Nucleotide-dependent assembly of the peroxisomal receptor export complex

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Pex1p and Pex6p are two AAA-ATPases required for biogenesis of peroxisomes. Both proteins form a hetero-hexameric complex in an ATP-dependent manner, which has a dual localization in the cytosol and at the peroxisomal membrane. At the peroxisomal membrane, the complex is responsible for the release of the import receptor Pex5p at the end of the matrix protein import cycle. In this study, we analyzed the recruitment of the AAA-complex to its anchor protein Pex15p at the peroxisomal membrane. We show that the AAA-complex is properly assembled even under ADP-conditions and is able to bind efficiently to Pex15p in vivo. We reconstituted binding of the Pex1/6p-complex to Pex15p in vitro and show that Pex6p mediates binding to the cytosolic part of Pex15p via a direct interaction. Analysis of the isolated complex revealed a stoichiometry of Pex1p/Pex6p/Pex15p of 3:3:3, indicating that each Pex6p molecule of the AAA-complex binds Pex15p. Binding of the AAA-complex to Pex15p in particular and to the import machinery in general is stabilized when ATP is bound to the second AAA-domain of Pex6p and its hydrolysis is prevented. The data indicate that receptor release in peroxisomal protein import is associated with a nucleotide-depending Pex1/6p-cycle of Pex15p-binding and release.

Peroxisomes are multifunctional dynamic organelles present in nearly all eukaryotic cells. A set of specific proteins, so called peroxins, are required for biogenesis of these organelles and the maintenance of their function. To date 34 peroxins are known, among them Pex1p and Pex6p, which both represent members of the AAA (ATPases associated with various cellular activities) protein family. In the early 90s, it was recognized that Pex1p, NSF (N-ethylmaleimide-sensitive factor) and p97/VCP (valosin-containing protein) share a highly conserved AAA domain. The AAA family today displays a group of at least 30,000 AAA-proteins found in all biological kingdoms. Pex1p as well as Pex6p belong to the group of classical type II AAA-ATPases, containing two AAA-domains, termed D1 and D2, located downstream to N-terminal domains (NTDs). First described in Pichia pastoris, the interaction of Pex1p and Pex6p was confirmed in other organisms like Homo sapiens, Hansenula polymorpha, and Saccharomyces cerevisiae. Studies on S. cerevisiae Pex1p and Pex6p revealed that ATP-binding to the second AAA-domain of Pex1p is essential for the interaction of both peroxins and the formation of a hetero-hexameric complex.

It is well established that Pex1p as well as Pex6p shows a dual localization in the cell, as they are located in the cytosol as well as at the peroxisomal membrane. Thereby, organellar association is facilitated by binding of the N-terminal region of Pex6p to the cytosolic domain of the tail-anchored membrane protein Pex15p. ATP-binding as well as ATP-hydrolysis at Pex6p D2 is crucial for the release of the AAA-complex from the peroxisomal membrane as corresponding

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mutations led to an increased association with peroxisomes\textsuperscript{19}. The data indicate that the assembly and disassembly of the AAA-complex as well as its Pex15p-mediated association and release from the membrane are dynamic processes possibly regulated by the nucleotide binding state of the proteins.

In order to gain more insight into this process, we analyzed membrane-attached peroxisomal AAA-complexes at different nucleotide states. Pex1/6p membrane complexes including a Walker B mutation in Pex6p resulted in an enhanced binding to its membrane anchor Pex15p, stronger interactions to different components of the import machinery and accumulation of polyubiquitinated Pex5p. A corresponding Walker B mutation in Pex1p revealed no such effects. We compared these \textit{in vivo} data with experiments using bacterial expressed proteins and reconstructed the binding of the Pex1/6p-complex to Pex15p \textit{in vitro}. Pex6p mediates the binding of the AAA-complex to the cytosolic part of Pex15p via a direct interaction and independent of other proteins. Moreover, our data clarify the direct correlation between ATP hydrolysis in Pex6p and the dissociation of the Pex1/6p-complex from Pex15p.

Based on these observations and considering the bipartite localization of the AAA-complex, we conclude that action of Pex1/6p at the peroxisomal import machinery is linked to an association and releasing cycle between the cytosol and the peroxisomal membrane.

**Results**

**Presence of ADP or ATP enables formation of the recombinant AAA-complex.** The AAA-peroxins Pex1p and Pex6p form a heterohexameric complex required in the late step of the peroxisomal matrix protein import. Both peroxins are characterized by the presence of two AAA-domains, termed D1 and D2, downstream to N-terminal domains (NTDs) (Fig. 1a). Two-hybrid studies with the yeast AAA-peroxins indicated that ATP-binding to the D2 AAA-domain of Pex1p is a prerequisite for its interaction with Pex6p\textsuperscript{17}. In line with this assumption, we recently demonstrated that the recombinant Pex1/6p-complex is formed only in the presence of ATP, whereas ATP-depletion results in the disassembly of the heterohexameric core complex into smaller subcomplexes\textsuperscript{18}. Since the AAA-complex performs its ATPase activity most likely at the peroxisomal membrane to drive the export of PTS-receptors, we addressed the question of how the complex is assembled when ATP is hydrolysed to ADP. To this end, recombinant yeast Pex1p and Pex6p both with an N-terminal hexahistidyl-tag (His6) and Pex1p in addition with a C-terminal glutathione-S-transferase (GST) tag were expressed separately in \textit{E. coli}. The cells were mixed and lysed and both AAA-peroxins were co-purified by affinity chromatography in the presence of either ATP or ADP. First, the His-tagged AAA-proteins were purified together by affinity chromatography with nickel-NTA-agarose (NiNTA). Subsequently, the GST-tagged AAA-complex was subjected to affinity chromatography on GSH-agarose. The bound AAA-complex was released by thrombin cleavage with the GST-tag remaining on the column, resulting in Pex1p/Pex6p complexes with high purity. For estimation of the rate of complex-formation, the recombinant AAA-complex was further subjected to size exclusion chromatography.
Figure 2. Nucleotide-dependent association of the AAA-complex to Pex15p. Protein complexes were isolated from 1% digitonin-solubilized membranes of wild-type (control) and wild-type cells expressing Pex15pTPA via IgG-Sepharose and subsequent TEV protease cleavage. Complex isolation was performed in the absence or presence of 5 mM ATP and ADP as indicated. Equal portion of solubilisate (1.2% of total) and eluate (20% of total) were subjected to SDS-PAGE and immunoblot analysis and probed with specific antibodies as indicated. Pex1p and Pex6p were part of the Pex15p complex when ADP or ATP was present, whereas a significant loss of binding to Pex15p was observed in the absence of nucleotides.

Nucleotide dependent in vivo binding of the AAA-complex to Pex15p. The Pex1p/6p-complex is localized in the cytosol and it is found associated with the peroxisomal membrane. The recruitment to yeast peroxisomes is mediated by the tail-anchored peroxisomal membrane protein Pex15p via binding of the N-terminal domain of Pex6p19. We addressed the question whether the nucleotide state affects the Pex15p binding in vivo as it was observed in vitro for the AAA-complex assembly. To this end, a wild-type strain expressing Pex15p genomically tagged with protein A (Pex15pTPA), which harbors a tobacco etch virus (TEV) cleavage site between both fusion-partners was used23. The Pex15pTPA complex was isolated after 1% digitonin extraction from total membranes by IgG-Sepharose affinity chromatography in the absence or presence of 5 mM ATP and ADP. Obtained TEV protease eluates were subjected to SDS-PAGE and analyzed by immunoblotting. None of the analyzed proteins was isolated from the untransformed wild-type strain, which served as control for the specificity of the isolation procedure (Fig. 2). Pex15p was isolated via its TPA-tag to the same extent independent of the nucleotide state of the samples. Pex1p as well as Pex6p were co-purified with Pex15p in presence of ATP and slightly reduced (to approximately 70% of ATP-conditions as judged by signal intensity measurements) in presence of ADP. In clear contrast, the amount of AAA-peroxins in the eluate was drastically reduced in the absence of ATP or ADP (Fig. 2). This demonstrates that the presence of either ATP or ADP is required for efficient binding of the AAA-complex to Pex15p. Moreover, it seems that less efficient interaction takes place in the presence of ADP compared to ATP.

Impact of Walker B mutations on Pex1/6p membrane complexes. Since the Pex1/6p complex consistently hydrolyses ATP to ADP and phosphate, we do not know the exact nucleotide state of the isolated complex in presence of ATP. To fix the complex in the ATP-bound state, we analyzed Walker B mutants of the membrane attached Pex1/6p complex. Recently, we showed that Pex1p and Pex6p contribute to the overall ATP hydrolysis rate of the AAA-complex in an unsymmetrical manner. While a mutation of the conserved glutamate in the Walker B motif of Pex1p (PEX1WBE798Q) resulted in a slight decrease of the ATPase activity, a corresponding mutation in Pex6p (PEX6WBE832Q) abolished hydrolysis activity completely11. In line with this observation, yeast strains harboring the Pex6p Walker B mutation PEX6WBE832Q do not grow on media with oleate as sole carbon source, whereas Pex1p Walker B mutants PEX1WB798Q are able to utilize oleic acid11. Interestingly, a mutation of the adjacent conserved aspartate in the Pex1p Walker B motif PEX1WB797Q showed a clear growth defect on oleic acid medium (Supplementary Fig. 1a). These data indicate that the glutamate E798 and thus ATP-hydrolysis at the conserved AAA-domain is not essential for Pex1 function, while mutation of the adjacent aspartate D797 is essential for the function of the protein. To investigate the reason for the different functional activity of the Pex1p mutants, we purified recombinant Pex1/6p, Pex1WB797Q/6p and Pex1WBE798Q/6p complexes by tandem affinity chromatography and size exclusion chromatography and tested the stability of the heterohexameric complexes after incubation at 37°C by a second gel filtration. Whereas, wild-type Pex1/6p and Pex1WBE798Q/6p complexes remained in a hexameric conformation, Pex1WB797Q/6p rapidly dissociates into typical pattern of trimeric Pex1p and monomeric Pex6p (Supplementary Fig. 1b). From this we conclude that mutation of the aspartate D797N has a negative effect not only on ATP hydrolysis, but also on the AAA complex stability, probably by interfering with a proper nucleotide binding in Pex1p D2. Thus, the observed phenotype of Pex1pWB797Q cells on oleic acid is not attributed to a defect of ATP hydrolysis in Pex1p, but to a defect in AAA

Figure 2:

- **WT**
- **ATP**
- **ATP**
- **ADP**

**Solubilisate**

- α Pex1p
- α Pex6p

**Eluate**

- α Pex1p
- α Pex6p

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complex formation. Therefore, we decided to use the Pex1pWBE798Q variant for further experiments. Exchange of this conserved glutamate to glutamine was shown for Pex6p11,22, and for many other AAA-proteins to inhibit ATP hydrolysis but not ATP binding8.

To investigate the effect of ATP hydrolysis of Pex1p and Pex6p on the formation and stability of AAA membrane complexes, we complemented genomically modified Δpex1Pex6pTPA and Δpex6Pex1pTPA strains with plasmids coding for PEX1 and PEX1WB E798Q or PEX6 and PEX6WB E832Q, respectively, under control of their own promoters. AAA-complexes were solubilized with 1% digitonin from total membrane pellets, isolated by IgG-Sepharose affinity chromatography in presence of 5 mM ATP and analyzed by SDS-PAGE and immunoblotting (Fig. 3). In all cases, eluates showed similar amounts of isolated Pex1p and Pex6p. The AAA-complexes were associated with its membrane anchor Pex15p and different members of the peroxisomal matrix protein import machinery, namely the PTS receptors Pex5p and Pex18p, the RING-finger complex represented by Pex12p and members of the importomer (Pex14p, Pex17p, Pex13p). In comparison to the wild-type situation (Δpex6Pex1pTPA + Pex6p, Δpex1Pex6pTPA + Pex1p), the Walker B mutation in Pex1p (Δpex1Pex6pTPA + Pex1pWBE798Q) did not show any detectable effect on membrane complex formation. In contrast, when Pex6p was mutated (Δpex6Pex1pTPA + Pex6pWBE832Q), a clear polyubiquitinylation pattern of accumulated Pex5p was discernible. This reflects the oleate growth defect and loss of ATPase activity of the Pex1/6p Walker B mutants11. Likewise, the increased amount of Pex18p in the solubilisate and complex eluate indicates that the WB mutation of Pex6p results in an import defect, which for Pex18p is known to result in its membrane association and decreased degradation24,25. Besides Pex5p and Pex18p, nearly all components of the import machinery exhibited an increased presence in the Pex1/6pWB complex when compared to wild type level. In particular, the abundance of the AAA-membrane anchor Pex15p was profoundly increased. Taken together, these results indicate that the
AAA-complex strongly associates to Pex15p when Pex6p is fixed in a constantly ATP bound state. Moreover, the loss of Pex1/6p ATPase activity blocks the export of the PTS receptors and stabilizes the interaction between the receptor import- and export-machinery.

Association of Pex15p with the recombinant AAA-complex. To gain more insight into the recruitment of the AAA-complex to and liberation from the peroxisomal membrane, we investigated the interaction of the recombinant Pex1/6p-complex with the cytosolic domain of Pex15p, comprising amino-acid residues 1 to 315 (Pex15p1-315). To this end, GST-Pex15p1-315His was expressed in *E. coli* and purified by GSH-agarose affinity chromatography. Glutathione, which was used to elute Pex15p1-315 from the affinity matrix, was removed by dialysis. Subsequently, equal portions of the obtained Pex15p1-315 were incubated with purified Pex1p and Pex6p or Pex1p/Pex6p-complex. The assays were again loaded on GSH-agarose columns and Pex15p-complexes were released by glutathione. Load and eluted fractions were subjected to SDS-PAGE and proteins were stained with Coomassie, the presence of Pex1p and Pex6p was analyzed by immunoblotting (Fig. 4a). The data show that a significant portion of Pex6p is retained on the column in the presence of GST-Pex15p, whereas Pex1p alone is not, indicating that isolated Pex6p but not Pex1p is capable to bind Pex15p directly. In the same experimental setup, the Pex1/6p complex is retained on the column, indicating that the AAA-complex is efficiently bound by Pex15p (Fig. 4a). The soluble AAA-complex is a heterohexamer formed by a trimer of Pex1/6p dimers. Equal portions of Pex1p and Pex6p bound to Pex15p, indicating that the Pex1/6p complex remains unchanged in composition, when recruited from the cytosol to the peroxisomal membrane and that no additional factors are required for its binding to Pex15p. Signal intensity measurements of Pex6p present in the load and in eluate fractions revealed striking increase when the Pex1/6p-complex was loaded in comparison to the single protein. This result indicates that the interaction of Pex15p and Pex6p is increased when Pex6p is in complex with Pex1p (Fig. 4a, lower panel).

To find out how many Pex15p molecules are associated with the Pex1/6p AAA-complex, we analyzed the composition of the purified complex of Pex15p and the AAA-peroxins in detail. The complex was isolated by affinity chromatography and by size exclusion chromatography. To exclude dimerization effects of GST-tagged Pex15p, the complex was released from the GSH-agarose by thrombin cleavage with the GST-tag remaining on the column. Gel filtration revealed that the complex of Pex1p, Pex6p and Pex15p has a size of about 700 kDa (Fig. 4b), whereas Pex15p alone was detected in fractions of about 100 kDa. A shift of the Pex1/6p-complex in size upon binding to Pex15p was not visible, due to the low resolution of the gel filtration column in this size area. However, when the eluted proteins were analyzed by immunoblotting with anti-His antibodies, which detects the remaining His-tag of all three proteins, HisPex1p, HisPex6p and Pex15pHis, it is evident that a portion of Pex15p shifted to the fractions of the AAA-complex. Similar intensities of the signals of the 700 kDa-complex indicate that the complex contains equal amounts of Pex1p, Pex6p and Pex15p. These data suggest that each Pex6p molecule is capable to bind one Pex15p, resulting in a 3:3:3 ratio of the Pex1p/Pex6p/Pex15p complex.

In order to verify the stoichiometry of the Pex1p/Pex6p/Pex15p complex, we performed the isolation procedure in higher quantity and analyzed the obtained complex on a preparative Superose6 column. Fractions of the size exclusion chromatography were analyzed by SDS-PAGE and Coomassie staining. As observed before, the AAA-complexes peaked in fractions of a size of about 700 kDa and contained a significant portion of co-migrating Pex15p1-315His (Fig. 4c, upper panel). In parallel, a complex with Walker B mutation in Pex6p was analyzed (Pex1/6pWB + Pex15p) (Fig. 4c, lower panel). Both wild-type and Pex1/6pWB/Pex15p complexes showed same elution profiles, although the amount of the mutated complex was substantially increased. Intensity measurements of Pex1p- Pex6p- and Pex15p- signals revealed a stoichiometric ratio of 1:0:9:0.9 for Pex1p, Pex6p and Pex15p in both formed complexes. Indeed, this data indicate that each Pex6p molecule is capable to bind one Pex15p, resulting in a 3:3:3 ratio of the Pex1p/Pex6p/Pex15p complex.

ATP-hydrolysis triggers the dissociation of Pex15p and the AAA-complex. We next studied the role of nucleotides in the binding of the recombinant Pex1/6p-complex to Pex15p. To this end, we analyzed the binding of isolated GST-Pex15p1-315His to wild-type Pex1/6p-complexes in presence of ATP and ADP, and analyzed whether mutations of Walker B motifs of the AAA-proteins have an influence on binding of the complex to Pex15p. Complexes were bound to GSH-agarose, eluted from the columns by addition of reduced glutathione and visualized by SDS-PAGE and Coomassie staining (Fig. 5a,b, upper panels). Signal intensity measurements of the AAA-proteins present in the load and the eluate fraction were performed and the ratio obtained for wild-type complex under ATP-condition normalized to a relative binding-capacity of one (Fig. 5a,b, lower panels). When ADP was present instead of ATP, the binding of the wild-type AAA-complex to the recombinant Pex15p was reduced (Fig. 5a), which corroborates the observed binding behavior of the isolated endogenous Pex15p-complex (Fig. 2). A reduced binding was also observed for the complex composed of wild-type Pex6p and mutated Pex1p (Pex1WB/6p, Fig. 5b). However, when ATPase activity of the AAA-complex was blocked by mutation of Pex6p (Pex1/6pWB), the binding capacity of the formed AAA-complex was significantly increased (Fig. 5b). This is in agreement with the observed accumulation of Pex15p at Pex1/6pWB-membrane complexes isolated from yeast (Fig. 3). We conclude that hydrolysis of ATP to ADP via the D2-domain of Pex6p weakens the interaction to Pex15p and thus might be a regulatory step in the AAA-cycle.

Driven by our observation that the *in vivo* and *in vitro* association of the AAA-complex with Pex15p is strengthened when ATP-hydrolysis in the Pex6p D2-domain is prevented, we analyzed the stability of the complexes over a longer period of time. Isolated wild-type Pex1/6p as well as the mutated Pex1/6pWB were bound to GSH-agarose loaded with GST-Pex15p1-315His. The Pex15p1-315His and bound AAA-peroxins were eluted by thrombin cleavage. In a third approach 2.5 mM ATPγS was added during thrombin incubation of Pex1/6p wild-type proteins bound to Pex15p1-315His. The eluted complexes were further purified by size exclusion chromatography. Subsequently, the 700 kDa fractions were pooled and kept on ice for 72h, before they were subjected...
Figure 4. In vitro assembly of the recombinant Pex1p/Pex6p/Pex15p-complex. (a) The cytoplasmic domain of purified recombinant Pex15p (GST-Pex15p1-315His) was combined with HisPex1p, HisPex6p, or with the hexameric Pex1/6p complex in presence of 5 mM ATP and subjected to GSH pull down assay. Load and 5x concentrated eluate fractions were separated by SDS-PAGE and proteins were visualized by Coomassie staining (upper panel) or with specific antisera as indicated (middle panel). Pex6p signals from load and eluate fractions were measured and relative binding efficiency determined (lower panel) by setting Pex1/6p binding-capacity to 1. Error bars represent standard error of mean (SEM) of four independent experiments. In complex with Pex1p, Pex6p binds three times stronger to Pex15p than single Pex6p. (b) Pex1/6p, Pex15p(1-315) or the Pex1/6p/Pex15p-complex of the three proteins were subjected to size exclusion chromatography in presence of ATP. Collected fractions were subjected to SDS-PAGE and immunoblot analysis and indicated proteins were detected via their His-tag with anti-His-antibodies. Pex15p was shifted to fractions of about 700 kDa in presence of the AAA-complex, demonstrating its association with this complex. (c) Size exclusion chromatography of the Pex1/6p/Pex15p-complex (upper panel) and the Pex6p Walker B mutated version Pex1/6pWB/Pex15p (lower panel) in presence of ATP after isolation of the proteins by GSH pull down assay as described in (a) and subsequent thrombin elution. Fractions were analyzed by SDS-PAGE and Coomassie staining and signal intensities of peak fraktions (dashed boxes) were evaluated using ImageJ. Taking into account the molecular weights of Pex1p (117 kDa), Pex6p (115 kDa) and Pex15p1-315 (36 kDa), both complexes consist of equal Pex1p, Pex6p and Pex15p ratios.
Figure 5. Nucleotide-dependent association and dissociation of the reconstructed Pex1p/Pex6p/Pex15p-complex. (a) Hexameric Pex1/6p complexes were isolated in presence of ATP or ADP and incubated with GST-Pex15p1-315His and then subjected to GST pull down assay. Load and 5x concentrates of eluate fractions were analyzed by SDS-PAGE and Coomassie staining (upper panel) and relative binding efficiency (lower panel) was estimated by measuring Pex6p signals from load and eluate fractions and setting Pex1/6p + ATP binding-capacity to 1. (b) GST-Pex15p1-315His pull down assay in presence of ATP using hexameric Pex1/6p wild type, Pex1WB/6p and Pex1/6pWB complexes. Binding efficiency was determined as in (a). In contrast to wild-type complex, Walker B-mutation of Pex6p leads to a significantly higher association of the complex with Pex15p, indicating that ATP-hydrolysis triggers complex-dissociation. (c) 700 kDa Pex1p/Pex6p/Pex15p and Pex1p/Pex6pWB/Pex15p complexes as well as Pex1p/Pex6p/Pex15p wild-type complexes, which had been incubated with 2.5 mM ATP γS during the isolation procedure, were obtained from size exclusion chromatography. Afterwards these complexes were incubated 72 h at 4 °C and subjected again to size exclusion chromatography. Presence of proteins was monitored by SDS-PAGE and immuno-blotting using anti-his-antibodies. Pex1p/Pex6pWB as well as wild-type complexes incubated with ATP γS exhibit a stabilized interaction to Pex15p, proving the direct correlation between ATP hydrolysis and complex-dissociation.
again to size exclusion chromatography. After this time-period, wild-type Pex1p and Pex6p were detected unaltered at 700 kDa fractions, whereas most of Pex15p/His was found in later fractions with a size of about 100 kDa (Fig. 5c, upper panel). Obviously, after incubation on ice, the AAA-complex remains assembled but dissociates from Pex15p. The complex composed of wild-type Pex1p and the Walker B mutant variant Pex6pWB behaved differently. Here a significant portion of Pex15p/His was still associated with the AAA-complex (Fig. 5c, middle panel). The Pex1/6/15p complex, which was incubated with a portion of ATP−γS displayed an intermediate state (lower panel). Signal intensity measurements of the ratio of associated and dissociated Pex15p molecules revealed that under wild-type conditions less than 10% of Pex15p was still bound to the complex. Block of ATP hydrolysis using the Pex6WB mutant or application of ATP−γS did result in a stabilization of the Pex15p association. Under these conditions, the amount of associated Pex15p increased to 40% or 20%, respectively. We conclude that a block of ATP-hydrolysis in the D2 AAA-domain of Pex6p prevents the release of the AAA-complex from its membrane anchor Pex15p.

Discussion

Pex1p and Pex6p belong to the AAA-protein family\textsuperscript{2,7} and are essential components of the peroxisomal protein import machinery\textsuperscript{26,27}. Despite a similar architecture, they do not perform overlapping functions and are both indispensable for peroxisomal matrix-protein import. They were shown to function as dislocases facilitating the import of peroxisomal matrix proteins\textsuperscript{28,29}. In contrast to the majority of AAA-ATPases, Pex1p and Pex6p assemble into a heteromeric complex\textsuperscript{23}. This complex exhibits a dual localization in the cell with a portion attached to the peroxisomal membrane via the membrane protein Pex15p (Pex26p in humans, APM9 in plants)\textsuperscript{30−32}, and a fraction localized to the cytosol.

Recently, we and others analyzed the structure of the heterohexameric Pex1/6p complex by electron microscopy\textsuperscript{10,22} and demonstrated that it is assembled as a trimer of Pex1p/Pex6p dimers, resulting in a tight organization with alternating Pex1p and Pex6p subunits. The D1− as well as the D2− domains of Pex1p and Pex6p are organized into two rings arranged on top of each other with a dynamic central pore. The presence of essential aromatic pore loops, which move up and down during the Pex1/6p ATP hydrolysis cycle led to the proposal that Pex5p is actively pulled out of the membrane by a threading mechanism through this central channel, similar to the mechanism of Clp-ATPases\textsuperscript{11,30−32}. Beside movement of the pore loops, it is likely that the ATPase-cycle also regulates the assembly of the AAA-complex as well as its association with the peroxisomal membrane. The first assumption is supported by the observations that ATP-binding is essential for Pex1/6p-association\textsuperscript{33}, whereas nucleotide depletion results in complex disassembly\textsuperscript{18,34}. Here we analyzed the influence of the nucleotide bound-state on assembly of the AAA-complex in the cytosol and on its interaction with Pex15p, which targets the complex to the peroxisomal membrane. We demonstrate that the heterohexameric complex arrangement is largely maintained even under excessive ADP conditions (Fig. 1b). This is in contrast to the nucleotide-free or AMP-bound states of the complex, which disassembles in short time\textsuperscript{18}. The stabilization of the complex by ADP indicates that the composition of the complex is not altered during the nucleotide cycle but that the complex remains assembled when ATP is hydrolyzed by the intrinsic ATPase activity of the Pex1/6p-complex. Nevertheless, in comparison to the ATP-bound form, the ADP bound AAA-complex showed a tendency to disassemble (Fig. 1b), which is in agreement with the considerably looser conformation as depicted by electron microscopy\textsuperscript{14} and keeps open a possibility for a regulative Pex1/6p disassembly mechanism. While in mammalian cells, the AAA-complex seems to disassemble in the cytosol to form a homotrimeric version of Pex1p\textsuperscript{33}, the yeast complex remains assembled in the cytosol\textsuperscript{23}. ATP hydrolysis in the D1 domain of human Pex1p is discussed to trigger dissociation of the Pex1/6p complex\textsuperscript{34}. However, the D1 domain in yeast Pex1p does not comprise a functional Walker B motif, which might explain that the cytosolic yeast complex remains assembled.

Here we studied the regulation of the recruitment of Pex1/6p to its membrane anchor Pex15p and analyzed the membrane association of AAA-complexes at different nucleotide states in vivo and the binding of the recombinant Pex1/6p-complex to Pex15p in vitro. Block of ATP-hydrolysis and therefore fixation of Pex6p in the ATP-bound form increased membrane association of the AAA-complex in vivo and binding to Pex15p in vitro (Figs 3 and 5b). In contrast to the Pex6p Walker B mutation, a corresponding mutation in Pex1p showed no impact on complex formation and composition (Figs 3 and 5b). This distinction reflects the different contributions of Pex1p and Pex6p to the overall ATPase activity of the AAA-complex, which is predominantly attributed to Pex6p\textsuperscript{22}. This seems to be a species-independent feature as the import of GFP-PTS1 into human peroxisomes is not affected by a Walker B mutation in Pex1p D2, while it was abolished in Pex6p Walker B cell lines\textsuperscript{33}. However, import of catalase was shown to be impaired for both Pex1/6p Walker B variants, indicating that ATP hydrolysis in Pex1p is somehow required at least for the transport of this human protein\textsuperscript{33}.

Reconstitution of the Pex1/6/15p complex revealed that Pex6p mediates the binding of the AAA-complex to Pex15p via a direct interaction and independent of other proteins (Fig. 4a), which is in line with previous reports\textsuperscript{12,23}. Binding assays and size exclusion chromatography indicated that stoichiometric amounts of Pex1p/Pex6p are bound to Pex15p with a ratio of 3:3:3 (Fig. 4b,c). Thus, each of the three Pex6p molecules within the Pex1/6p-complex is capable to anchor the AAA-complex to the peroxisomal membrane by Pex15p-binding. As the affinity of the Pex1/Pex6-complex to Pex15p is lower under ADP condition, we cannot exclude a change of the stoichiometry upon ATP hydrolysis. However, for p97, a similar binding ratio of three p47 adaptor molecules to the homohexameric AAA-complex was found\textsuperscript{35} and also binding of three α-SNAP molecules to three N-terminal domains of the NSF hexamer is discussed\textsuperscript{36,37}.

Our data suggest that ATP hydrolysis in Pex6p and the dissociation of the Pex1/6p complex from Pex15p is directly correlated. Obviously, the intrinsic ATPase activity of the complex hydrolyses ATP and converts the complex into the ADP-bound form, which exhibits lower Pex15p-binding affinity. Interestingly, also the Pex1WB/6p complex showed lower affinity to Pex15p compared to the wild-type complex, which might indicate that Pex6p preferably is situated in a post-hydrolysis conformation when Pex1pWB is constantly bound to
ADP by ATP enables a new round of the AAA-complex cycle. Dissociation of Pex5p from other pore constituents. Finally, the complex is released to the cytosol and exchange of segregates the SNARE complex. At the peroxisomal membrane, such a rotational process might trigger the of rotating and pulling movements is discussed for NSF, which together with its adaptor α complex might hydrolyze ATP at the same time, leaving room for a sequential binding and releasing of the three binding to Pex15p, which weakens the Pex15p-association of the complex. However, not all Pex6p-subunits of the are available upon request. Targets of ATP-hydrolysis are included. The current data suggest the following scenario for the Pex1/6p-cycle in yeast (Fig. 6). In the cytosol, newly synthesized Pex1p assembles into a trimer, whereas Pex6p remains monomeric. Binding of ATP to the subcomplexes promotes the assembly of the heterohexameric Pex1p/Pex6p-complex, which represents the dominant state in the cell. The ATP-loaded complex attaches to the peroxisomal membrane via its membrane-anchor protein Pex15p. Contact to Pex15p is mediated by the N-terminal domains of Pex6p, which are located at the three vertices of a triangular shaped structure. At the membrane, the complex functions as dislocase for the PTS1-receptor Pex5p, and its de-ubiquitinating enzyme Ubp15p in yeast or the export stimulation factor AWP1 in human cells. In particular, contact of substrate to the AAA-machinery might trigger its activity, similar to other AAA-ATPases. The intrinsic ATPase activity of NSF for instance is very low. Binding of NSF to α-SNAP stimulates its ATPase activity and maximum stimulation is achieved when both α-SNAP and SNARE complexes are included.

Material and Methods

Protein purification. Expression of recombinant Pex1p and Pex6p proteins was performed in Escherichia coli (Tuner (DE3) cells (Merck, Darmstadt, Germany) at 20 °C in the presence of 0.4 mM IPTG. Time of expression was 20 h for Pex1p and 5 h for Pex6p. Cells were harvested and homogenized by sonication either separately or as a mixture of Pex1p- and Pex6p-expressing strains. The homogenization was performed in AAA-buffer I (50 mM Tris, 300 mM NaCl, 5 mM MgCl₂, 5 mM ATP-γS/ADP-γS, and 1 mM DTT, pH 7.4) containing 40 mM imidazole and selected protease inhibitors (1 mM PMSF, 8 mM Antipain, 0.3 mM Aprotinin, 1 mM Bestatin, 10 mM Chymostatin, 5 mM Leupeptin, 15 mM Pepstatin). The homogenate was centrifuged at 45,000g (rotor SS34) for 60 min. The resulting supernatant contained soluble proteins and was loaded to NiNTA-Agarose (SPRIME, Hamburg, Germany) according to manufacturer’s instructions. The column was washed with 20-fold column
volume of AAA-buffer I supplemented with 40 mM imidazole and bound proteins were eluted in two steps with AAA-buffer II (50 mM Tris, 300 mM NaCl, 5 mM MgCl₂, 5 mM ATP-Na₂/ADP-Na₂ and 10 mM DTT, pH 7.4) containing 100 mM and 300 mM imidazole, respectively.

For further purification of the HisPex1pGST/HisPex6p-complex, NiNTA eluates containing Pex1p and Pex6p were loaded onto a Glutathione Agarose 4B column (Macherey-Nagel, Düren, Germany). Unbound proteins were removed by washing of the matrix with 6-fold column volume of AAA-buffer II. The column with bound fusion-proteins was subsequently incubated with thrombin overnight at 4 °C and the released Pex1/6p-complex was separated from the agarose matrix by centrifugation.

GST-Pex15p1-315His was expressed in BL21 (DE3) cells (Merck, Darmstadt, Germany) from plasmid pKE15/2. Induction was performed for 5 h at 30 °C in the presence of 0.4 mM IPTG. Cells were harvested and homogenized in AAA-buffer I (without nucleotides, with selected protease inhibitors) by sonication. The supernatant of a 45,000 × g centrifugation (rotor SS34, 60 min.) was loaded to Glutathione Agarose 4B (Macherey-Nagel, Düren, Germany). Unbound proteins were removed by washing of the matrix with 6-fold column volume of AAA-buffer II. The column with bound fusion-proteins was subsequently incubated with thrombin overnight at 4 °C and the released Pex1/6p-complex was separated from the agarose matrix by centrifugation.

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Size exclusion chromatography. To obtain the hexameric Pex1/6p complex and to analyze the size of Pex1/6p/Pex15p complexes, gel filtration analysis was performed with an ÄKTApurifier system (GE Healthcare, Freiburg, Germany). For small-scale approaches, 50 μl of recombinant proteins were loaded on Superose™6 PC 3.2/30 column (GE Healthcare, Freiburg, Germany) equilibrated with AAA-buffer II and subsequently 50 μl fractions were collected. For preparative gel filtration, 500 μl of recombinant proteins were subjected to a Superose™6 10/300GL column and 500 μl fractions were collected. Calibration of the columns was performed with the HMW-Gel Filtration Calibration Kit (GE Healthcare, Freiburg, Germany) containing thyreoglobulin (669 kDa), ferritin (440 kDa) and aldolase (158 kDa).
Pex15p complexes and membrane attached Pex1/6p complexes were isolated according to 23 in the absence or presence of 5 mM ATP or ADP. Immuno-reactive complexes were visualized using Pex1p-, Pex6p-, Pex18p-, Pex15p-, Pex12p-, Pex13p-, Pex14p-, Pex17p-, or His6-specific primary antibodies and a IRDye 800CW goat anti-rabbit IgG or IRDye 680 goat anti-mouse secondary antibody (LI-COR Bioscience, Bad Homburg, Germany) followed by detection using the "Infratol Imaging System" (LI-COR Bioscience, Bad Homburg, Germany). ImageJ or LI-COR Odyssey application software 3.0 was used for signal intensity measurements.

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Author Contributions

I.G. and D.S. performed and analyzed the experiments. I.G., D.S., W.G. and R.E. designed the study and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Additional Information

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