REVIEW ARTICLE

Protein Translocation Across the Peroxisomal Membrane

Jorge E. Azevedo,[1,2,]* João Costa-Rodrigues,[1,2] Carla P. Guimarães,[1,2] Márcia E. Oliveira,[1,2] and Clara Sá-Miranda[1,3]

[1]*Instituto de Biologia Molecular e Celular, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal;* [2]*Instituto de Ciências Biomédicas de Abel Salazar, Largo do Prof. Abel Salazar, 2, 4099-003 Porto, Portugal; and* [3]*Instituto de Genética Médica Jacinto de Magalhães, Praça Pedro Nunes, 88, 4050-466 Porto, Portugal*

Abstract

Peroxisomal matrix proteins are synthesized on free cytosolic ribosomes and posttranslationally imported into the organelle. Translocation of these newly synthesized proteins across the peroxisomal membrane requires the concerted action of many different proteins, the majority of which were already identified. However, not much is known regarding the mechanism of protein translocation across this membrane system. Here, we discuss recent mechanistic and structural data. These results point to a model in which proteins en route to the peroxisomal matrix are translocated across the organelle membrane by their own receptor in a process that occurs through a large membrane protein assembly.

Index Entries: Peroxisome; protein targeting; protein translocation; membrane; peroxin; biogenesis.

INTRODUCTION

Peroxisomes are single-membrane-bound organelles ubiquitous in the eukaryotic kingdom. Although their shape, size, and number

*Author to whom all correspondence and reprint requests should be addressed. E-mail: jazevedo@ibmc.up.pt

can vary widely according to cell type or environmental conditions, typical peroxisomes are spherical with a diameter of 0.1–1 μm (reviewed in ref. *1*). In mammals, peroxisomes participate in many biochemical processes, such as β-oxidation of long-chain and very long-chain fatty acids, synthesis of ether-linked phospholipids and bile acids, and the catabolism of purines *(2–5)*. The vital importance of these biochemical pathways in human

development and health is underscored by the existence of a group of genetic diseases, collectively called peroxisomal biogenesis disorders, in which peroxisomes are partially or completely nonfunctional. The Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and rhizomelic chondrodysplasia punctata type I are members of this class of diseases (reviewed in refs. *6–9)*. Peroxisomal biogenesis disorders are caused by mutations in genes coding for proteins involved in peroxisomal maintenance and inheritance. In the most severe cases, a mutation in one such gene can lead to the complete absence of peroxisomes in the cells of these patients *(10–15)*.

Research on the topic of peroxisomal biogenesis has been intensive during the last decade. Using many different organisms as experimental models (from *Saccharomyces cerevisiae* to mammalian cells), several laboratories have been quite successful in the task of identifying the genes involved in peroxisomal biogenesis. Almost 30 different genes involved in this process are known *(3,5,9,16,17)*. The proteins they encode are designated by the word peroxin (Pex) followed by a number reflecting the order of their discovery *(18)*. Although some of the identified peroxins seem to be specific to one or a few organisms, a great number is found in all the organisms characterized until now. Clearly, the mechanism of peroxisomal biogenesis has been fairly conserved through evolution.

Peroxins are divided into three groups by most authors (reviewed in refs. *3* and *5)*: (1) those that are necessary for the assembly/stability of the peroxisomal membrane; (2) those implicated in the transport of newly synthesized proteins into the matrix of the organelle; and (3) those that regulate peroxisome proliferation. Here, we will discuss data regarding the second group of peroxins, focusing our attention on the mechanism of protein translocation across the peroxisomal membrane. Excellent reviews providing a more general perspective on the topic of peroxisomal biogenesis have been published recently *(1,3–5,9,19)*.

GENERAL PROPERTIES OF PROTEIN TRANSLOCATION ACROSS THE PEROXISOMAL MEMBRANE

Peroxisomal proteins are synthesized on free ribosomes and posttranslationally imported into the organelle (reviewed in refs. *1, 3*, and *5)*. This property provides the foundations for the so-called "growth and division model" for peroxisome biogenesis (reviewed in ref. *20)*. According to this model, peroxisomes increase their masses by importing proteins from the surrounding cytoplasm; new peroxisomes arise by fission of preformed ones (*see* ref. *19)*.

Correct targeting of newly synthesized peroxisomal matrix proteins to the organelle is achieved by the existence of peroxisomal targeting signals (PTS). There are two well characterized types of PTS. By far, the vast majority of peroxisomal proteins possess the so-called peroxisomal targeting signal (PTS) type 1 (PTS1), a tripeptide with the sequence S-K-L (or a variant) present at the extreme C-terminus of these proteins *(21–25)*. A very small group of peroxisomal matrix proteins has a PTS type 2 (PTS2) *(26)*. PTS2 sequences are degenerated nonapeptides generally present at the N-terminus of these proteins that comply to the consensus sequence $(R/K)-(L/I/V)-X_5-(H/Q)-(L/A/F)$ *(27,28)*. Unlike the S-K-L signal, PTS2 sequences are often cleaved in the peroxisomal matrix *(26,27,29)*.

Undoubtedly the most striking property of protein translocation across the peroxisomal membrane is related to the folding state of the proteins that are transported across this membrane system. Indeed, peroxisomes are capable of importing already folded proteins *(30)*. Many peroxisomal enzymes are oligomeric proteins and, at least in some cases, it has been demonstrated that oligomerization takes place in the cytosol before the translocation step across the organelle membrane *(31,32)*. The elasticity of the peroxisomal import machinery (PIM) in accepting large passengers is best illustrated by the observation that 4–9 nm gold particles coated with PTS1-containing proteins can be imported into the organelle matrix in vivo *(33)*. This characteristic of the PIM is in sharp contrast with the

Table 1
The Core Components of the PIM

Peroxin	Main features (domains)	Subcellular localization	References
Pex1p	Member of the AAA ATPase family of proteins	Cytoplasmic/peroxisomal (membrane associated)	*(92,99,125–129)*
Pex2p	Contains a zinc RING finger domain	Peroxisomal intrinsic membrane protein	*(130–134)*
Pex5p	Member of the tetratricopeptide (TPR) family of proteins	Cytoplasmic and peroxisomal	*(130–134)*
Pex6p	Member of the AAA ATPase family of proteins	Cytoplasmic/peroxisomal (membrane associated)	*(93,99,138–141)*
Pex7p	Member of the beta-transducin-related family of proteins, also known as the WD-40 family	Cytoplasmic and peroxisomal	*(68,142–145)*
Pex10p	Contains a zinc RING finger	Peroxisomal intrinsic membrane protein	*(82,86,146,147)*
Pex12p	Contains a zinc RING finger	Peroxisomal intrinsic membrane protein	*(83–85)*
Pex13p	Contains a Src homology 3 (SH3) domain	Peroxisomal intrinsic membrane protein	*(52–54,71,148)*
Pex14p	Contains a coiled-coil motif	Peroxisomal intrinsic membrane protein	*(71,74,80,149)*

properties of protein translocation across other membrane systems. Indeed, the general rule for protein translocation across the membranes of mitochondria, chloroplasts, and endoplasmic reticulum is that proteins have to be in an unfolded conformation to be translocated across these membrane systems (reviewed in ref. *34*).

Pulse-chase experiments and peroxisomal import assays revealed two other important properties of the peroxisomal protein import process: the requirement for energy and cytosolic components *(35–37*; *see* The Mechanism of Protein Translocation Across the Peroxisomal Membrane).

PEROXINS DIRECTLY INVOLVED IN PROTEIN TRANSLOCATION ACROSS THE PEROXISOMAL MEMBRANE

From the almost 30 peroxins known, the majority has been suggested to be involved in the process of protein translocation across the peroxisomal membrane. However, from this group, only nine peroxins have been strictly conserved throughout the evolution (Table 1). This observation together with recent protein purification results (*see* The Architecture of the PIM) strongly suggest that these nine proteins constitute the core of this machinery. We will restrict our discussion to these peroxins.

Both functional and protein interaction data have been used to classify a peroxin as a member of the peroxisomal import machinery. First, in mutant cell lines lacking a component of this machinery PTS1- or PTS2-containing proteins are partially or completely mislocalized to the cytosol (reviewed in ref. *6*). The targeting of peroxisomal intrinsic membrane proteins is not (greatly) affected in these cell lines because the machinery catalyzing the insertion of these proteins into the peroxisomal membrane involves a different set of peroxins (reviewed

in refs. *1* and *5*). Second, several independent experimental approaches have revealed the existence of an intricate network of protein-protein interactions between components of the PIM (*see* ref. *38* and references cited therein). As discussed later, most peroxins belonging to this machinery can be coisolated as a stable protein complex *(39–41)*.

The most relevant properties of the PIM core components are presented in the following section.

Pex5p and Pex7p: The Shuttling Receptors

Newly synthesized PTS1-containing proteins are specifically recognized by Pex5p, the PTS1 receptor. Structurally, Pex5p can be divided into two domains. The carboxyl-terminal half of Pex5p contains seven tetratricopeptide repeats and binds the PTS1 signal present in the majority of the proteins to be transported into the matrix of the peroxisome *(24,42–49)*. The N-terminal half of Pex5p is involved in numerous protein interactions with other members of the PIM. The strongest interacting partner is Pex14p. Indeed, both in yeast and mammals, the Pex5p-Pex14p interaction is the only one resisting stringent solubilization conditions (*see* The Architecture of the PIM). One reason for the strength of this interaction is related to the fact that the N-terminal half of Pex5p has multiple Pex14p-binding sites, the so-called di-aromatic motifs. There are at least two motifs in yeast Pex5p and seven in mammalian Pex5p *(49–51)*. It was shown that each of the mammalian motifs binds Pex14p with high affinity (K_d values in the low nM range) *(51)*. The other reason stems from the fact that Pex14p interacts with itself (*see* the following section). Thus the Pex5p-Pex14p interaction is mutually multivalent. Pex13p is another PIM component binding to this region of the PTS1-receptor *(52–55)*. Data indicating that this interaction involves some of the di-aromatic motifs present in Pex5p have been reported *(49,55)*.

The third PIM component binding to the N-terminal half of Pex5p is Pex7p. This interaction was only observed with the mammalian

and plant peroxins *(56–60)*. In mammals, because of alternative splicing of the Pex5p transcript, two isoforms of Pex5p are produced, the so-called Pex5L and Pex5S *(56)*. Pex5L (the largest isoform) has an insertion of 37 amino acid residues when compared with Pex5S. This insertion creates a Pex7p-binding site in the N-terminal half of Pex5L *(56–59)*.

Our knowledge on the PTS2 import pathway is still very limited. It is generally accepted that PTS2-containing proteins are recognized by Pex7p, a member of the WD-repeat protein family *(28,61,62)*. However, it seems that Pex7p *per se* is not sufficient to target PTS2-containing proteins to the peroxisomal compartment. Indeed, in mammals this process requires Pex5L, suggesting that PTS2-containing proteins are also targeted to the organelle by the PTS1-receptor *(56–58)*. In this scenario, Pex7p could be seen as an adapter protein increasing the range of targeting signals recognized by mammalian Pex5p. In lower eukaryotes, the Pex7p-mediated protein import does not depend on Pex5p. Instead, two apparently redundant peroxins, Pex18p and Pex21p, in *S. cerevisiae (63)* or Pex20p in *Yarrowia lipolytica (64)* and *Neurospora crassa (65)* have been implicated in the PTS2 import pathway. Interestingly, these three peroxins have on their primary structure some of the features observed in mammalian Pex5p (e.g., a Pex7p-binding domain and di-aromatic motifs; *see* the following section) *(59,66)*. This observation and that no mammalian homologues of Pex18p, Pex20p, or Pex21p are known, led to the suggestion that, during evolution, the functions carried out by these proteins were transferred to Pex5p *(59,66)*.

One of the most interesting properties of both Pex5p and Pex7p concerns their subcellular localization. Indeed, using a variety of cell biology techniques, these receptors have been shown to exist both in the cytosol and peroxisomal compartment (reviewed in ref. *67*). These observations provided the cornerstone for the so-called cycling receptor model, a concept first applied to the peroxisomal biogenesis field by Kunau and coworkers *(68)*. According to this model, newly synthesized peroxisomal matrix

proteins are recognized by Pex5p or Pex7p while still in the cytosol. The receptor-cargo protein complex is then recognized by some components of the peroxisomal membrane. After releasing their cargo into the peroxisomal matrix, Pex5p and Pex7p are recycled back to the cytosol to catalyze further rounds of transportation. Data supporting this model were first provided by Dodt and Gould *(69)*: by manipulating the temperature and adenosine triphosphate (ATP) levels in cultured mammalian cells, the subcellular distribution of Pex5p could be reversibly altered. More recently, Dammai and Subramani have shown that Pex5p goes through multiple rounds of cycling between the cytosol and the peroxisomal compartment *(70)*. Additional evidence supporting this model will be discussed in the section The Mechanism of Protein Translocation Across the Peroxisomal Membrane.

Intrinsic Membrane Components of the PIM

Most components of the PIM are intrinsic proteins of the peroxisomal membrane. One of the best characterized is Pex14p. As stated previously, Pex14p interacts strongly with the di-aromatic motifs present in the N-terminal half of Pex5p *(49,71,72)*. The Pex14p domain involved in this interaction has been mapped to the first 78 amino acid residues of the human peroxin *(50)* and further refined in *Arabidopsis thaliana* and *Trypanosoma brucei* Pex14p *(60,73)*. Besides Pex5p, Pex14p also interacts with Pex13p, a SH3-containing intrinsic protein of the peroxisomal membrane *(71,74,75)*. The Pex14p-Pex13p interaction involves the SH3-domain of Pex13p and a P-X-X-P motif (the classical SH3 ligand) present in the N-terminal third of Pex14p *(55,71,76,77)*. Finally, Pex14p interacts with itself *(74,75)*. This di(or oligo)-merization of Pex14p is mediated by a coiled-coil domain *(38,78)*.

The primary structures of virtually all Pex14p proteins characterized to date reveal another interesting feature: the existence of a putative membrane-spanning domain. This property has been the subject of much contro-

versy between researchers in the field and, because of its importance in our understanding of Pex14p function, we will take more than just a few words in discussing this issue. There are both experimental and conceptual arguments feeding the debate of whether or not Pex14p is an intrinsic membrane protein. First, although Pex14p from most organisms behaves as an intrinsic membrane protein, some authors have suggested that yeast Pex14p is a peripheral membrane protein *(55,74,76)*. Such a conclusion derives from the observation that yeast Pex14p can be extracted from the peroxisomal membrane at alkaline pH *(74)*. Alkaline treatment of membranes is probably the most used method to classify a membrane protein as either peripheral (extractable) or intrinsic (nonextractable) to the membrane. It relies on the principle that molecular interactions occurring inside a biological membrane are not exposed to water and thus are pH-insensitive. For most membrane proteins, this premise is correct: their membrane-spanning domains are completely shielded from the aqueous environment either by membrane lipids or by other hydrophobic protein domains. However, the same may not apply to transmembrane proteins involved in channel formation across a biological membrane. In these cases, membrane-spanning domains are partially accessible to the aqueous environment. The behavior of such proteins on alkaline extraction may not be easily predictable and may depend on subtle factors. Thus inferring that a protein is not intrinsic to the membrane because it is extractable by alkaline treatment is a conclusion that should be taken with caution.

But the problem raised by the existence of a putative membrane-spanning domain in the primary structure of Pex14p goes beyond the interpretation of experimental results. Indeed, if we consider the order in the primary structure of Pex14p of the four domains described previously (i.e., NH2-Pex5p-binding domain, Pex13p-binding domain, putative membrane spanning domain, coiled-coil domain-COOH) and that the coiled-coil domain and the remainder carboxyl-terminal amino acid are exposed into the cytosol

(78–80), then the existence of a single membrane-spanning domain would imply that both the Pex5p- and Pex13p-binding domains of Pex14p face the lumenal side of the peroxisomal membrane. This domain topology may seem quite strange because the SH3 domain of Pex13p to which Pex14p binds is exposed into the cytosol *(52–54,81)* and Pex5p is supposed to dock at the peroxisomal membrane from the cytosolic side. Again, we would be assuming that Pex14p and all the other PIM components are typical membrane proteins having their membrane-spanning domains completely surrounded by the peroxisomal membrane. If, instead, we assume that these peroxins are subunits of a large protein translocase capable of accepting passenger proteins of different sizes and shapes, then there is no reason to exclude the possibility that many of these peroxin-peroxin interactions occur in the channel itself away from the surface of the membrane. It is likely that this is the reason why so many different membrane topologies have been proposed for the N-terminus of mammalian Pex14p *(78–80)*.

In addition to Pex13p and Pex14p, the PIM from all the organisms studied thus far contain three other intrinsic membrane proteins: Pex2p, Pex10p, and Pex12p. The main characteristic of all these three proteins is the existence of RING (really interesting new gene)-finger domain at their C-terminus. Membrane topology studies indicate that the RING-finger domains of Pex2p, Pex10p, and Pex12p are exposed into the cytosolic compartment *(82–89)*. Several protein-protein interactions involving these components of the PIM have been observed (reviewed in ref. *5*). It is likely that the strongest interactions are established between themselves (*see* The Architecture of the PIM). In addition, a direct interaction between Pex12p and the tetratricopeptide repeats-containing domain of Pex5p has been described *(90,91)*.

Extrinsic Membrane Components of the PIM

Structural and functional data regarding extrinsic membrane components of the PIM are still scarce. In fact, the function and the subcellular localization of two of these proteins, Pex1p and Pex6p, is still a matter of debate (reviewed in ref. *5*). Pex1p and Pex6p are members of the family of adenosine triphosphatase associated with various cellular activities (AAA ATPases) *(92,93)*. Considering that many of the proteins belonging to this family are involved in the disassembly of protein complexes (e.g., *N*-ethylmaleimide-sensitive factor; reviewed in ref. *94*), it has been proposed that Pex1p and Pex6p could be involved in recycling Pex5p back to the cytosolic compartment *(95)*. The attractiveness of this hypothesis resides basically in the fact that Pex1p and Pex6p are the only known peroxins having ATP-binding/hydrolysis domains, thus providing a possible explanation for the need of ATP in the process of peroxisomal protein import. However, a completely different role for these peroxins has also been proposed. According to Titorenko and Rachubinski *(96)*, both Pex1p and Pex6p could be involved in membrane fusion/fission events. Besides interacting with each other in several organisms *(97–99)*, Pex1p and Pex6p seem to bind to Pex5p, Pex10p, and Pex12p in *S. cerevisiae* (unpublished observations in ref. *100*). Two other peroxins in the protein-interaction map of Pex1p and Pex6p are Pex15p in *S. cerevisiae (101)* and Pex26p in mammals *(102)*. Both Pex15p and Pex26p are intrinsic components of the peroxisomal membrane and thus are thought to mediate the interaction of the AAA ATPases with this membrane system.

THE ARCHITECTURE OF THE PIM

Practically all the protein–protein interaction data presently known in the field of peroxisomal biogenesis were obtained using the yeast and bacterial two-hybrid system, in vitro binding assays with recombinant proteins, and nonquantitative immunoprecipitation experiments (*see* ref. *38* and references cited therein). These approaches revealed the existence of a complex network of protein–protein interactions between peroxins and, in many cases, it

was even possible to define the specific protein domains involved in these interactions. However, because of the nature of these techniques, no distinction between transient and stable protein interactions could be made. Thus, until very recently, our knowledge on the architecture of the PIM was very limited.

Data on this issue have been obtained by purifying and characterizing peroxin-containing protein complexes from the peroxisomal membrane of different model organisms. The first peroxin-containing complex to be isolated was the Pex5p-Pex14p protein assembly from rat liver peroxisomes *(39)*. Characterization of this complex revealed a Pex5p:Pex14p stoichiometry of approximately 1:5. No other component of the PIM was copurified with these two peroxins, an observation related to the stringent membrane solubilization conditions used in that work. Indeed, in a later study, solubilization of peroxisomal membrane proteins using the mild detergent digitonin led to the identification of a protein complex comprising, in addition to Pex5p and Pex14p, two RING-finger peroxins, Pex2p and Pex12p *(40)*. Pex13p was also detected in this protein complex but only in substoichiometric amounts. Similar findings were recently described for the *S. cerevisiae* peroxins *(41)*. Yeast Pex5p strongly interacts with an oligomeric Pex14p-Pex17p protein complex containing several Pex14p molecules (Pex17p homologues are not known in mammals) *(103)*. Under stringent solubilization conditions, no other peroxin was found in this protein complex. However, when digitonin was used in the same type of experiments, a much larger complex containing Pex5p, Pex14p, Pex17p, the RING-finger peroxins, and Pex8p (a peroxin found only in lower eukaryotes) *(104)* could be identified. Again, only a minor fraction of Pex13p was found in this protein complex. Finally, characterization of these peroxins in a mutant strain lacking Pex8p revealed the existence of a subcomplex containing only the RING-finger proteins *(41)*.

Although data of this kind are still missing for Pex1p and Pex6p, the perspective that emerged from these studies is that protein translocation across the peroxisomal membrane takes place through a single large protein assembly. This may seem a simple concept, but for many years hypothetical models assuming the existence of a cascade of protein–protein interactions resulting in independent Pex5p-containing complexes were frequent in the literature.

THE MECHANISM OF PROTEIN TRANSLOCATION ACROSS THE PEROXISOMAL MEMBRANE

Characterization of the mechanism of peroxisomal protein import has been quite a difficult task. The main reason for the lack of data regarding this topic is related to the fact that in vitro import systems have not been very popular in the field of peroxisomal biogenesis, a tendency that only now starts to change. Thus until very recently, our knowledge on the mechanism of protein translocation across the peroxisomal membrane was derived from data obtained using two different experimental approaches: steady-state level analysis of peroxisomal Pex5p in mutant cell lines lacking a particular PIM component and peroxin–peroxin interaction studies using the nonquantitative techniques cited previously. For instance, if deletion of the gene encoding a given Pex5p-interacting peroxin resulted in no detection of Pex5p at the peroxisomal membrane, then it was proposed that that peroxin could be involved in the docking step of the PTS1 receptor. Conversely, if Pex5p was detected at the peroxisomal membrane in a given peroxin-null mutant, then it was concluded that the missing peroxin was not involved in the docking step of Pex5p at the peroxisomal membrane but rather in some subsequent step. These kind of studies led to the classification of membrane PIM components into one of three families: peroxins involved in the docking step of the receptor-cargo protein complexes (Pex14p and Pex13p); peroxins involved in protein translocation across the peroxisomal membrane (the RING-finger peroxins); and peroxins promoting the

recycling step of the receptors back to the cytosol (Pex1p and Pex6p) (*see* ref. *95* and references cited therein). There are, however, some limitations inherent to this strategy. The major one is that the absence or malfunction of a given component of the PIM will cause also direct effects on other components of the protein complex. Thus even if all the remainder peroxins could be detected in these mutant cell lines at their normal levels (which is seldom the case; *see* refs. *69* and *95*), the possibility that the observed cell phenotypes result from a conformational alteration of some other PIM component cannot be ruled out. Although this uncertainty should not be taken as an argument to refute the results described previously, it clearly emphasizes the need of using additional independent approaches to define the function of each of the PIM components.

Surely one strategy is to characterize in detail peroxin–peroxin interactions using more refined in vitro and in vivo binding assays *(105)*. A good example of this is provided by Pex13p. This Pex5p- and Pex14p-binding peroxin was considered for a long time a member of the docking complex *(52,54,76)*. However, reexamination of the Pex5p-binding properties of Pex13p using a multicomponent in vitro binding assay revealed that Pex13p binds more strongly to free Pex5p than to cargo-loaded Pex5p *(49,72)*. Thus Pex13p seems to be involved not in the peroxisomal docking step of Pex5p but rather in some subsequent stage of the transport cycle.

Valuable information on the mechanism of protein translocation across the peroxisomal membrane has also been obtained by defining the membrane topology of the PIM components. Biochemical analysis of the peroxisomal pool of Pex5p illustrates this point. Peroxisomal Pex5p from several organisms are tightly bound to the peroxisomal membrane *(44,45,106)*. Surprisingly, protease protection assays using purified rat liver peroxisomes revealed that this pool of Pex5p is a transmembrane protein having the majority of its mass exposed into the lumen of the organelle and only a small N-terminal fragment facing the cytosol *(39,107)*. This finding supports a transport mechanism in which PTS1-containing proteins are translocated across the peroxisomal membrane by Pex5p itself.

But undoubtedly the most powerful approach to study the mechanism of protein translocation across any biological membrane relies on the use of in vitro import systems. Basically two different experimental systems have been described for peroxisomes: those that use mammalian semipermeabilized cells and those that employ isolated organelle fractions. Using these systems it has been shown that protein import into peroxisomes requires ATP hydrolysis, cytosolic components, and Zn^{2+}, and can be inhibited by anti-Pex14p antibodies *(36,71,108–117)*.

More recently, an in vitro system particularly suited to study the cytosol-peroxisomal trafficking of mammalian Pex5p was described *(107,118)*. It was shown that insertion of Pex5p into the peroxisomal membrane is cargo-protein and Pex14p-dependent. Strikingly, cargo-dependence on this process is only observed with the full-length version of Pex5p. Indeed, C-terminal truncated forms of the PTS1-receptor lacking any known cargo-protein binding domain are substrates for the peroxisomal machinery catalyzing docking and insertion of Pex5p into the peroxisomal membrane *(118)*. This observation confirmed and extended earlier findings made in living cells expressing similar truncated versions of Pex5p *(59)*. Perhaps the best explanation for this phenomenon is to assume that binding of cargo proteins to Pex5p induces conformational alterations on the PTS1 receptor resulting in the exposure of its peroxisomal targeting domain. That free Pex5p and cargo-loaded Pex5p display different binding affinities for both Pex13p and Pex14p *(49,72)*, together with data suggesting that the cargo-binding and peroxisomal targeting domains of Pex5p interact with each other *(119)*, strongly support this interpretation.

At the peroxisomal membrane level, two different populations of Pex5p were identified. In the presence of ATP, conditions in which Pex5p enters and exits the peroxisomal compartment *(107,120)*, the vast majority of peroxisomal

Pex5p displays the membrane topology described previously for Pex5p present in purified rat liver peroxisomes. This is the so-called stage 2 Pex5p. In the absence of exogenous nucleotides or in the presence of nonhydrolyzable ATP analogues, the existence of a second peroxisomal Pex5p population becomes apparent on protease protection assays. This population, the so-called stage 3 Pex5p, is completely resistant to proteinase K. In vitro pulse-chase experiments revealed that stage 2 is the precursor of stage 3 Pex5p and that this population of the PTS1-receptor leaves the peroxisomal compartment rapidly in the presence of ATP *(107)*.

Interestingly, both stage 2 and stage 3 Pex5p can be immunoprecipitated using anti-Pex14p antibodies. This observation led to the proposal that Pex14p remains in contact with Pex5p during most of the steps (if not all) occurring at the peroxisomal membrane *(107)*—a conclusion that, in the light of the protein interaction data discussed previously, is no longer surprising. It should be noted that this observation does not mean that Pex14p is the essential component of the PIM. In fact, the concept of "essential component" is probably not applicable to the membrane peroxins involved in protein translocation across the peroxisomal membrane. Indeed, the absence of any such peroxin results in a dramatic decrease in the process of peroxisomal protein import but, on the other hand, residual import activities can be detected in several peroxin null (Pex14p included) mutants *(83,99,104,121,122)*.

Finally, recent in vitro import experiments have shown that insertion of the PTS1-receptor into the peroxisomal membrane is ATP-independent *(120)*. This observation strongly suggests that the ATP-requiring step in protein import into the peroxisome occurs after the membrane translocation step. Thus it is possible that the driving force for protein translocation across the peroxisomal membrane resides on the several protein–protein interactions that Pex5p establishes with the membrane components of the PIM.

A working model for the process of protein translocation across the peroxisomal membrane is presented in Fig. 1. Free Pex5p interacts with already folded PTS1-containing proteins in the cytosol. In mammals, it is possible that PTS2-containing proteins also bind to Pex5p via Pex7p (*see* Pex5p and Pex7p: The Shuttling Receptors). Binding of cargo proteins to Pex5p induces conformational alterations in the PTS1 receptor exposing its peroxisomal targeting domain. In the second step, Pex5p-cargo protein complex docks at the peroxisomal membrane. The identity of the first peroxisomal peroxin contacting this Pex5p-containing complex is still unknown. Pex14p is a good candidate to perform this function: as stated previously, anti-Pex14p immunoglobulin G blocks peroxisomal protein import and the insertion of Pex5p into the peroxisomal membrane. However, other possibilities are equally feasible. After docking, the Pex5p cargo protein complex is partially translocated across the peroxisomal membrane. This step could involve a rotational movement of Pex5p at the level of the peroxisomal membrane and some rearrangement of the other components of the PIM.

After this movement, the cargo-protein binding domain of Pex5p, together with its passenger protein, are now exposed into the peroxisomal matrix. It seems reasonable to assume that at this stage the affinity of Pex5p for its cargo has to be decreased. In *S. cerevisiae*, it has been proposed that Pex8p could fulfill this role by acting as a cargo-protein releasing factor *(104)*. However, considering the Pex5p-binding properties of Pex13p (see previous section) and the proposed membrane topology for the peroxisomal pool of Pex5p it is obvious that Pex13p is also a good candidate to promote this step. Last, Pex5p is recycled back to the cytosol, a step that requires hydrolysis of ATP. This is surely the most obscure step in all this process. Besides all the doubts regarding the involvement of Pex1p and Pex6p in this step (*see* Extrinsic Membrane Components of the PIM), no data regarding the mechanism of this step are available. *A priori*, two different mechanisms leading to the release of Pex5p from the peroxisomal membrane could be envisaged: Pex5p could be pulled
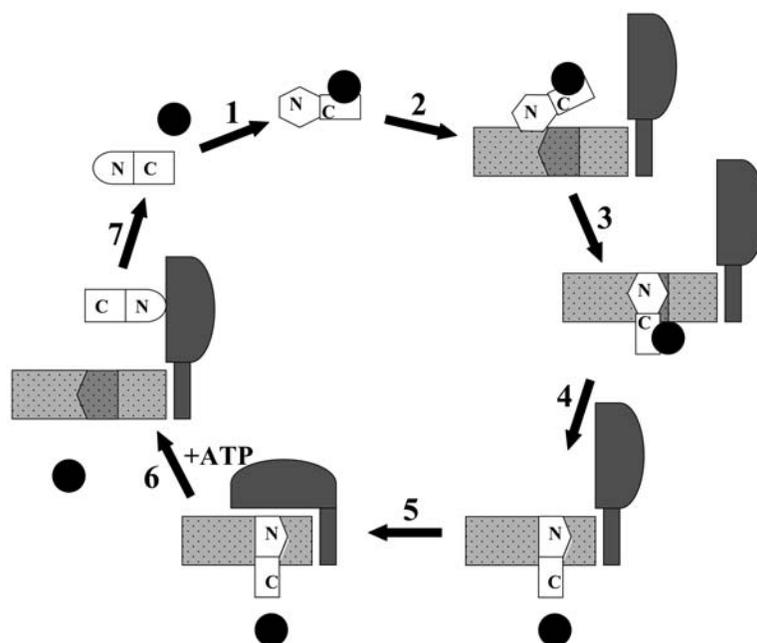
Fig. 1. Hypothetical model for protein translocation across the peroxisomal membrane. Step 1: Pex5p (white) binds PTS1-containing proteins (black circle) at its C-terminal domain (C in figure), resulting in a conformational alteration at the N-terminal half of Pex5p (N in figure). This change in the conformation exposes the peroxisomal targeting signal of Pex5p. In mammals, a Pex7p-PTS2-containing protein complex can also bind to the N-terminal half of the large isoform of Pex5p (not shown for simplicity). It is assumed that the fate of this Pex5p-Pex7p-cargo protein complex is the same as the one shown here for a Pex5p-cargo protein complex. Step 2: Docking of the receptor-cargo protein complex at the PIM membrane complex (gray dotted box). Core components of the PIM membrane complex are Pex2p, Pex10p, Pex12p, Pex13p, and Pex14p. The protein domain(s) involved in this initial interaction remain(s) unknown. Step 3: Translocation of the cargo protein across the peroxisomal membrane. Such an event could result from a rotational movement of Pex5p. Protein-protein interactions provide the driving force for this step. Step 4: Cargo release is accomplished because the affinity of Pex5p for its cargo is drastically diminished. Pex13p in mammals and Pex13p or Pex8p in yeast could be the releasing factor(s). Binding of Pex13p to the N-terminal half of Pex5p could change the conformation of this Pex5p domain. Step 5–7: Recycling of Pex5p back to the cytosol. This process requires ATP. Although the identity of the ATPase catalyzing this step (gray lollipop-like structure) has not been established, Pex1p and Pex6p are possible candidates. These peroxins are anchored to the peroxisomal membrane by Pex15p in yeast and Pex26p in mammals.

from the membrane by the ATPases or these ATPases could partially disassemble the PIM membrane complex.

Obviously, only after positive identification of the ATPase involved in this step and characterization of its protein-linkage map can the two mechanisms be discriminated.

The correlation between the peroxisomal Pex5p intermediates proposed in the model of Fig. 1 and the experimentally detected stage 2 and stage 3 Pex5p of mammalian peroxisomes is still speculative. It is possible that stage 2 Pex5p corresponds to the intermediate(s) obtained after step 3 or 4; stage 3 Pex5p could

correspond to the Pex5p intermediate(s) obtained after step 4 or 5.

FINAL REMARKS

The model presented in this article accounts for the vast majority of the data published. However, it should be noted that some authors have proposed that Pex5p is completely translocated across the peroxisomal membrane during its transport cycle (reviewed in ref. *123*). To the best of our knowledge, the only data that could support such mechanism excluding, at the same time, the one proposed previously, derive from the observation that, in *Hansenula polymorpha*, a small amount of Pex5p is detected in the peroxisomal matrix by immunogold electron microscopy *(121,124)*. However, this labeling technique also reveals the presence of Pex5p in the nucleus and in other membrane-bound compartments. Thus the biological meaning of this intraperoxisomal pool of Pex5p remains enigmatic.

It is evident that our knowledge on the process of peroxisomal protein import has increased dramatically in the past few years. Most peroxins have been identified and their interactions are being characterized. The new methodologies developed recently are providing new insights into the structure and function of the PIM components. Particularly promising is the protein purification strategy described recently for *S. cerevisiae* peroxins *(41)*. The availability of unlimited amounts of peroxin-containing complexes opens a completely new door in the peroxisomal biogenesis field. Reconstitution experiments, the ultimate goal in molecular biology, can now be started. Only then can we believe in and understand all the protein interaction data published.

In the meanwhile, we can only recognize that we are still very far from understanding all the facts of protein translocation across the peroxisomal membrane and that many fundamental questions are still waiting for an answer. What is the reason for two peroxisomal import pathways? What is the advantage in having cycling receptors? How many of the peroxins identified to date are mechanistically involved in protein translocation across the peroxisomal membrane? Are there quality control systems monitoring this process? Research on this topic will continue to be a great challenge.

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