact with PEX3, as our ITC measurements clearly show that a PEX19-derived peptide corresponding to these amino acids does not bind sPEX3 (supplemental Table S1). The functional relevance of the observed interaction between sPEX3 and PEX19 is further supported by the presence of highly conserved residues in the peptide-binding groove. A conserved region including PEX3 residues Lys100 to Arg114 was predicted to contribute to PEX19 binding (49). Our structure shows that many of these residues indeed form key contacts with PEX19. Residues Lys100, Leu101, Trp104 and Leu107 are all part of the largest binding region (Fig. 3B). Trp104 can even be considered a central residue as it interacts with several hydrophobic PEX19 side chains, including Phe29. Furthermore, the tryptophan helps to orient side chains of additional sPEX3 residues (Leu93, Lys100) to form contacts with PEX19. Consistent with the central role of Trp104 in complex formation, it was reported that its replacement with alanine reduces the affinity of PEX3 for PEX19 significantly. Moreover, the W104A mutant of PEX3 was unable to restore peroxisomes in a PEX3-deficient cell line (49). Our surface analysis reveals a mostly hydrophilic surface, and strongly argues against the insertion of PEX3 into the peroxisomal membrane. Instead, our structure supports a model in which PEX3 is anchored to peroxisomes with a single sequence located at the N-terminus, but has no other direct contact with the lipid bilayer. An earlier study reported an interaction of the cytosolic domain of PEX3 with membrane lipids, based on the observation that PEX3 forms high molecular mass aggregates in the presence of mild detergents (50). This lipid binding property was assigned to hydrophobic residues in several predicted amphipathic helices. However, our structure shows that these hydrophobic residues are in fact part of the solvent-inaccessible core of the protein, and are thus not available for interactions with lipids. As the lipid binding activity can be abolished by the addition of recombinant PEX19, a competing interaction between PEX3 and lipids on one side and PEX3 on the other side has been suggested (50). We note that the borders of the PEX19-binding groove, as well as a region just beyond the groove, exhibit a basic character due to the presence of highly conserved positively charged amino acids (Arg96, Lys100, and Lys324) (Fig. 4 and supplemental Fig. S4). Thus, we cannot rule out the possibility that these three residues form favorable charge-charge interactions with membrane phospholipids, which are abolished once PEX19 engages PEX3 with high affinity. Although such interactions with phospholipids explain the observed lipid binding capacity of the cytosolic domain of PEX3 in vitro, they are not likely to be relevant under physiological conditions. It is known that PEX19 is recycled back to the cytosol after cargo release into the peroxisomal membrane (32). Thus, the high-affinity interaction between PEX3 and PEX19 must be disrupted in order to start a new insertion cycle. The structure of the complex...
does not offer any plausible scenarios for how this might occur. Subtle changes in pH are
known to drive the association and dissociation of other large complexes. However, as the
sPEX3-PEX19Pep interface is largely hydrophobic, changes in pH are unlikely to
affect its stability. Instead, we consider it more likely that other peroxins might play a role in
this process.

Our results can serve as a model of the general mechanism of peroxisomal membrane
protein import. Membrane insertion of PMPs has to involve the recruitment of PMP-loaded
PEX19 to the peroxisome, followed by positioning of the PMP directly adjacent to the
membrane and its subsequent insertion. While the PEX3-PEX19 complex formation is clearly
explained by the structure presented here, the membrane-positioning and -insertion of PMPs
remain speculative. It is known that PEX19 is composed of a flexible N-terminal and a
compact, farnesylated C-terminal domain (51), with the farnesylation being crucial for correct
PMP targeting to peroxisomes (52). It is likely that upon PEX19 binding to PEX3, the cargo-
loaded C-terminus of PEX19 is oriented close towards the peroxisomal membrane. This could
either involve additional interactions with PEX3,

perhaps mediated by the farnesy group, or a
direct insertion of the prenyl anchor into the
peroxisomal membrane. The mechanism of PMP
insertion could also involve PEX16. The release
of cargo from PEX19 could be triggered by a
conformational change in PEX19, or by binding
of PMPs to PEX3. This might be sufficient for
dissociation of the PEX3-PEX19 complex. One
other possibility is that the affinity for PEX3 is
reduced once PEX19 has unloaded its cargo. In
support of this, it has been shown that the
affinity of PEX3 for PEX19 carrying
GFP-PMP24 is higher than for cargo-free
PEX19 (27). Thus, cargo-loaded PEX19 could
displace cargo-free PEX19 from PEX3 at the
peroxisomal membrane to initiate a new
insertion cycle.

In conclusion, our structure provides one of
the first views of a complex between two
peroxins at high resolution. It reveals essential
structural features of sPEX3, and establishes a
platform for understanding the parameters that
guide its interactions with PEX19. Moreover,
surface conservation analysis of sPEX3 provides
a basis for potential interaction with other
molecules and for the general role of PEX3 in
peroxisomal membrane biogenesis.

REFERENCES


FOOTNOTES

Coordinates and structure factors have been deposited with the RCSB Protein Data Bank (accession code 3MK4).

The abbreviations used are: PEX, peroxisomal biogenesis factor; ER, endoplasmic reticulum; COP, coat protein; PMP, peroxisomal membrane protein; ITC, isothermal titration calorimetry; TEV, tobacco etch virus; IPTG, isopropyl-beta-thiogalactopyranoside.

ACKNOWLEDGMENTS

The authors would like to thank the beamline staff of BESSY (Berlin, Germany) for support during data collection. The structure determination of PEX3 was initiated in the laboratory of Prof. Dr. Georg E. Schulz (University of Freiburg, Germany). ITC measurements of PEX3 with full-length PEX19 were carried out by Dr. Carsten Kintscher at the Max-Planck Institute for Developmental Biology in Tübingen. H.K. acknowledges support from SFB 685.

FIGURE LEGENDS

FIGURE 1. ITC affinity measurements of complex formation between PEX3 and PEX19. Single experiments were carried out at 25 °C. Binding data for A, PEX326-373(C235S) with full-length PEX19, B, sPEX3 with PEX19\(^{\text{apo}}\) and C, sPEX3 with Pex19\(^{\text{apo}}\) F29A. D, Integrated heat values for the different ITC experiments. PEX326-373(C235S) and full-length PEX19 (closed circles), sPEX3 and PEX19\(^{\text{apo}}\) (closed squares), sPEX3 and Pex19\(^{\text{apo}}\) F29A (open squares), see also Table S1.

FIGURE 2. Overall structure of sPEX3 (green) in complex with PEX19\(^{\text{apo}}\) (orange). sPEX3 folds into an all-helical bundle, with one central helix \(\alpha_3\) surrounded by nine \(\alpha\)-helices and a short \(3_{10}\)-helix. The N-terminus of helix \(\alpha_1\) faces towards the peroxisomal membrane. Helices were assigned with DSSP (53) and numbered sequentially. A Top view of the sPEX3-PEX19\(^{\text{apo}}\) complex. B
Side view of the sPEX3-PEX19<sup>pp</sup> complex. Representation in A is rotated 90 degrees around a horizontal axis. C Topology of sPEX3. Helices 1-10 are represented as cylinders. The central helix α3 is surrounded by five helical segments (α1+α2, α4, α5+3<sub>10</sub>, α6+α7, α8+α9+α10) arranged in nearly parallel fashion. Regions not included in the structure are shown as dashed lines. D Schematic view of the secondary structure elements of sPEX3. Residues involved in PEX19<sup>pp</sup> binding are highlighted in bold, and residues not present in the crystal structure are shown in italics. The cysteine-serine mutation at position 235 is boxed.

FIGURE 3. Interactions between sPEX3 (green) and PEX19<sup>pp</sup> (orange). A-C, The magnified regions show details of the three major contact areas. Labels in regular font correspond to sPEX3, labels in italics correspond to PEX19<sup>pp</sup>.

FIGURE 4. Surface distribution of conserved amino acid residues. A, Overall side views of surface representation of sPEX3 differing by 180 degrees along a vertical axis. PEX19<sup>pp</sup> is shown as an orange ribbon. B, Magnified view of the binding groove. PEX19<sup>pp</sup> is displayed as ribbon. Alignments were performed with ClustalW (54) and displayed in Jalview (55). Residues are colored according to the conservation score (supplemental Fig. S4) indicated at the lower right. Residues with a conservation score ≥ 9 (9, 10*, 11*) are displayed in the same color. Residues conserved in sPEX3 are colored in shades of green, residues conserved in PEX19<sup>pp</sup> are colored in shades of orange.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
</tr>
<tr>
<td>Beamline</td>
<td>BL14.1, BESSY</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.91841</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>38.48, 65.68, 61.59</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90.0, 91.52, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>25 – 2.42 (2.48 – 2.42) *</td>
</tr>
<tr>
<td>R&lt;sub&gt;meas&lt;/sub&gt;</td>
<td>5.9 (55.9)</td>
</tr>
<tr>
<td>I / σI</td>
<td>20.2 (3.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.8 (99.8)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.7 (3.7)</td>
</tr>
<tr>
<td>Wilson B (Å²)</td>
<td>46.7</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>25 – 2.42</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>11779 (848)</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt; / R&lt;sub&gt;free&lt;/sub&gt;</td>
<td>0.194/0.234 (0.289/0.290)</td>
</tr>
<tr>
<td>No. atoms</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>2249</td>
</tr>
<tr>
<td>Peptide</td>
<td>122</td>
</tr>
<tr>
<td>Water</td>
<td>67</td>
</tr>
<tr>
<td>B-factors (Å²)</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>39.2</td>
</tr>
<tr>
<td>Peptide</td>
<td>58.7</td>
</tr>
<tr>
<td>Water</td>
<td>40.8</td>
</tr>
<tr>
<td>R.m.s. deviations</td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.01</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.08</td>
</tr>
</tbody>
</table>

*Values in parentheses are for highest-resolution shell.
Figure 1
Figure 2

A

B

C

D

41 QEREAAYIAQARRQYHFESNQRTCNMTVLSMLPTREALMQQLNSESLLTTLLKNRFSNKLEIWEELKIISFRSTTVAYSTVCMYSLVLLR

α1 α2 α3

131 VOLNNIGGYIYLDNAAVGHHRNTTILAPDFQQQYILSSIQHLGGLTGELITLVQKAQQKVLTGSLVLGSVSLNLKHSLSLLDDLEQKLIKIEIRNLRVQHK

α4 α5

221 SSSWINKGSMFLILHMYMPDEETFLAVQQACGLSPRDITKLLNETROMLSPFDSTVLNCTLNRGSRLLDNMAERHRPETQODLQHGN

α6 α7

311 SMNSLSVSLAKIPIVQGHSVCTEPTSHFQOQDLLTMEQVDFANVYEAFASTPOQLEK 373

α8 α9 α10
Insights into peroxisome function from the structure of PEX3 in complex with a soluble fragment of PEX19
Friederike Schmidt, Nora Treiber, Georg Zocker, Sasa Bjelic, Michel O. Steinmetz, Hubert Kalbacher, Thilo Stehle and Gabriele Dodt

J. Biol. Chem. published online June 16, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.138503

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/06/16/M110.138503.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2010/06/16/jbc.M110.138503.full.html#ref-list-1