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MiniReview

Protein translocation machineries: How organelles bring in matrix proteins

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Abstract

Eukaryotic cells contain several thousands of proteins that have to be accurately partitioned over the components of the cytoplasm (cytosol or any of the known organelles) to allow proper cell function. To this end, various specific topogenic signals have been designed as well as highly selective protein translocation machineries that ensure that each newly synthesized polypeptide reaches its correct subcellular destination or, in case of secretory proteins, is exported to the cell exterior.

This contribution gives an overview regarding the principles of the main examples of polypeptide sorting and translocation, with emphasis on the function of cofactor binding in peroxisomal matrix protein import.

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Keywords: Protein translocation; Cofactor binding; Peroxisome

1. Introduction

In general, protein translocation machineries are designed to transport either unfolded or folded proteins. Two of such mechanisms have been analyzed in detail in prokaryotes, namely the secretion (Sec) pathway and the twin-arginine translocation (Tat) pathway, that mediate secretion of unfolded (Sec) or folded (Tat) proteins across the plasma membrane. In eukaryotes, secretory proteins are not directly transported across the cell membrane but travel via the endoplasmic reticulum (ER) and Golgi apparatus. This apparent roundabout is of major importance to assure the tightly controlled protein modification processes and quality control systems involved in eukaryotic protein secretion. Eukaryotic cells also harbor a variety of intracellular protein

translocation systems because of their subcellular compartmentalization.

This contribution presents an overview of the various known protein translocation machineries. Special attention is given to the translocation of proteins into peroxisomes and the importance of cofactor binding in this process.

2. Unfolded-protein translocation machineries

2.1. The Sec machinery

The Sec translocation machinery of bacteria is designed to accommodate unfolded protein transport. Typical for unfolded protein transport is that the newly synthesized polypeptides have to be prevented from premature folding by the function of molecular chaperones. A second general property is that such proteins contain a cleavable targeting signal that is located at the extreme

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N-terminus of the protein and is recognized by a specific cytosolic receptor that mediates routing of the precursor protein to its translocation site. Most proteins to be transported via the Sec system possess a conserved class-1 N-terminal signal sequence that shows at least three structural characteristics namely (i) a positively charged N-domain, (ii) a hydrophobic H-domain and (iii) an uncharged polar C-domain [1].

The receptor that recognizes this sequence is SecA. Together with the heterotrimeric membrane protein complex SecYEG, SecA forms the translocase. The precursor polypeptide may travel either directly to SecA or via the function of a molecular chaperone, SecB. Most probably, the translocation channel (translocon) is formed by the assembly of several copies of the SecYEG complex [2]. SecA is an ATPase and functions as molecular motor during protein translocation. The N-terminal sequences of precursor proteins take part in protein–protein and protein–lipid interactions, which results in the initiation of protein translocation through the translocon. ATP hydrolysis by SecA as well as the proton-motive force that is formed by the membrane potential ($\Delta\Psi$) and a pH gradient (ΔpH), generate the driving power to allow translocation. After translocation the signal sequences are cleaved-off by a signal peptidase, followed by folding/activation of the mature protein.

It should be noted that the SecYEG translocon can accommodate unfolded proteins in two distinct manners namely (i) via post-translational translocation, as depicted above or (ii) co-translationally. In the latter mode protein synthesis and translocation are coupled events that require the function of the universally conserved SRP (signal recognition particle) to sort the evolving precursor protein via the SRP receptor, FtsY, to the SecYEG translocon. For further molecular details of the Sec protein translocation machinery the reader is referred to recent reviews [3,4].

2.2. The endoplasmic reticulum

The counterpart of the bacterial Sec machinery in eukaryotes is the unfolded-protein import machinery of the ER. Newly synthesized proteins are sorted to the translocation pore by an N-terminal targeting sequence that is characterized by a core of mostly hydrophobic amino acids that are often preceded by one or a few basic ones. The pore is formed by a Sec61 complex that in mammals is composed of Sec61α and two other proteins, Sec61β and Sec61γ, the actual pore being lined up by three or four Sec61 complexes.

A major difference between the prokaryotic and eukaryotic Sec translocation machinery is the location of the ATPase that drives the translocation process. In bacteria SecA is localized at the cytosolic (*cis*)-side of the cell membrane whereas in the ER system the ATPase that forms the motor in protein translocation is localized in the lumen (at the *trans* side of the membrane). The ATPase in the ER that fulfils this role is a member of the hsp70 protein family (also termed BiP or Kar2p) [6]. ATP required for protein translocation in the ER lumen is imported via an ATP/ADP antiporter. BiP binds newly synthesized proteins as they are translocated into the ER lumen and maintains them in a state competent for subsequent folding and oligomerization [5]. BiP is thought to prevent sliding back of the precursor polypeptide chain as it protrudes through the channel ("molecular ratchet model"), but might also actively pull the polypeptide chain across the ER membrane [6].

Besides the above post-translational translocation also co-translational protein import is an important mode of unfolded-protein import into the lumen of the ER. This pathway starts with an unattached ribosome that synthesizes a secretory protein precursor. After emerging of the N-terminal signal sequence, the signal recognition particle (SRP) binds to this sequence. The complex thus formed, consisting of the ribosome, the nascent polypeptide chain and the SRP, binds to the α-subunit of the SRP receptor at the ER membrane. At this step the translocon is still closed. In the next step the SRP and its receptor are released and recycled at the expense of GTP hydrolysis, a process that is associated with the direction of the nascent chain via the translocon into the translocon gate. Entrance of the signal sequence opens the gate and allows further elongation of the nascent polypeptide. Upon entrance in the lumen, the signal sequence is processed by a signal peptidase. Finally, the imported protein assumes its native conformation.

2.3. Unfolded-protein import into double-membrane bound organelles: mitochondria and chloroplasts

The characteristics of post-translational translocation of nuclear-encoded proteins into mitochondria and chloroplasts basically resemble those of the prokaryotic Sec system. Also in these cases an N-terminal signal sequence is required, an organelle docking site, a translocation pore, and ATP as the main driving force. However, in the case of mitochondria and chloroplasts the cells had to cope with the problem of the double-boundary membrane and, in case of chloroplast thylakoids, a triple membrane passage. In case of mitochondrial matrix proteins this is solved by designing two translocation machineries present in the outer membrane (termed TOM complex) and in the inner membrane (termed TIM complex), whose functions are precisely orchestrated to allow efficient protein import. The N-terminal signal sequence of mitochondrial matrix proteins forms an amphipathic α-helix that is recognized at the outer organelle surface by its receptor, Tom40. In the cytosol the protein is kept in an import-competent conformation via the function of cytosolic chaperones

(cytosolic Hsp70). After recognition, the protein is guided through the TOM pore, consisting of various interacting proteins and handed over to the TIM complex in the inner membrane. Also the TIM complex is composed of various interacting proteins. Transport of the precursor protein through the TIM complex to the organelle matrix requires a membrane potential and ATP. When emerging in the matrix, the precursor protein binds to mitochondrial Hsp70 (mtHsp70) that is associated with the TIM complex. MtHsp70 facilitates import to be completed at the expense of ATP and prevents the precursor protein from premature folding or aggregation. During import the signal sequence is removed and degraded, followed by assembly of the mature protein into its active native conformation, eventually with the help of matrix-localized molecular chaperones (mtHsp60) [7].

The translocation of the precursor proteins into the chloroplast stroma is directed by the TOC complex in the outer membrane (OM) and the TIC complex in the inner membrane (IM), which allow the translocation of proteins stimulated by ATP and GTP [8,9]. The cleavable signal sequence for the chloroplast stroma is positively charged and enriched in the amino acids serine and threonine. Cytosolic chaperones (Hsp70) are required to keep the protein in an import-competent conformation. Precursor proteins can either directly, or indirectly after modification by phosphorylation, associate with their corresponding GTP-dependent receptor protein to be delivered to the TOC complex. Following passage across the OM, the precursor passes the TIC complex to reach its final destination, the stroma. This is an ATP-dependent process and requires the function of molecular chaperones (Hsp70) to provide the driving force and Hsp60 for subsequent protein assembly (comparable to the processes in the mitochondrial matrix).

Proteins destined for the thylakoid lumen have to pass three membranes and to this end contain cleavable bipartite targeting signals [8,9], consisting of an N-terminal stroma-targeting domain followed by a thylakoid lumen-targeting domain. After entering the stroma, the signal sequence is removed by a stromal signal protease to expose the thylakoid signal sequence. At this stage import diverges over a folded- and an unfolded-import machinery, which in many respects strongly resemble protein secretion in prokaryotes. A number of proteins (e.g. plastocyanin) are kept unfolded by stromal molecular chaperones. Their signal sequences contain a core of hydrophobic amino acids that direct the protein to the thylakoid lumen via binding and passage through a complex of receptors and channel proteins in a ΔpH-independent manner. This pathway highly resembles the Sec system in bacterial inner membranes. In the thylakoid lumen the signal sequence is removed by a specific protease, followed by folding and assembly. Others (e.g. metal-binding proteins) fold in the stroma and are imported via a ΔpH-dependent pathway (similar to the Tat pathway in bacteria, see below, paragraph 3.1) [10]. The pH gradient across the thylakoid membrane (internally acid) presents the driving force for this protein translocation process.

3. Translocation of folded proteins

3.1. The Tat pathway

The twin-arginine translocation (Tat) pathway has been identified in prokaryotes and the thylakoid membrane of chloroplasts to facilitate translocation of partially or fully folded proteins [11]. Originally this pathway was thought to be used solely by redox proteins that have to incorporate a cofactor prior to translocation. These proteins therefore fold before translocation and hence are incompatible for the Sec pathway. However, substrate predictions from genomic approaches have suggested that certain bacteria secrete mainly non-redox proteins via the Tat pathway [12]. The driving force for translocation of non-cofactor-containing proteins by this pathway is not understood yet.

Proteins that are translocated by the Tat-pathway are proposed to pass a proteinaceous pore, composed of the three functionally distinct integral membrane proteins TatA, TatB and TatC. The initial recognition step of the precursor protein is most likely mediated by the TatBC complex (based on studies of the thylakoid Tat system), which targets the precursor protein to the membrane, whereas TatC might form a specific binding site for the Tat signal peptide. The Tat signal peptide resembles typical Sec signal peptides and consists of the classical tripartite structure (N-, H- and C-domain, including the cleavage site for a signal peptidase). However, characteristic for Tat signal peptides is the twin-arginine motif between the N- and H-domains, consisting of the originally defined motif: (Ser/Thr)–Arg–Arg–Xaa–Phe–Leu–Lys, in which the two arginines are essential. Upon docking, the precursor protein interacts with TatA to initiate translocation through the Tat pore. The structure of the active translocon is unknown yet. Because the channel has to maintain its barrier function to preserve the transmembrane proton gradient, while translocating folded proteins, it is likely that the translocon functions as a 'zipper' structure. Consequently, at the first stage of translocation the pore is closed at the *trans*-side of the membrane, whereas the *cis*-side is opened. During translocation, the translocated protein itself may function as barrier to maintain the proton gradient, and at the end of the process the pore is closed again.

3.2. The cytoplasm-to-vacuole-targeting pathway

As for other organelles, also the vacuole may acquire its proteins via various pathways. An important route is the vacuole protein-sorting pathway (VPS) that involves the function of the ER and Golgi apparatus and vesicle-mediated transport (e.g. for carboxypeptidase Y). For details of this pathway the reader is referred to [13].

In bakers' yeast folded-protein transport machinery is described for vacuoles as well. This pathways is involved in sorting of a limited number of vacuolar hydrolases and designated cytoplasm-to-vacuole-targeting (CVT) pathway [14]. Characteristic for the CVT pathway is that the proteins are incorporated in the cytosol in double-membrane vesicles. The most extensively studied protein that is imported via this pathway is aminopeptidase 1 (Ape1). Precursor Ape1 (prApe1) molecules assemble into dodecamers in the cytosol and are, upon binding to the soluble receptor protein Atg19, delivered to the pre-autophagosomal structure (PAS). The PAS most likely represents the origin of the membranes that sequester the cargo proteins to form the CVT vesicle. Upon closure of the CVT vesicle, it fuses with the vacuolar membrane, resulting in the delivery of its content in the vacuole lumen, where the precursors is processed and activated.

4. Matrix protein translocation into peroxisomes

Peroxisomes constitute an important functional class of the microbody family of cell organelles [15,16] that have been detected in most eukaryotic cells. Characteristic for peroxisomes is their unprecedented functional versatility and their inducible nature that allows the cells to adequately adapt to developmental [17] or varying environmental conditions [18].

In normal wild-type cells peroxisomes develop by growth and fission of pre-existing organelles. Proteins destined for the peroxisome lumen are synthesized in the cytosol and post-translationally incorporated into the organelles. For most proteins sorting depends on either of the two targeting signals, PTS1 or PTS2 [19], but exceptions do exist [15,20–22]. The cytosolic routing machineries do not basically differ from those described above for protein import into other organelles. However, the actual translocation event may proceed via a novel, yet unknown, mechanism. Fact is that a constitutive peroxisomal pore has not yet been observed, despite extensive research. However, most if not all matrix proteins enter the organelle lumen in an oligomeric or at least partly folded state. Below, relevant recent data on the topic of peroxisome matrix protein import are discussed.

4.1. The PTS1-pathway

Most peroxisomal matrix proteins are routed to their target organelle via the PTS1 pathway. The PTS1 signal is located at the extreme C-terminus of proteins and consists of three amino acids: -SKL or conserved variants thereof. These proteins are recognized by the C-terminal tetratricopeptide repeat (TPR) domain of the soluble PTS1 receptor protein, Pex5p. Besides the general model of protein translocation, where one receptor binds one cargo protein, for PTS1 protein import a new model has been presented, termed the pre-implex model (Fig. 1).

This model is based on the fact that Pex5p forms tetramers and hence can bind multiple PTS1 proteins. By multiple-binding events between various Pex5p tetramers and oligomeric PTS1 cargo proteins, large protein complexes may be formed prior to import [23]. These complexes may also include monomeric proteins and oligomeric matrix proteins that contain subunits lacking a PTS1. Indeed, it has been shown that the absence of a PTS1 does not affect import when also subunits of the same protein are synthesized that do contain a PTS1. This mode of import has also been termed piggy-backing (see below) [24].

The recent discovery that certain peroxisomal matrix proteins do not bind to the carboxyterminal TPR domains of Pex5p but, via still unknown novel PTS sequences, to the N-terminal domain of Pex5p (e.g. alcohol oxidase and acyl-CoA oxidase [20,22]), is not in conflict with the pre-implex model.

Proteins known to be essential for docking of Pex5p at the peroxisomal membrane are Pex13, Pex14p and Pex17p. The subsequent protein translocation step is fully unclear. It has been proposed that the three zinc finger proteins (Pex2p, Pex10p and Pex12p) may form a (transient) translocation pore. An attractive alternative is that import proceeds via a pore formed by Pex5p molecules themselves, eventually facilitated by the Pex5p-docking protein Pex14p. This speculation is consistent with recent findings that Pex5p can behave like an integral membrane protein [25]. However, other options, e.g. pinocytosis-like manners, cannot be excluded (e.g. suggested by McNew and Goodman [26]). Also, it is not known whether the putative pre-implex disassembles prior to translocation or whether supercomplexes of Pex5p molecules and PTS1 cargo are imported. However, the fact that large structures can be incorporated is clear from the finding that the peroxisomal protein import machinery can accommodate large rigid structures, including gold particles with a diameter of 9 nm [27].

Pex5p is proposed to enter the peroxisome together with its cargo (the so-called extended-shuttle model). Recycling of Pex5p to the cytosol requires the function of Pex4p [28], a ubiquitin-conjugating enzyme that is
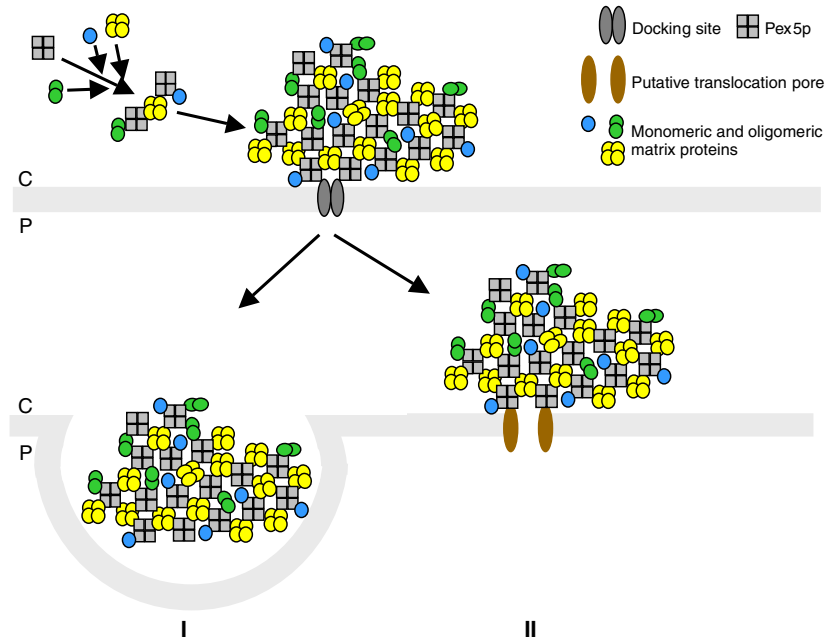
Fig. 1. Hypothetical model of translocation of peroxisomal PTS1 matrix proteins via the putative pre-implex complex [23]. At the vicinity of the peroxisomal membrane a pre-implex complex is formed via numerous protein–protein interactions between newly synthesized peroxisomal matrix proteins that contain a PTS1 (circles) and the tetrameric receptor protein Pex5p (squares). The pre-implex complex may dock to the target membrane by binding of Pex5p to components of the receptor docking site (Pex13p, Pex14p, Pex17p). Subsequently, translocation of the PTS1-proteins and receptors might occur through a transient pore, the structure of which is still unknown (II). Alternatively, import might proceed via a pinocytosis-like machinery (I). C – cytosol, P – peroxisomal matrix.

bound to the peroxisomal membrane via Pex22p. Removal of Pex5p molecules that are not properly recycled (for instance those molecules that got stuck at the export site) involves the ubiquitin – proteasome degradation pathway. Remarkably, this process is independent of Pex4p [29,30] but involves other Ub-conjugating enzymes. The luminal protein Pex8p promotes the dissociation of the Pex5p–PTS1 cargo complex in the peroxisomal matrix [31,32]. Recycling of Pex5p requires ATP [33,34]. Most likely, the two membrane-bound AAA-ATPases, Pex1p and Pex6p, play a role in this process [35].

4.2. The PTS2-pathway

Relatively few peroxisomal matrix proteins use an N-terminal PTS2-targeting sequence. The PTS2 consists of a nonapeptide with the consensus (R/K)-(L/V/I)-X_5-(H/Q)-(L/A). PTS2 matrix proteins are recognized by the soluble protein receptor Pex7p [15]. However, the PTS2 import machinery differs from its PTS1 counterpart in that the PTS2 receptor Pex7p requires additional, auxiliary proteins. So far, four of these have been characterized. Pex18p and Pex21p occur in *Saccharomyces cerevisiae*. *Yarrowia lipolytica*, *Neurospora crassa* and *Hansenula polymorpha* lack these peroxins but contain Pex20p, while in human cells

the long isoform of the PTS1-receptor, Pex5pL, fulfils an auxiliary function in PTS2 protein import. These proteins share a number of properties: (i) they play an essential role in PTS2 protein import, (ii) they contain a conserved Pex7p-binding box [36–38] and (iii) they contain WXXXF motifs that are involved in binding to the docking proteins Pex13p and Pex14p [36,37]. ScPex18p and ScPex21p show redundancy, because PTS2 import is only blocked when both proteins are absent (i.e. in a *pex18/pex21* double-deletion strain). It has been proposed that the auxiliary proteins form the actual PTS2 receptor together with Pex7p.

Indeed, recent in vitro binding experiments using purified *H. polymorpha* proteins indicated that the affinity of a hetero-oligomeric Pex7p–Pex20p complex for PTS2 proteins is higher than that of Pex7p or Pex20p alone (Wang, D.Y., and van der Klei, I.J., unpublished data).

Like Pex5p, Pex20p has been shown to interact with the matrix-localized peroxin Pex8p. Therefore, most likely Pex20p also enters the peroxisomal lumen. Moreover, recent data have indicated that also Pex7p shuttles between the peroxisomal matrix and the cytosol [39]. Hence, Pex7p together with the auxiliary proteins may also function according to the extended shuttle model.

4.3. Non-PTS1 and non-PTS2 proteins

Some peroxisomal matrix proteins lack a typical PTS1 sequence, but are still dependent on the function of Pex5p for import. Examples are malate synthase (MAS) in *H. polymorpha* [21] and acyl-CoA oxidase (Pox1p) in *S. cerevisiae* [22]. For the latter protein it has been shown that it associates to the N-terminal domain of Pex5p, in contrast to the PTS1 signal, which binds the TPR-domain in the Pex5p C-terminus. Moreover, it has recently been shown that a truncated version of Pex5p, which lacks the C-terminal TPR domain, is sufficient for import of Pox1p, but not for other PTS1 proteins [40].

Proteins that lack a PTS can also be imported upon the formation of a hetero-oligomeric complex with a PTS-containing matrix protein. An example of this piggy-back import mechanism is import of *S. cerevisiae* Dci1p and Eci1p, peroxisomal proteins that belong to the isomerase/hydratase family. These two proteins can be imported as hetero-oligomeric complex, in which Eci1p that lacks a PTS is co-transported with the PTS1-containing protein Dci1p [41].

4.4. Cofactor-containing matrix enzymes

As peroxisomal matrix proteins are now generally believed to be imported as folded proteins, the quality control mechanisms for these proteins are likely to reside predominantly in the cytosol. This may in particular be important for complex multimeric enzymes that require cofactor binding for their function.

The first studies on cofactor binding to peroxisomal matrix proteins were reported by Lazarow and de Duve [42], who studied import and assembly of the heme protein catalase. They proposed that in human cells catalase is synthesized in the cytosol as apomonomer, which is subsequently transported into the peroxisome, where heme addition and assembly/activation of catalase tetramers takes place. An unsolved problem is how and where soluble heme-containing proteins acquire their heme moiety. Heme is a highly hydrophobic molecule that is synthesized in the mitochondrial matrix. Probably heme carrier proteins do exist that serve in transfer of heme to other cellular locations.

The peroxisomal matrix is not obligatory for heme binding to and tetramerization of catalase, as peroxisomal catalase is normally activated and assembled in the cytosol of peroxisome-deficient cells. Moreover, in bakers' yeast a cytosolic catalase (catalase T) is present that is highly homologous to the peroxisomal isoenzyme catalase A. This implies that, in case of intraperoxisomal assembly of catalase A in bakers' yeast, a system must exist that allows catalase T assembly in the cytosol, but at the same time prevents this for catalase A. Since both catalase variants are very similar, this is difficult to envisage. Therefore, it is more likely that, like other peroxisomal enzymes, catalase is also imported into peroxisomes as folded, cofactor-containing tetramers.

A second peroxisomal protein that is extensively studied with respect to cofactor binding and translocation is *Y. lipolytica* acyl-CoA oxidase (Aox), a flavoprotein [43]. From this extensive and elegant study it is clear that the protein is folded in the cytosol prior to translocation. Aox is a heteropentamer composed of five different Aox monomers (termed Aox1p–Aox5p) that each can bind one FAD molecule. Two of these isoforms, Aox2p and Aox3p, have been shown to assist in assembly of the Aox complex but are not required for acquisition of the cofactor FAD by other components of the complex. The authors have shown convincingly that import of Aox requires cofactor binding and preassembly of the pentamer in the cytosol [43].

The molecular mechanisms involved in FAD-binding to peroxisomal matrix proteins remained unsolved. The first clues came from studies on the biosynthetic pathway of peroxisomal alcohol oxidase (AO) in methylotrophic yeasts. Enzymatically active AO protein is a homo-octamer, with one FAD molecule non-covalently bound to each monomer. In excellent studies by Goodman and co-workers [44,45] it was shown that *Candida boidinii* AO initially assembles at the peroxisomal membrane and forms instable octamers, followed by stabilization in the matrix. These authors also showed that import and activation of this peroxisomal protein is an energy-dependent process [45]. Later studies demonstrated that AO monomers are imported into peroxisomes, followed by oligomerization of the protein within the organellar matrix [46]. In line with these observations are data from Faber et al. [47] that revealed that octameric AO cannot be imported into peroxisomes, because this form of the protein fails to bind to its receptor Pex5p.

Studies by Ozimek et al. provided the first clues on FAD binding to the AO monomers in *H. polymorpha*. In this yeast it was shown that FAD binding to AO is not a spontaneous process, but requires the function of the cytosolic protein pyruvate carboxylase (Pyc1p) [48]. Pyc1p is an anapleurotic enzyme that replenishes the citric acid (TCA) cycle with oxaloacetate generated from pyruvate. Unexpectedly, not the enzyme activity of Pyc1p but a second function of the protein is required for AO assembly. Identical results were observed in the related yeast *Pichia pastoris*.

FAD binding is essential for AO octamerisation and import into peroxisomes, as was evident from the analysis of an *H. polymorpha* mutant affected in riboflavin biosynthesis [49]. In this mutant AO accumulated in the cytosol as monomers that lacked FAD. A similar phenotype was observed in cells of *H. polymorpha* *PYC1* deletion strains. This indicated that AO monomers, which contain the PTS1 sequence-ARF, are not

imported into peroxisomes because of the lack of FAD binding. Indeed, import of AO is independent of its PTS1, but requires the function of the PTS1 receptor Pex5p in an alternative way [20]. For this pathway the TPR motifs in the C-terminal part of Pex5p are not required, but the N-terminal domain of Pex5p is essential and sufficient to mediate import of AO. Our current data suggest that FAD binding to AO monomers results in the formation of an internal PTS. After FAD-binding and recognition of the FAD-containing monomers by Pex5p, the monomers are transported into the peroxisomal matrix (Fig. 2). The obvious advantage of this unique import and assembly machinery is that it prevents premature AO assembly/activation in the cytosol. This is in fact a prerequisite for the survival of the cell, because the presence of cytosolic AO activity has severe energetic disadvantages, related to alternative, energy-consuming hydrogen peroxide degradation pathways that prevent normal cell growth [50].

A third peroxisomal enzyme whose activity is dependent on a specific cofactor is dihydroxyacetone synthase (DHAS), a dimer that contains thiamine pyrophosphate (TPP) as cofactor. In the peroxisomal membrane a carrier protein is present, PMP47, that was initially suggested to be involved in transport of TPP across the peroxisomal membrane [51]. This assumption was based on the finding that deletion of the gene encoding PMP47 caused a specific protein import defect for DHAS. Later studies, however, convincingly demonstrated that DHAS enters the peroxisomes as dimers [46]. Whether these dimers contained TPP remained unclear. But in vivo studies showed that dimeric, enzymatically active DHAS can efficiently cross the peroxisomal membrane [47].

Clearly, a general picture of the biosynthetic pathway of cofactor-containing peroxisomal matrix proteins has not emerged yet. It is tempting to speculate that folding and oligomerisation of such proteins, and hence also binding of the respective cofactors, takes place outside the organelle in the cytosol or at the organelle membrane. However, more research is needed to elaborate these pathways.

5. Concluding remarks

The different protein sorting pathways described in this contribution share some general properties. Specific N-terminal targeting sequences are required to mediate their correct sorting, driving forces (e.g. membrane potential, GTP, ATP) are essential, and the proteins are usually translocated via distinct pore structures.
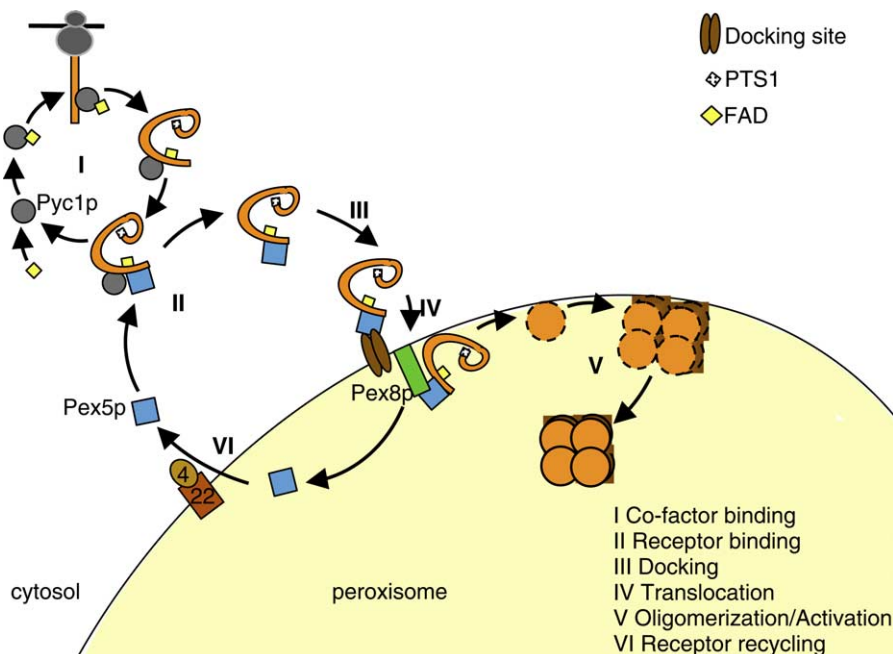

Fig. 2. Hypothetical model of import and assembly of AO in peroxisomes of the yeast *Hansenula polymorpha*. Newly synthesized AO monomers, emerging from the ribosome (orange), interact with pyruvate carboxylase (Pyc1p, grey circles) in the cytosol, which mediates FAD binding to AO monomers (I). FAD binding is believed to result in partial folding of AO polypeptides, thereby exposing a novel, yet unknown peroxisomal targeting signal that allows recognition and binding of Pex5p (blue squares) via its N-terminal domain under simultaneous release of Pyc1p (II). The Pex5p-AO complex may take part in pre-implex formation (see Fig. 1) or interact directly with the organelle docking site (III). After translocation (see Fig. 1 and IV), AO dissociates from Pex5p via the function of Pex8p (green rectangle), followed by formation of an unstable octamer that is subsequently stabilized to form enzymatically active AO octamers that may form crystals in the peroxisomal matrix (V). Recycling of Pex5p requires ATP and the function of Pex4p, that is recruited to the peroxisomal membrane by Pex22p (VI).

Exception to this rule seems to be the CVT pathway, which involves the formation of double-membrane bound vesicles and peroxisomal matrix protein import.

PTS1-peroxisomal matrix protein import is unusual in that the targeting signal is located at the extreme C-terminus. Also, the PTS1 signal is the smallest known targeting sequence (only three amino acids). This signal is sufficient and essential to sort soluble protein molecules of high complexity to peroxisomes. It becomes more and more clear that the actual translocation process does not take place via a distinct, rigid pore but may be mediated by a transient pore that is formed when sufficient molecules have arrived at the organellar docking site. Indeed, Gould and co-workers have proposed the so-called pre-implex model, which in fact brings all seemingly fragmentary and sometimes conflicting data of peroxisomal matrix protein import together. In view of recent data of Azevedo and co-workers [25], who observed that the PTS1 receptor Pex5p may behave as an integral component of the peroxisomal membrane, one may speculate that the PTS1 receptor in fact may form the temporary pore to allow import of a supercomplex of matrix proteins. This hypothesis may also add to an explanation of very recent data of Kiel et al. and Erdmann et al. [29,30], who showed that Pex5p molecules that are stuck in the peroxisomal membrane may be removed via poly-ubiquination for degradation by the proteasome. If these Pex5p molecules would have represented a transient pore, it may also explain how Pex5p molecules that were not trapped in the membrane may have escaped from the degradation process by recycling to the cytosol. However, other options for an import pore are also still possible, e.g. the involvement of the zinc finger proteins Pex2p, Pex10p and Pex12p. Clearly, much research is still required to solve this primary question in modern peroxisome research.

Acknowledgments

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