Chaperone proteins and peroxisomal protein import

Wim de Jonge, Henk F. Tabak, and Ineke Braakman

Abstract

Peroxisomes are ubiquitous organelles present in most eukaryotic cells. Their role in cellular metabolism is diverse among species. An array of genes involved in the formation and maintenance of peroxisomes has been discovered, and can be categorised into genes important for protein import into the peroxisome and genes involved in the maintenance of the organelles' size and abundance. Thorough cell biological and biochemical studies revealed great detail about the process of peroxisomal protein import. Although involvement of several classes of molecular chaperone proteins in peroxisomal protein import has been demonstrated, details regarding the mechanistic aspects of chaperone involvement in this process are not known yet. This review aims to discuss peroxisomal maintenance, with the emphasis on protein import. A general overview of chaperone proteins and their role in protein import processes will be used as context to discuss the - possible - roles of chaperone proteins in peroxisomal protein import.

1 Introduction

When a protein is synthesized, it faces many difficulties on the pathway to its native, fully folded structure and proper subcellular localisation. Although the roadmap of this pathway is determined by the linear amino-acid sequence of a protein, without help, its maturation will not occur at a high efficiency in a living cell. At this stage, chaperone proteins come into play. Analogous to their human equivalent, they protect the “infant” protein from unwanted inter- and intramolecular interactions in a crowded cellular milieu, ensuring efficient maturation. Chaperones come in various flavours (Hartl and Hayer-Hartl 2002) and similar chaperone systems are found in all kingdoms (Fig. 1).

It is, therefore, easy to envisage that chaperone proteins play an essential role in the living cell. A number of biomolecular processes are supported by chaperone proteins, such as folding and oligomerisation of newly synthesized proteins, protection of proteins from denaturation by environmental stress, protein import into organelles, targeting of proteins for degradation, modulation of protein-protein interactions, and regulation of translation, transcription, and signal transduction pathways.

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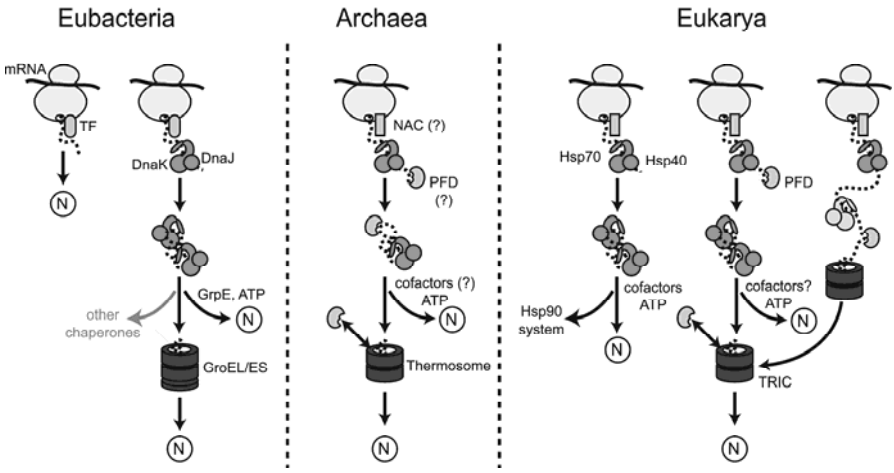

Fig. 1. Variety in chaperone systems. Newly synthesised proteins exit the ribosome, where they first meet ribosome associated chaperones such as trigger factor (TF) in Eubacteria, and nascent chain associated complex (NAC) in Eukarya and possibly in Archaea. Hsp70/40 (DnaJ/K in Eubacteria and Archea) are involved in folding of a part of the pool of newly synthesised proteins, and hand over some proteins to either GroEL/ES in Eubacteria, or similar chaperone systems in Archaea (thermosome) and Eukarya (TRIC). In the latter case, prefoldin serves as a 'targeting factor' for newly synthesised and folding proteins that are client proteins for the TRIC chaperone. Finally, in Eukarya a specific subset of proteins depends on the Hsp90 system for maintaining a proper conformation (Figure adapted and modified after Hartl and Hayer-Hartl 2002).

Chaperones are located in the cytosol and the lumenal cavities of various organelles with the possible exception of peroxisomes. Proteomics studies did not reveal chaperones, except a Lon homolog in mammalian peroxisomes (Kikuchi et al. 2004). Here the chaperones already contribute in the cytosol, which is made possible by the fact that the peroxisomal protein import machinery can accommodate proteins that have attained some conformation. The focus of attention in this review is particularly devoted to the role of chaperones in the formation and maintenance of these remarkable organelles.

2 Peroxisomes

2.1 Formation, maintenance, and function

Peroxisomes are single-membrane bounded organelles present in all eukaryotic kingdoms, with a variety in appearance and metabolic function. Originally, the organelle was defined as containing hydrogen peroxide (H_2O_2) producing oxidases, and the H_2O_2 detoxifying enzyme catalase, hence the name '*peroxi*some'. Examples of peroxisomal metabolic processes are β-oxidation of fatty acids, parts of the

Table 1. Peroxins and their main characteristics Abbreviations used: (species) Mamm - Mammals, Y - Most yeast species, Sc - *Saccharomyces cerevisiae*, Yl - *Yarrowia lipolytica*, put - putative. (localisation) lum - peroxisomal lumen (matrix), im - integral membrane, pm - peripheral membrane, cyt - cytosol, ? - unresolved or conflicting literature reports.

Peroxin	Remarks	Localisation	Species
Pex1p	AAA ATPase	cyt, pm	Y, Mamm
Pex2p	RING protein, translocase?	im	Y, Mamm
Pex3p	Membrane protein stability	im	Y, Mamm
Pex4p	Ubiquitin conjugating enzyme	pm	Y
Pex5p	PTS1 receptor	cyt, pm, im, lum (?)	Y, Mamm
Pex6p	AAA ATPase	cyt, pm	Y, Mamm
Pex7p	PTS2 receptor	cyt, pm, lum (?)	Y, Mamm
Pex8p	PTS1 & PTS2	pm, lum	Y
Pex9p	Unknown function	im	Yl
Pex10p	RING protein, translocase?	im	Y. Mamm
Pex11p	Proliferation	im, pm	Y, Mamm
Pex12p	RING protein, translocase?	im	Y, Mamm
Pex13p	SH3 domain, docking complex	im	Y, Mamm
Pex14p	Docking complex	im, pm	Y, Mamm
Pex15p	Phosphorylated	im	Sc
Pex16p	Membrane protein import	im	Yl, Mamm
Pex17p	Docking complex	im	Sc
Pex18p	Pex7p co-receptor	cyt	Sc
Pex19p	Membrane protein import	pm, cyt	Sc
Pex20p	Thiolase import	cyt, pm	Yl
Pex21p	Pex7p co-receptor	cyt	Sc
Pex22p	Pex4p anchoring	im	Sc
Pex23p	Protein import	im	Yl, Sc (put)
Pex24p	Protein import	im	Yl, Sc (put)
Pex25p	Protein import, proliferation	pm	Sc
Pex26p	Protein import	im	Mamm
Pex27p	Protein import, proliferation	pm	Sc
Pex28p	Proliferation	im	Sc
Pex29p	Proliferation	im	Sc
Pex30p	Proliferation	im	Sc
Pex31p	Proliferation	im	Sc
Pex32p	Proliferation	im	Sc
Djp1p	Hsp70 co-chaperone, protein import	cyt	Sc

glyoxylate cycle or the glycolytic pathway, photorespiration, methanol catabolism, and penicillin synthesis (Hettema 1998). Although the metabolic functions of peroxisomes are diverse among species, the basic mechanism to maintain the organelle in the cell is conserved throughout evolution. This became apparent upon characterisation of the first serendipitously identified peroxisomal targeting signal (PTS1) of luciferase, an enzyme located in the lantern organ of fireflies, which

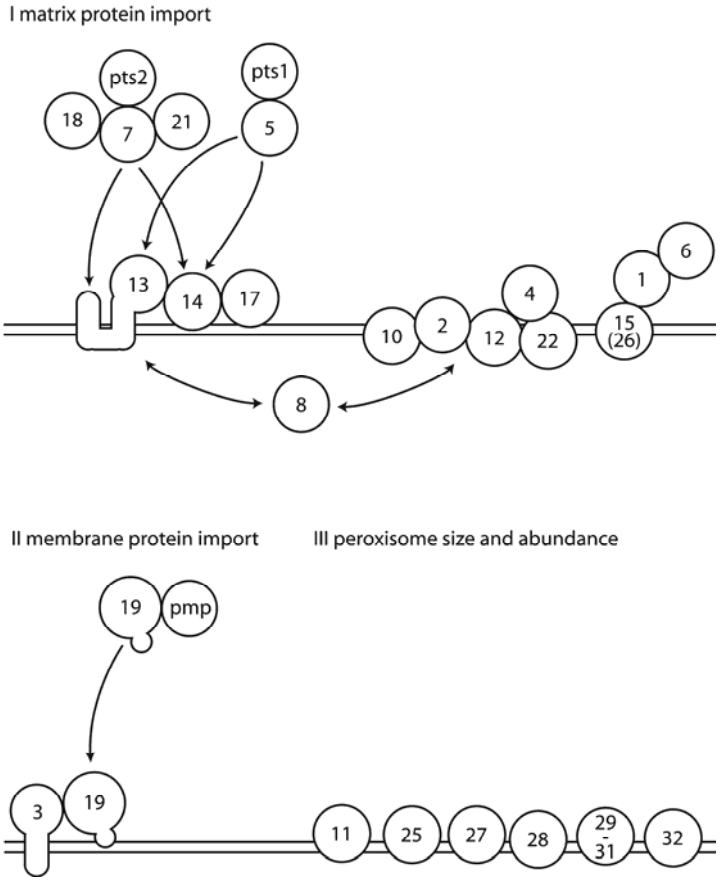

Fig. 2. Overview of peroxins and their interactions. Panel I shows peroxins involved in the import of lumenal proteins. The role of *S. cerevisiae* Pex15p, i.e. tethering Pex1p/Pex6p to the membrane, is performed by Pex26p in mammals. Panel II shows the few proteins involved in peroxisomal membrane protein import. Panel III lists the peroxins that can influence size and abundance of the organelle. Peroxins are indicated by their number (e.g. Pex7p is indicated by a 7). Arrows indicate reported interactions. PTS1 - peroxisomal targeting signal type 1, PTS2 - peroxisomal targeting signal type 2.

was shown to be able to direct a reporter protein to peroxisomes of yeast, mammalian, plant, and insect peroxisomes (Gould et al. 1989, 1990).

Since these initial studies, a series of genetic screens resulted in the characterisation some thirty genes involved in the maintenance of peroxisomes, collectively called peroxins (Distel et al. 1996). A summary of peroxins and their characteristics can be found in Table 1 and Figure 2.

From the early steps in import to the translocation of proteins into the peroxisome, several steps must occur, separated in space and time. In each of these steps, different complexes form and dissociate. In the following paragraphs, an

overview of the general properties of peroxisomal protein import is presented. Next, some of the discrete steps in the import process are discussed.

2.2 General properties of protein import

What are the general requirements for peroxisomal protein import? When ATP is depleted import cannot occur in *in vivo*, and in semi *in vitro* experimental systems (Bellion and Goodman 1987; Behari and Baker 1993; Soto et al. 1993; Wendland and Subramani 1993). Because Hsp70, Hsp90, Pex1p, Pex6p, and ANT1 (PMP34) all are ATPases involved in maintenance of, or import into, peroxisomes this is not a surprising finding.

Reports about the intraperoxisomal pH give conflicting results, the intraperoxisomal pH varying from alkaline in human fibroblasts (Dansen et al. 2000), filamentous fungi (van der Lende et al. 2002), and yeast (Van Roermund et al. 2004) to neutral in CHO cells and fibroblasts (Jankowski et al. 2001). It is unclear what causes these differences in pH measurements. Different approaches were used, employing fluorophores coupled to a PTS1, and pH sensitive forms of GFP fused to a PTS1.

In mitochondria, the pH gradient across the outer membrane is essential for the import of mitochondrial precursor proteins. Ionophores did inhibit peroxisomal protein import *in vivo*, suggesting the involvement of a proton gradient in peroxisomal protein import (Bellion and Goodman 1987). Ionophores, however, also diminish the proton gradient across the mitochondrial outer membrane, and thereby, block ATP generation. Therefore, as the authors suggested, the effects observed *in vivo* may be indirect, via ATP depletion. This result is supported by the observation that, in a mammalian peroxisomal import assay using semi-permeabilised cells, ionophores did not inhibit import (Wendland and Subramani 1993). In human fibroblasts, in the absence of import of a subset of peroxisomal proteins, the pH gradient is diminished. In this situation, a peptide containing a peroxisomal targeting signal coupled to a fluorophore could still be imported into peroxisomes (Dansen et al. 2000). Although it is as yet unclear whether a pH gradient across the peroxisomal membrane exists, it is unlikely that it plays a role in the import of peroxisomal proteins.

2.3 Proteins involved in protein import

2.3.1 PTS1 protein import

Genes encoding lumenal and membrane peroxisomal proteins are encoded by the nuclear DNA, implying the requirement for a transport machinery that can specifically recognise these proteins and direct them to the peroxisome. At least two different targeting signals have been identified. The canonical peroxisomal targeting signal type 1 (PTS1) consists of the C-terminal tripeptide Serine-Lysine-Leucine or a conserved variant thereof. PTS1 is able to direct reporter proteins to the per-

oxisomal compartment of many species, but variants of the signal are not always functional across species (Motley et al. 1995; Elgersma et al. 1996b). Detailed mutagenesis studies on the tolerance of deviations from the generic -SKL sequence revealed a role for amino acids upstream of less conserved variants of PTS1 (Elgersma et al. 1996b; Lametschwandtner et al. 1998; Neuberger et al. 2003).

The PTS1 signal can be recognised by Pex5p, a soluble protein that is mainly present in the cytosol. *In vitro* binding studies show convincingly that Pex5p can bind PTS1 independently of other proteins (Fransen et al. 1995; Terlecky et al. 1995; Wiemer et al. 1995a; Klein et al. 2001). The Pex5p-PTS1 interaction appears to be the first specific step in peroxisomal protein import.

The domain responsible for PTS1 recognition is formed by two sets of three TPR motifs in the COOH-terminal half of Pex5p, connected by a TPR motif-like linker region. TPR motifs are formed by 34 amino acids long antiparallel pairs of alpha-helices, connected by loops, that form a superhelical structure. Mutational analysis of the TPR motifs, combined with homology modelling, revealed a role for residues in the intra-repeat loops of TPR2 and TPR3 in anchoring the lysine at position 2 of SKL, and for nearby residues in recognising the additional residues of the PTS1. In TPR4-7, a set of asparagine residues can be found that could be involved in interactions with the peptide backbone (Klein et al. 2001). These results were in agreement with the structure of the TPR region of human Pex5p, co-crystallized with a PTS1-peptide (Gatto et al. 2000). In the light of more recent results on the importance of residues upstream of PTS1, this is not the complete picture. Co-crystallisation of a PTS1 protein with Pex5p will explain how proteins with a low affinity PTS1 use accessory amino acid residues to ensure proper recognition by Pex5p.

2.3.2 PTS2 protein import

A less common targeting signal is the PTS2, defined as a nonapeptide close to or at the N-terminus with the consensus sequence R/K-L/V/I-X_5-H/Q-L/A (Subramani 1996). PTS2 is necessary and sufficient to direct a reporter protein to peroxisomes (Swinkels et al. 1991; Gietl et al. 1994; Tsukamoto et al. 1994). The few proteins possessing a PTS2 are transported to the peroxisome via the PTS2 receptor Pex7p, characterised by the presence of WD-40 repeats. Pex7p mediated PTS2 import is assisted by either Pex18p/Pex21p (*S. cerevisiae*), Pex20p (*Y. lipolytica*) or by an alternatively spliced form of Pex5p (mammals). For details, see Section 2.4. Mutational analysis of Pex7p was performed in two independent studies, yet in contrast to the PTS1 - Pex5p studies, detailed information on structural aspects of Pex7p – PTS2 recognition is not available yet.

Besides PTS1- and PTS2-mediated import, additional targeting signals have been reported. This concerns rare cases where proteins have found alternative ways to enter peroxisomes. (Interested readers are referred to Klein et al. 2002; Titorenko et al. 2002)

2.3.3 mPTS protein import

Initial research on defining the targeting signal for membrane proteins gave an incomplete picture (Dyer et al. 1996; Jones et al. 2001). Recently, more details about targeting signals for peroxisomal membrane proteins (PMPs) emerged (Rottensteiner et al. 2004). The core sequence was identified as a helical nonapeptide, containing hydrophobic and positively charged amino acids. Although this core sequence is necessary for proper targeting, at least one additional transmembrane region is required for specific insertion into the membrane.

Peroxisomal membrane protein (PMP) insertion depends on Pex3p and Pex19p; in the absence of either one of them, PMPs are mislocalised to the cytosol, and rapidly degraded (Hettema et al. 2000; Jones et al. 2004). Pex3p is an integral membrane protein, and Pex19p is predominantly cytosolic with a small fraction located at the peroxisomal membrane. In cells lacking Pex3 or Pex19p, peroxisomes are absent, in contrast to the peroxisomal 'ghosts' observed when matrix protein import is blocked. When Pex3p, respectively Pex19p, is re-introduced in these cells, peroxisomes arise again (Hettema et al. 2000; Sacksteder et al. 2000). Pex19p has been shown to transiently bind newly synthesized PMPs, and requires Pex3p for membrane localisation (Fang et al. 2004; Jones et al. 2004). The current view is that of Pex19p as a combined targeting receptor – specialised chaperone for PMPs, which uses Pex3p as a docking factor at the acceptor membrane.

2.4 Formation of peroxisomes

The formation of peroxisomes has been a matter of debate since decades, the two extremes being a division model, where peroxisomes multiply by growth and division, and a pathway where the peroxisomal membrane is derived from pre-existing membranes of the endoplasmic reticulum (ER) (Lazarow and Fujiki 1985).

Support for the ER-peroxisome connection was found in morphological studies employing electron microscopy, showing close proximity of peroxisomes to the ER (Novikoff and Novikoff 1972). More recently, direct connections between rough ER and peroxisome precursor structures were detected, shedding new light on the ER-peroxisome connection (Geuze et al. 2003; Tabak et al. 2003).

Attempts to implicate factors involved in classical secretory vesicle transport in the formation of peroxisomes failed (South et al. 2000, 2001; Voorn-Brouwer et al. 2001). In addition, time lapse fluorescence microscopy revealed that, in *S. cerevisiae*, peroxisomes divide and are transported to the daughter cell, dependent on Vps1p (a member of the dynamin family, and the motor protein Myo2p, respectively (Hoepfner et al. 2001). A similar role in peroxisomal fission was found for Dlp1p in mammalian cells (Koch et al. 2003; Li and Gould 2003).

Logically, a model emerges combining both ideas: peroxisomal membranes are derived from the ER via a non-classical secretion route, grow in size, and divide using a machinery dependent on dynamin-like proteins (Vps1p in yeast, Dlp1p in mammals).

2.5 Recycling receptor import model

2.5.1 The Pex5p shuttle

Discussion about functioning of Pex5p has mainly focused on localisation of the protein. Most groups report a predominantly cytosolic localisation for Pex5p, with a small fraction associated with peroxisomes (Van der Leij et al. 1993; Dodt et al. 1995; van der Klei et al. 1995; Wiemer et al. 1995b). The dual localization of Pex5p led to the proposal of the recycling receptor model: Pex5p guides peroxisomal proteins from the cytosol to the peroxisomal membrane, where import can occur. After delivery of the peroxisomal protein, Pex5p recycles to the cytosol, where it can pick up another peroxisomal protein.

ATP depletion and low temperature block peroxisomal protein import *in vivo*. Gould and co-workers found that, when studying import into peroxisomes of fibroblasts, these conditions led to a shift in Pex5p localisation from the cytosol to the peroxisome. Taking away these import-blocking conditions resulted in redistribution of Pex5p to the cytosol (Dodt and Gould 1996). Another condition that caused Pex5p location to the peroxisome is mutation of *PEX2* or *PEX12*.

2.5.2 The Pex5p extended shuttle

Pex5p was also found inside the peroxisome matrix, as shown by electron microscopy (Szilard et al. 1995; van der Klei et al. 1995). These results led to the notion of an extended recycling receptor model, where Pex5p traverses the membrane twice: once with PTS1-containing protein into the organelle, and the second time empty to return to the cytosol (Dodt and Gould 1996). This would be a unique situation since, thus far, only the nuclear pore complex has been shown to allow reversible transport of proteins involved in protein cargo movement.

One study aiming to support the extended recycling receptor model used a protease-recognition site, which is specifically cleaved by an intraperoxisomal protease, combined with an epitope tag fused to the NH_2-terminus of mammalian Pex5p (Dammai and Subramani 2001). Using antibodies that exclusively recognised the cleaved, exposed epitope tag, the authors showed that Pex5p is exposed to the inside of peroxisomes, and recycles back to the cytoplasm. The authors used biochemical criteria to show that Pex5p is present in the peroxisomal matrix, but these results were not conclusive. They did show that the NH_2-terminal region of Pex5p is exposed to the peroxisomal lumen during the import cycle. As an alternative explanation for this result, Pex5p could function similarly to the bacterial SecA protein. SecA "pushes" proteins through the SecYEG translocon at the cytoplasmic membrane of bacteria by a piston-like motion (Economou and Wickner 1994). Perhaps the NH_2-terminus of Pex5p functions as such a piston, explaining why it is at least partially exposed to the peroxisomal lumen. Results from elegant *in vitro* studies, showing the presence of Pex5p inserted into the peroxisomal membrane in different “stages” characterised by differential sensitivity to proteases, might reflect this piston-like motion in the translocation process of peroxisomal proteins (Gouveia et al. 2003).

2.5.3 The Pex7p shuttle

As Pex5p, Pex7p was reported to be present in the cytosol, at the peroxisomal membrane, and inside the peroxisome (Marzioch et al. 1994; Zhang and Lazarow 1994; Elgersma et al. 1998). Upon deletion of *FOX3*, the gene encoding 3-ketoacyl-CoA thiolase, the only PTS2 protein in yeast, Pex7p could not be detected in the peroxisomal fraction anymore, suggesting that binding of Fox3p enhances peroxisomal localisation of Pex7 (Marzioch et al. 1994). As expected, Pex7p and Fox3p could be co-immunoprecipitated from a yeast lysate, indicating that Pex7p and Fox3p interact (Rehling et al. 1996).

Pex7p was co-immunoprecipitated with peroxins that are involved in later steps of the import pathway: Pex14p, Pex17p, and Pex13p. Interestingly, deletion of any of these genes increases the pool of Pex7p-associated Fox3p, strongly suggesting the accumulation of Fox3p in a pre-import stage prior to docking (Girzalsky et al. 1999).

Pex18p and Pex21p are two other proteins involved in the PTS2 pathway. Because they are redundant in function, they were not identified in screens selecting for import defects, but instead by using a two-hybrid approach to identify binding partners of Pex7p. Upon deletion of both *PEX18* and *PEX21*, import of Fox3p is abolished, and Pex7p is not found in the peroxisomal fraction upon subcellular fractionation (Purdue et al. 1998). These results led the authors to suggest a chaperone-like role for Pex18p/Pex21p in guiding Pex7p to the peroxisomal membrane. Because loss of PTS2 protein also abolishes Pex7p association to the peroxisomal fraction, Pex18p and Pex21p, alternatively, may be involved in enhancement of the PTS2-Pex7p interaction, thereby, indirectly enhancing peroxisomal association of Pex7p. Pex18p and Pex21p are not found in higher eukaryotes, where an alternatively spliced, longer form of Pex5p (Pex5L) appears to take over their role (Dodt et al. 2001). In the absence of Pex5L, PTS2 containing proteins are not imported, and Pex5Lp interacts directly with Pex7p (Braverman et al. 1998; Matsumura et al. 2000).

The PTS2 pathway is not conserved throughout all eukaryotes. It is absent in *Caenorhabditis elegans*, and proteins that contain a PTS2 in other organisms have acquired a PTS1 in C. elegans (Motley et al. 2000). Another example of evolutionary divergence in the PTS2 pathway occurs in the yeast *Yarrowia lipolytica*. Here, peroxisomal thiolase is targeted to the peroxisome in the form of a hetero-tetramer containing two thiolase molecules, and two Pex20p molecules. Pex20p is only found in *Y. lipolytica*, and is the only protein that interacts with thiolase in this particular model organism (Titorenko et al. 1998). Pex20p was suggested to be the ortholog of *S. cerevisiae* Pex18p/Pex21p, and Pex5L in mammals (Einwachter et al. 2001). This is likely because Pex18p and Pex21p were duplicated in *S. cerevisiae* (Dietrich et al. 2004). In addition, *Neurospora crassa* Pex7p acts together with a *Y. lipolytica* Pex20p in PTS2 import (Sichting et al. 2003). A Pex7p homolog has not yet been discovered in *Y. lipolytica,* however, allowing a role of Pex20p as the principal import receptor for PTS2 proteins.

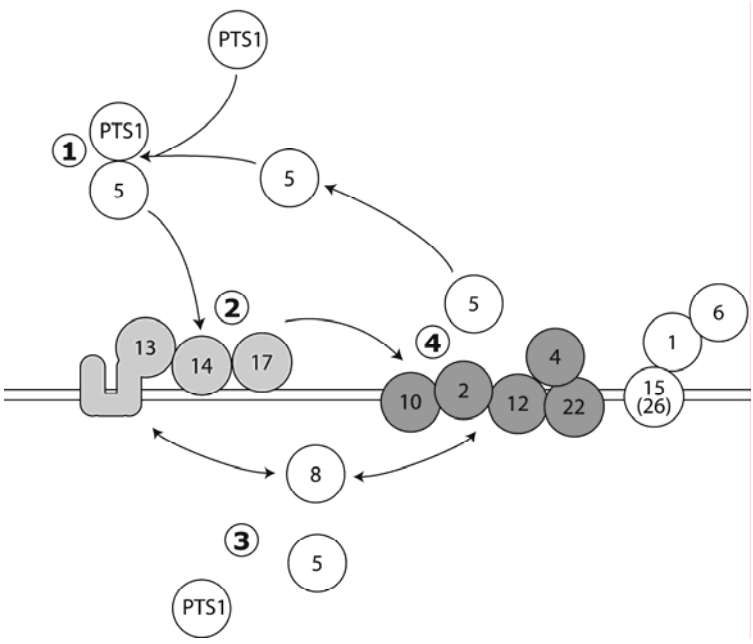

Fig. 3. The peroxisomal protein import cycle. 1. Binding of PTS1 containing cargo to the import receptor Pex5p. 2. Docking of the cargo-receptor complex onto a membrane protein complex formed by Pex13, Pex14p, and Pex17p. 3. Translocation of cargo and receptor across the peroxisomal membrane and subsequent retro-translocation of receptor. Alternatively, translocation of cargo, and 4. recycling of receptor to the cytosol for a new round of import. Arrows indicate movement and formation/dissociation of protein complexes as cargo and receptor progress through the import cycle. Import receptor and the complexes indicated in light grey and dark grey correspond to membrane subcomplexes I and II (See Section 2.6) whose interactions are orchestrated by the intraperoxisomal Pex8p.

2.6 Docking and translocation of peroxisomal proteins

After the initial recognition of the PTS-containing cargo proteins by the cognate receptor, the cargo-receptor complex can interact with proteins at the peroxisomal membrane, a stage in import referred to as docking. Subsequently, the PTS protein will enter the peroxisome, and the receptor will return to the cytosol. This requires two other functionalities: translocation of the cargo, and recycling of the import receptor.

Initial studies suggested that the "docking protein" for Pex5p is Pex13p, an integral peroxisomal membrane protein that contains an SH3 domain (Elgersma et al. 1996a; Erdmann and Blobel 1996; Gould et al. 1996). Later studies revealed that Pex14p is the principal protein on which receptor-cargo complexes dock, and where the PTS1 and PTS2 pathways converge: Pex14p can bind both Pex5p and Pex7p (Albertini et al. 1997). Several interaction studies revealed a complex net-

work of protein-protein interactions, suggesting a cascade of interactions performing the aforementioned functionalities (Agne et al. 2003) (Fig. 3).

A recent investigation using purified components showed that Pex5p loaded with PTS1 peptide preferentially interacts with Pex14p, whereas the empty receptor preferentially interacts with Pex13p (Urquhart et al. 2000). This result suggests that when PTS-containing proteins are "handed over" to the translocation machinery, Pex5p possibly relocates to Pex13p. This study used isolated proteins, and might therefore not represent the events as they occur in protein complexes at the peroxisomal membrane. This caveat becomes more apparent when we consider the complexity of protein assemblies at the peroxisomal membrane, as characterised recently and discussed below (Agne et al. 2003).

Gould and co-workers used the differential instability of Pex5p in some *pex* mutants in an attempt to dissect the sequence of events at the peroxisomal membrane (Collins et al. 2000). The authors classified the import mutants into three major groups: the “docking proteins” (Pex13p, Pex14p), the “translocating” proteins (Pex2p, Pex10p, Pex12p), and the proteins involved in recycling of Pex5p (Pex4p, Pex22p, Pex1p, Pex6p).

Studies on purified protein complexes from rat liver peroxisomes show the existence of two large protein complexes within the peroxisomal membrane. Pex5p and Pex14p form a "core complex", which is more stable than the larger complex found by the same group, which contains Pex2p, Pex12p, Pex5p, and Pex14p (Gouveia et al. 2000; Reguenga et al. 2001). Similar complexes have been found in yeast using affinity purification of subcomplexes from peroxisomal membranes, in an attempt to dissect the sequence of events at the peroxisomal membrane (Girzalsky et al. 1999; Johnson et al. 2001; Hazra et al. 2002). A recent study in *S. cerevisiae* demonstrated a central role for the intraperoxisomal peroxin Pex8p in organising the import-complexes in the peroxisomal membrane (Agne et al. 2003). The authors suggest a working model where the Pex14p/Pex5p/Pex13p/Pex17p complex (I) is the actual translocon and the Pex2p/Pex10p/Pex12p complex (II) likely plays a role in recycling or in exporting Pex5p to the cytosol. Pex8p was suggested to transfer Pex5p from complex I to complex II, while Pex5p is present in the peroxisomal lumen. A model, combining most results discussed above, is shown in Figure 3.

The models are drawn using Pex5p as the cycling receptor. Pex7p likely follows a similar route, depending on the model organism used. In mammals, Pex7p probably moves with Pex5p through the import cycles, since Pex5p and Pex7p physically associate (see previous paragraph). In yeast, at least the early steps in import are similar, with a protein assembly like subcomplex I, where Pex7p replaces Pex5p. The topology of Pex7p in this membrane complex is unknown as yet. It will be informative to know whether Pex7p, like Pex5p, is inserted in a membrane complex, or remains peripherally associated to the import complexes. Both PTS1 and PTS2 are translocated into the peroxisomal lumen, and therefore, Pex5p and Pex7p likely share the machinery for translocation. Within the context of current knowledge about the two import processes, the core translocon is probably formed by (a) component(s) of subcomplex I.

Although many protein interactions have been charted, insight into the real mechanics of protein translocation across the peroxisomal membrane is lacking. A reliable *in vitro* protein import system is much needed to increase our knowledge.

2.7 Folding state and import of peroxisomal proteins

As most peroxisomal lumenal proteins possess a C-terminal targeting signal, import is a posttranslational event. Already in 1973, Lazarow and de Duve showed that most peroxisomal proteins are synthesised on free polyribosomes (As discussed in Lazarow and Fujiki 1985). This result is underlined by *in vivo* pulse-chase experiments: the import substrates are soluble for quite a long time before they get imported, dependent on the protein concerned (Ruigrok et al. in preparation). Although the protein import machinery of peroxisomes can accommodate at least partially folded proteins, it remains an open question to which extent proteins are folded as they cross the peroxisomal membrane.

At least some degree of folding appears to be tolerated by the peroxisomal import machinery *in vivo*. Convincing experiments showed that, in *S. cerevisiae*, the homodimeric enzyme peroxisomal thiolase is imported as a dimer, and that subunits do not mix during the import process. Tagged thiolase lacking a targeting signal could only be recovered as a heterodimer in the peroxisomal fraction, an observation that is highly indicative of "piggy back" translocation of the thiolase lacking the targeting signal (Glover et al. 1994). An often used experimental approach to study the impact of folding on import into mitochondria and the endoplasmic reticulum employs dihydrofolate reductase (DHFR), a protein of which the structure can be stabilised *in vivo* by aminopterin, and *in vitro* by methotrexate. Aminopterin was shown not to prevent import into peroxisomes of DHFR fused to a PTS (Hausler et al. 1996).

Another indication that the translocon of peroxisomes is substantially different from ER and mitochondrial import pores is demonstrated by Danpure's work on alanine:glyoxylate aminotransferase 1 (AGT). AGT is a dimeric protein containing both a peroxisomal and a mitochondrial import signal. Wild type AGT dimerises quickly in the cytoplasm, and is imported into peroxisomes. A naturally occurring mutant, causative for the human disease primary hyperoxaluria 1, prevents dimerisation, resulting in mistargeting of all AGT into mitochondria (Leiper et al. 1996).

An indication of low efficiency of "piggy back" import are experiments performed on peroxisomal malate dehydrogenase (Mdh3p). A massive overexpression of PTS-less tagged Mdh3p was required to force formation of hetero-oligomers and demonstrate "piggy back" import, perhaps because Mdh3p is one of the faster translocating proteins *in vivo* (Elgersma et al. 1996b; Ruigrok et al. in preparation). A more recent report shows that Eci1p, a peroxisomal lumenal protein, stripped of its PTS1 can also be transported in a “piggy-back” fashion by oligomerisation with another peroxisomal protein, Dci1p (Yang et al. 2001). Our own studies on kinetics of protein import into peroxisomes *in vivo* suggest that

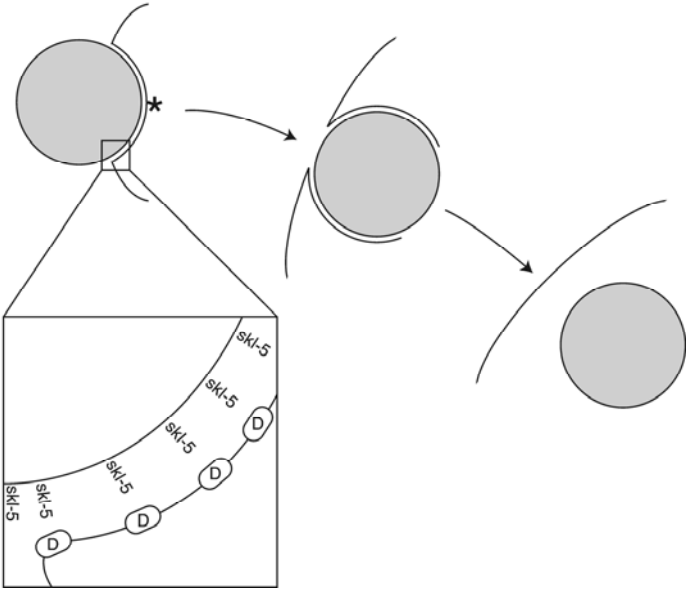

Fig. 4. Putative mechanism for gold particle import. The inset in the first stage of import shows the PTS1-Pex5-Docking complex interaction, which folds the membrane over the gold particle. The asterisk indicates mechanical shearing of the peroxisomal membrane. The membrane slides over the gold particle, and reseals in the last stage, allowing import of the gold particle. Gold particles are depicted in light grey. Abbreviations used: skl-5 - PTS1-Pex5p complex, D - Docking proteins at peroxisomal membrane.

newly synthesised proteins destined for the peroxisome are highly import-competent, as opposed to 'older' import substrate that is not imported yet and gets trapped in an off-pathway state (Ruigrok et al. in preparation).

An example favoured by many authors to support the notion of import of fully folded proteins is the observation that gold particles coated with PTS1-peptides can be imported. Gold particles may not be representative for proteinaceous import substrates; however, it has been reported that hepatocytes take up 17 nm gold particles from their environment, although, these cells do not display phagocytosis (Hardonk et al. 1985). Perhaps such particles traverse the peroxisomal membrane by physical means. When gold particles are coated with PTS1 peptides, Pex5p can interact with both PTS1 and peroxisomal membrane proteins, folding the membrane over the particle, and eventually allowing entry by mechanical shearing (see Fig. 4). The physical properties of gold particles (a rigid, non-deformable structure) might allow this type of import, but these are properties that natural import substrates do not possess.

The idea of the peroxisomal membrane engulfing proteins destined for the peroxisome was already postulated by the endocytosis like hypothesis of McNew and Goodman (McNew and Goodman 1996). A more recent opinion paper on the mechanistics of peroxisomal protein import defined the concept of a "preimplex":

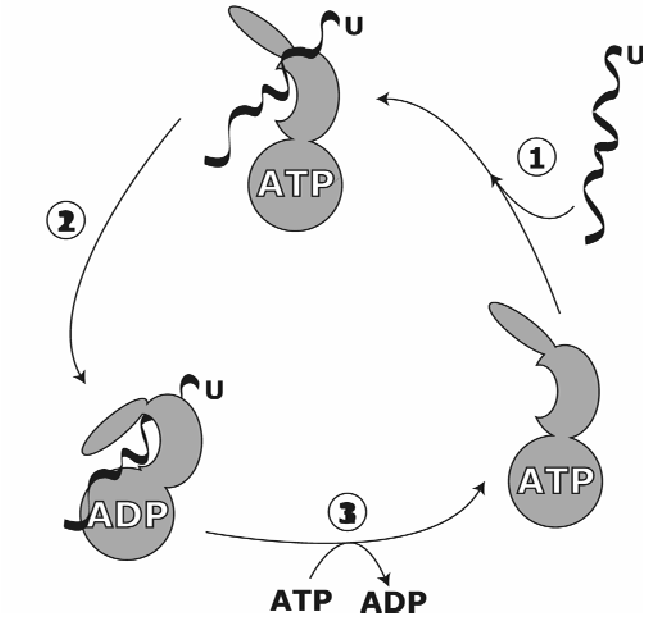

Fig. 5. The chaperone cycle of Hsp70. 1. Binding of substrate to the low-affinity, ATP-bound form of Hsp70. 2. Stimulation of ATPase activity by J-proteins induces the conformational change to a tightly bound, ADP-bound form of Hsp70. The client protein is allowed to fold. 3. Nucleotide exchange stimulates the release of the client protein, and returns Hsp70 to the low-affinity, ATP-bound state. See text for further details. Abbreviations used: U - Unfolded, folding, or misfolded client protein, which exposes regions that can be bound by Hsp70. Hsp70 is depicted in dark grey, and "ATP" or "ADP" indicates the nucleotide state within the schematic representation of Hsp70.

an assembly consisting of multiple receptor-cargo complexes with peroxisomal membrane proteins (Gould et al. 2002). Such large complexes would allow import of discrete 'packages' of peroxisomal proteins via an endocytosis-like process, the 'piggy-back' import of oligomerised peroxisomal proteins, and the occurrence of intra-peroxisomal import receptors.

3 Involvement of Hsp70 in peroxisomal protein import

3.1 Hsp70 family introduction

Hsp70 is an ATPase, and this enzyme activity, located in the N-terminal 44 kDa ATPase domain, is coupled to its chaperone function. When Hsp70 is bound to ATP, it has low affinity for its substrate (folding, or unfolded proteins), whereas the ADP bound state of Hsp70 is characterised by high binding affinity (Schmid et al. 1994). The Hsp70-substrate interaction is mediated by the peptide binding

Box 1. Hsp70 regulatory proteins

After hydrolysis of ATP by Hsp70, the final part of the Hsp70 cycle takes place. Here, the exchange of ADP for ATP, which goes hand in hand with the release of substrate is stimulated by regulatory proteins.
In *E. coli*, chloroplasts, and mitochondria, this function is performed by GrpE (Harrison et al. 1997). In the eukaryotic cytosol and the endoplasmic reticulum, a GrpE-like function is fulfilled by BAG-like proteins like BAG1 (Snl1p in *S. cerevisiae*) in the cytosol, and BAP (Sls1p in *S. cerevisiae*) in the endoplasmic reticulum (Takayama et al. 1997; Kabani et al. 2000; Chung et al. 2002; Sondermann et al. 2002). BAG-like proteins, which contain the Hsp70-regulating "BAG-domain", may be comparable to J-proteins: they use the "BAG-domain" as a module that regulates Hsp70's activity at a specific site in the cell.
In the cytosol, HspBP1 (Fes1p in *S. cerevisiae*) acts as a negative regulator of Hsp70 nucleotide binding (Raynes and Guerriero 1998; Kabani et al. 2002a). It prevents ATP binding to Hsp70 by stimulating the release of this nucleotide before it can be hydrolysed (Kabani et al. 2002b). In mammals, another protein called HIP can interact with the ATPase domain of Hsp70, stabilising the ADP state (Hohfeld et al. 1995).
Finally, two additional classes of proteins, which interact with the COOH-terminus of Hsp70, play a role in the substrate binding by Hsp70. HOP (Sti1p in *S. cerevisiae*) plays an organising role in handing over substrate from Hsp70 to the Hsp90 chaperone system, and thus, promotes substrate release from Hsp70 (Nicolet and Craig 1989; Chen and Smith 1998). CHIP negatively regulates substrate release from Hsp70 (Ballinger et al. 1999), and a homologue of CHIP (small glutamine-rich protein/viral protein U-binding protein (SGT/UBP)) also inhibits substrate release from Hsp70 (Angeletti et al. 2002). The latter protein has a homolog in *S. cerevisiae* (*SGT1*), but the Hsp70-regulating capabilities of this protein have not yet been determined.

domain, which is located C-terminal to the ATPase domain and binds short, mostly hydrophobic, amino acid stretches that are exposed in folding or unfolded proteins (Blond-Elguindi et al. 1993; Rudiger et al. 1997). This on/off cycle of binding prevents unwanted interactions of these hydrophobic segments with the environment (membranes, other proteins), enhancing the chance of productive folding. At the C-terminus of Hsp70 a third domain can be found, often referred to as the 'lid domain'. Upon binding to ATP, Hsp70 is folded such that the peptide-binding domain is freely accessible, allowing the substrate to bind. This interaction is transient in nature, and only upon ATP hydrolysis the conformation of Hsp70 changes such that the peptide is held in the peptide-binding domain, covered by the 'lid domain'. ADP-ATP exchange then changes the conformation of Hsp70, allowing release of the substrate (Buchberger et al. 1995; Mayer et al. 2000) (Fig. 5).

Although Hsp70 can perform this chaperoning cycle on its own, its activity is highly regulated. The best-studied regulators of Hsp70 are the J-proteins, a family of proteins that is characterised by a so-called J-domain of approximately 70 amino acids. These J-proteins, also referred to as Hsp40s, owe their name to DnaJ, the first identified *E. coli* family member. J-domains specifically interact with Hsp70s, and stimulate their ATP hydrolysis activity (Liberek et al. 1991). The peptide-binding domain of Hsp70 shows little substrate specificity (James et al. 1997). This substrate specificity is provided by J-proteins, present at specific subcellular locations or associated to client proteins, recruiting the Hsp70 chaperone to its target (Silver et al. 1993; Cyr et al. 1994). Several other proteins can influence the ATP-ADP state and substrate binding affinity of Hsp70 (Box1).

Hsp70 has duplicated many times in evolution. Whereas the prokaryote *E. coli* only contains two Hsp70s besides DnaK: HscA and HscC (Kawula and Lelivelt 1994; Itoh et al. 1999), *S. cerevisae* contains several Hsp70 families, each consisting of one or more family members. During the ancient genome duplication in *S. cerevisiae*, most Hsp70 families were duplicated (Dietrich et al. 2004) (Table 2). The *SSB* family is ribosome-associated, and presumably acts in early (co-translational) protein folding. The *SSB* family binds to nascent chains, as they emerge from the ribosome, an interaction stimulated by Ssz1p in conjunction with the J-protein Zuo1p. A four-membered family of Ssa proteins forms another group of cytosolic Hsp70. *SSA* comprises an essential gene family, and members of the *SSA* family are functionally redundant, i.e. expression of any of the Ssa proteins can support cell growth in the absence of the other three members (Werner-Washburne et al. 1987). Binding of Hsp70 to substrate is not specific across the *SSA* and *SSB* families, since exchange of the peptide-binding domain of these chaperone families did not affect their function (James et al. 1997). The *SSE* family members are Hsp70-like, but contain an insertion, and are thought to function as co-factors in the Hsp90 chaperone machinery. Finally, one or more intra-organellar Hsp70s can be found in ER and mitochondria (see also Section 3.3).

Less detailed information is known about mammalian Hsp70 subfamilies. The distinction between ribosome-bound and cytosolic Hsp70 is not conserved from yeast to mammals, and stress-inducible Hsp70 as well as constitutively expressed Hsc70 isoforms can be found in the cytosol. Besides differential gene expression, no indication exists of functional differences between Hsc70 and Hsp70. For the purpose of simplicity, Hsc70 and Hsp70 will be referred to as Hsp70. As in yeast, an *SSE*-like family (Hsp105) exists, and intra-organellar Hsp70s can be found in ER, mitochondria, chloroplasts, nucleus, and lysosomes, but not in peroxisomes.

The number of different proteins that can influence the Hsp70 chaperone cycle, Hsp70s' subcellular localisation, and the presence of redundant isoforms of Hsp70 itself reveals an intricate network of regulation, where Hsp70s' activities are regulated in a localised and precise way. A great challenge lies in determining the interplay of these different regulatory networks to ultimately map the way Hsp70s' specificities are achieved in the cell.

Table 2. Duplications in Hsp70s of *S. cerevisiae*

gene	localisation	duplicated (Dietrich et al. 2004)
SSA1	cytosol	no data
SSA2	cytosol	no
SSA3	cytosol	yes (*SSA4*)
SSA4	cytosol	yes (*SSA3*)
SSB1	cytosol	yes (*SSB2*)
SSB2	cytosol	yes (*SSB1*)
SSZ1	cytosol	no
SSE1	cytosol	yes (*SSE2*)
SSE2	cytosol	yes (*SSE1*)
KAR2	ER	no
LHS1	ER	no
SSC1	mitochondria	yes (*ECM10*)
ECM10	mitochondria	yes (*SSC1*)
SSQ1	mitochondria	no

3.2 *In vivo* roles of Hsp70

Hsp70 plays a role in many processes in the cell. Beautiful pulse-chase studies in *E. coli* showed that the *E. coli* Hsp70 (DnaK) transiently associates with newly synthesized proteins, indicating an important role for Hsp70 in *de novo* folding of proteins. In yeast, one class of Hsp70s (Ssb) associates to the ribosome and binds the emerging nascent chain of newly synthesized proteins (Nelson et al. 1992; Pfund et al. 1998). Ssb consists of two almost identical family members. Recently, a ribosome-associated J-protein (Zuo1p) was found in a stoichiometric complex with an Hsp70-like protein (Ssz1p or Pdr13p), which also localises to the ribosome. Ssz1p itself does not bind nascent chains, but the Ssz1p/Zuo1p complex stimulates nascent chain binding to Ssb (Gautschi et al. 2002; Hundley et al. 2002). In mammals, Hsp70 also associates to nascent chains, as was shown in a mammalian translation system (Frydman et al. 1994). Not all proteins, however, depend on Hsp70 for proper cotranslational folding (Nicola et al. 1999).

In yeast, Hsp70s of the cytosolic Ssa family assist at the level of posttranslational folding and perhaps oligomerisation. Maturation of the cytosolic enzyme ornithine transcarbamoylase was followed in a temperature sensitive *ssa1* mutant, in the absence of the other three family members *SSA2*, *SSA3*, and *SSA4*. At the non-permissive temperature, a large decrease of specific activity of the enzyme resulted and a monomeric species accumulated, suggesting delayed oligomerisation or oligomer disassembly (Kim et al. 1998). The tetrameric enzyme peroxisomal catalase (Cta1p) heavily aggregates in the absence of cytosolic Hsp70 (our own unpublished observations). This also implies a role for Hsp70 in folding of cytosolic proteins.

It is clear that Hsp70 also acts on fully folded proteins. One example is the J-protein auxillin, which is associated with clathrin-coated vesicles. Auxillin recruits Hsp70 to depolymerise the clathrin coat into free subunits, releasing the vesicle to join the endosomal system (Ungewickell et al. 1995; Pishvaee et al. 2000). An-

other example of the capability of Hsp70 to bind folded substrates was demonstrated in elegant studies using surface plasmon resonance by Rapoport and colleagues (Misselwitz et al. 1998). Using purified BiP, an ER Hsp70, and the J-domain of its cognate J-protein Sec63, they showed that BiP can be activated to bind fully folded proteins, provided that the activation of BiP takes place in close proximity to the substrate proteins. Since they could not detect binding to a tightly folded model protein, they assume that BiP binds portions of the protein that are exposed by "thermal breathing".

3.3 Hsp70 and import of proteins into organelles

The role of Hsp70 in protein import processes is diverse and extensively studied. In the next paragraphs, we will highlight some key findings on the role of Hsp70 chaperones in different protein import processes. Our aim is to uncover possible generic mechanisms in Hsp70s functioning in protein import, and to discuss them in the context of peroxisomal protein import. Since peroxisomes do not appear to contain lumenal Hsp70, we will focus our discussion on cytosolic processes prior to import.

Proteins that are destined for import into the ER and mitochondrion traverse the membrane of their target organelle in a partially folded/unfolded state. Two classes of Hsp70 are involved in the import into these organelles: cytosolic and lumenal Hsp70. Cytosolic Hsp70 plays a role in keeping some import substrates in a loosely folded, import competent conformation, whereas lumenal Hsp70 is involved in ensuring unidirectional transport of the polypeptide chain through the translocon.

The role of cytosolic Hsp70 was established in *in vivo* studies in *S. cerevisiae*. Partial depletion of cytosolic Hsp70 by deleting one of the four cytosolic Hsp70s *SSA1*, already resulted in an inability to respire at 37°C (Deshaies et al. 1988). This suggests mitochondrial malfunction, possibly caused by diminished import in the absence of sufficient cytosolic Hsp70. To investigate this, later experiments made use of cells where the complete *SSA* subfamily was deleted, and *SSA1* was expressed under control of a regulated promotor to deplete Hsp70 completely *in vivo* (Deshaies et al. 1988). Pulse-chase experiments revealed precursor accumulation of some but not all mitochondrial and ER precursor proteins. Perhaps some proteins are more easily unfolded, and do not require Hsp70 to keep them loosely folded enough to be translocation competent. Similar observations were made with an *ssa1-45* temperature sensitive (t_S) mutant, also in combination with a t_S mutant of Ydj1p, one of the cytosolic DnaJ proteins. These results again confirmed the involvement of Ssa proteins in mitochondrial and ER import, and showed that Ydj1p works together with Ssa proteins in this process (Becker et al. 1996).

Some *in vitro* evidence also underlines a possible role for Hsp70 to keep proteins in an import-competent state. The precursor to mitochondrial aspartate aminotransferase (pmAAT) was chemically denatured, and refolded in the presence of lysate. Hsp70 was shown to associate to pmAAT, which resulted in a form of

pmAAT that could be imported into isolated mitochondria. However, upon depletion of Hsp70, pmAAT was not competent for import anymore (Artigues et al. 2002). Although this situation is different from that in the living cell - a denatured full-length polypeptide chain instead of vectorially translated protein is used as starting material - it does show that Hsp70 in principle can maintain a protein in an import-competent state. Hsp70 appears to act specifically because cytosolic Hsp90, another cytosolic chaperone, also bound to pmAAT and kept it in a partially folded state but did not allow import of pmAAT into mitochondria. Additional evidence for maintenance of import competence by Hsp70 and/or Hsp40 is the observation that Hsp70/Hsp40 stimulate import of prepro α–factor into isolated microsomes by preventing aggregation (Ngosuwan et al. 2002). Using clever experimentation to specifically manipulate levels of ATP outside mitochondria, Asai et al. (2004) showed that cytosolic ATP (and likely cytosolic Hsp70) was important for keeping mitochondrial precursor proteins in an import-competent state.

Although proteins can be presented in a folded state to isolated mitochondria and still be imported efficiently, import occurs more efficiently when the polypeptide chain is (partially) unfolded (Verner and Schatz 1987). A clear example is formed by adenylate cyclase (Adk1p) in yeast. Adk1p is present in the cytosol and mitochondria, a result of competition between Adk1p folding and import (Strobel et al. 2002). Translocation into mitochondria requires unfolded polypeptide, and several factors, including the "pulling" or “trapping” by mitochondrial lumenal Hsp70 and the membrane potential, play a role in actively unfolding the protein at the membrane during translocation (Matouschek et al. 2000; Huang et al. 2002). Studies on the import of a larger set of mitochondrial precursor proteins *in vivo* would provide us with more insight into the specific requirements of Hsp70 in import into mitochondria.

Unlike the above-discussed classical translocation pores, import into several other organelles allows the imported substrate to be folded and even assembled into oligomers. In nuclear import, medium sized proteins can passively diffuse across the nuclear envelope through an aqueous channel of 9 nm diameter formed by the nuclear pore. Even larger structures (at least 20 nm in size) can passage the nuclear pore by active transport. Involvement of Hsp70 in nuclear import was demonstrated using the same conditional mutant in *SSA1* (*ssa1-45*) as used to characterise mitochondrial and ER import.

The lysosome (vacuole in yeast) can accommodate import of folded proteins, and different import mechanisms exist. Lysosomes contain lumenal Hsp70s, and a fraction of cytosolic Hsp70 is associated with the lysosomal membrane (Terlecky and Dice 1993; Agarraberes et al. 1997). When mammalian cells are starved for serum, cytosolic proteins containing a KEFRQ amino-acid sequence, are targeted to the lysosome for degradation. Hsc73 was found to bind to these peptides, and to stimulate lysosomal degradation of a protein containing this sequence in two different *in vitro* assays (Chiang et al. 1989).

Import of a subset of proteins into yeast vacuoles can involve membrane inclusion of oligomeric protein into small vesicles, termed VID vesicles, which are subsequently targeted to the vacuole. When investigating the influence of Hsp70

in an *in vivo* degradation assay, and in an *in vitro* import assay using isolated VID vesicles, a specific requirement for Ssa2p was found (Brown et al. 2000).

3.4 Hsp70 and peroxisomal protein import

Several lines of evidence have implicated Hsp70 in peroxisomal protein import. In a micro-injection based assay, polyclonal antibodies directed against Hsp70 inhibited import of a co-injected PTS1-containing protein. Hsp70 was found to be associated to purified rat liver peroxisomes, and under peroxisome inducing conditions, more Hsp70 associated with peroxisomes (Walton et al. 1994). The PTS2 pathway relies on Hsp70 and Hsp40 for efficient import (Legakis and Terlecky 2001).

Several reports exist on Hsp70s' role in peroxisomal protein import in plants. In cucumber seedlings, two glyoxisomal membrane proteins, PMP73 and PMP61, were shown to be immuno-related to Hsp70 and a DnaJ homologue also present in other plant species (Corpas and RN 1997). Studies in watermelon reveal a Hsp72 gene that could be initiated at two distinct methionines, resulting in a form targeted to plastids, and a form present in the glyoxisomal lumen. The latter localisation was dependent on a PTS2, which was shown to be functional in the yeast *H. polymorpha* (Wimmer et al. 1997). Another group independently reported membrane-associated and lumenal Hsp70 and DnaJ homologs in glyoxisomes (Diefenbach and Kindl 2000). None of the yeast or mammalian Hsp70s carry resemblance to a PTS in their primary structure, suggesting that intraperoxisomal Hsp70 occurs only in the plant kingdom.

In pumpkin seedlings, Hsp70 was found to be peroxisome-associated. The amount of Hsp70 associated with peroxisomes correlated well with the import efficiency: in heat-shocked seedlings more Hsp70 was associated with peroxisomes, and import efficiency increased. As expected, immunodepletion caused a decrease of import into peroxisomes. Hsp70 was suggested to act on the import pathway in the cytosol because prior treatment of peroxisomes with anti-Hsp70 antibody did not affect import. Hsp70 depletion during synthesis of the protein affected import more than Hsp70 depletion after protein synthesis. Taken together with the co-immunoprecipitation of two different import substrates with Hsp70, Hsp70 was concluded to act directly on the import substrate already during its synthesis (Crookes and Olsen 1998).

A recent report directly implicates Hsp70 in peroxisomal protein import via its interaction with Pex5p (Harano et al. 2001). Using the expressed and purified mammalian PTS1 protein acyl-CoA oxidase as bait, both Pex5p and Hsp70 were fished out of mammalian cell lysates. Part of the associated Hsp70 could be released from the complex(es) by ATP, a property indicative of Hsp70-client protein interaction, perhaps reflecting the association of acyl-CoA oxidase with Hsp70.

Other interaction studies, employing PTS1-peptides and purified human Hsp70 and Pex5p, showed that Hsp70 did not have an effect on the binding of Pex5p to PTS1-peptide. The authors concluded that Pex5p-PTS1 interaction is independent

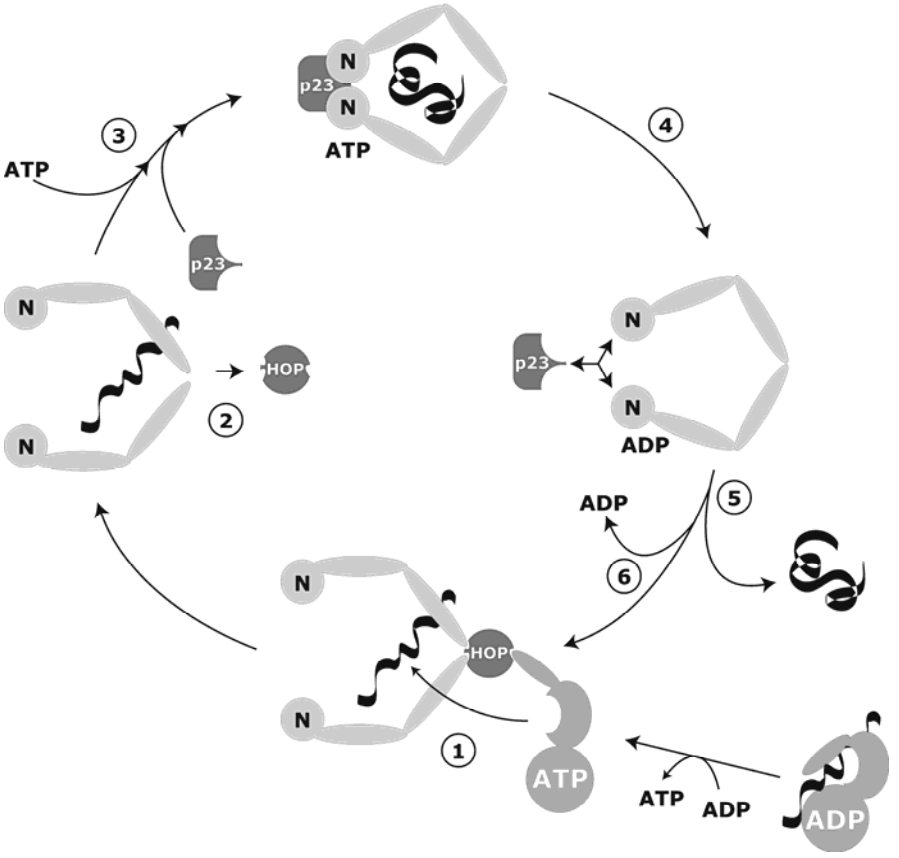

Fig. 6. The chaperone cycle of Hsp90 (After Young et al. 2002). 1. ADP-ATP exchange and substrate release of Hsp70 is stimulated by HOP, which connects Hsp70 to Hsp90. The client protein is next transferred from Hsp70 to Hsp90. 2. HOP dissociates from Hsp90-client protein complex, and ATP binds Hsp90, which induces dimerisation of the NH_2-terminal domains of Hsp90. 3. p23 binds to the dimerised N-termini of Hsp90. 4. Slow ATP hydrolysis by Hsp90 causes the NH_2-termini to monomerise, p23 dissociates from Hsp90. 5. The substrate is released. 6. ADP dissociates, and Hsp90 returns to its nucleotide-free state, ready to accept new client proteins. Abbreviations used: HOP - Hsp90-Hsp70 Organising Protein. Hsp90 dimers are depicted in light grey, cofactors in dark grey, and Hsp70 in medium grey. The NH_2-terminus of Hsp90 is indicated with an N.

of Hsp70, and that Hsp70 may assist folding and oligomerisation of PTS1 proteins. Because Pex5p exists as a tetramer, oligomeric proteins might have higher affinity for Pex5p than a monomeric protein subunit, resulting in more efficient recognition by Pex5p (Harper et al. 2002).

A general problem with Hsp70 immunodepletion is the specificity of the observed effect. All, or at least a subclass, of Hsp70 is removed prior to the start of an import reaction, which can possibly result in aggregation of the import sub-

strate, or misfolding of proteins needed for import. Although the *in vitro* import systems give a rough indication that Hsp70 is involved, more detailed analysis focussing on the interactions between Hsp70 and the different components of the import machinery will be required to understand the role of this chaperone in peroxisomal protein import. Addition problems arise when studying Hsp70s' influence in *S. cerevisiae*. Here several Hsp70 subfamilies, consisting of redundant isoforms, can be found, which makes it difficult to pinpoint which Hsp70 acts in peroxisomal protein import (our unpublished observations).

4 Involvement of Hsp90 in peroxisomal protein import

4.1 Hsp90 family introduction

The Hsp90 molecular chaperone works in concert with a set of co-factors and with the Hsp70 chaperone system to assist in maintaining the conformation of a specific set of proteins, often involved in signalling pathways, thereby, regulating their function. This protein complex is called the "foldosome" (Hutchison et al. 1994). As for Hsp70, the chaperone function of Hsp90 is coupled to its intrinsic ATPase activity. An outline of the Hsp90 chaperone cycle is depicted in Figure 6.

In initial stages of client protein interaction with Hsp90, Hsp70 hands over the client protein to Hsp90 via a bridging protein called HOP (Hsp70-Hsp90 Organising Protein). HOP contains two domains, consisting of TPR repeats, which bind to a conserved EEVD-COOH motif present on both Hsp70 and Hsp90 (Scheufler et al. 2000). Hsp90 in its nucleotide-free, or ADP bound state is dimeric through interactions in the COOH-terminal region of the protein. HOP is then released from the complex and ATP is bound, inducing dimerisation of the NH_2-terminal ATPase domain of Hsp90. This state has a high affinity for p23. Now ATP hydrolysis occurs, which is a slow step in the Hsp90 cycle. p23, substrate, and ADP are released, and Hsp90 is again in the nucleotide-free state, ready to accept a new client protein.

This "minimal Hsp90 chaperone cycle" is a simplified depiction of the actual situation. The p23-bound state of Hsp90, also referred to as the "mature complex", can contain immunophilins, or immunophilin-like proteins, which act in the maturation of specific substrates.

4.2 *In vivo* roles of Hsp90

Unlike Hsp70, Hsp90 does not appear to function in early events of protein folding at the level of the nascent chains (Frydman 2001). Although refolding studies of denatured model proteins showed that Hsp90 can prevent aggregation and can assist in more efficient refolding (Freeman and Morimoto 1996), the same model proteins are not affected in *de novo* folding.

Instead, Hsp90 acts on folded or partially folded proteins to keep them in an active state. This role is exemplified by the well-characterised involvement of Hsp90 in the maturation of signalling proteins such as nuclear hormone receptors, where binding of the receptor to the Hsp90 chaperone machinery ensures that these proteins can be activated by hormones (Picard et al. 1990). Different kinases, such as src- and raf-like kinases and Ste11p in yeast also require Hsp90 for their activity (Xu and Lindquist 1993; Schulte et al. 1995; Louvion et al. 1998). The prominent role of Hsp90 in chaperoning signal transduction pathways explains its being essential in eukaryotes.

4.3 Hsp90 and import of proteins into organelles

Demonstrations of the involvement of Hsp90 in protein import processes are scarce. Hsp90 is involved in import of at least some mitochondrial proteins. Hsp90 interacts with various TPR domain-containing proteins, one of which is Tom70p. Tom70p is an outer membrane protein involved in the import of a subset of mitochondrial proteins (Hines et al. 1990). When the interaction between Tom70p and Hsp90 is disturbed, import of Tom70p dependent mitochondrial preproteins does not occur (Young et al. 2003). The authors concluded that Hsp90 targets mitochondrial preproteins to Tom70p. Tom34p, another protein involved in mitochondrial import also binds Hsp90 (Young et al. 1998). The functional significance of this interaction remains to be established.

Hsp90 is involved in regulating the activity of many signalling proteins. Two examples of signalling proteins, the glucocorticoid receptor (GR), and the dioxin receptor (DR) are targeted to the nucleus in an Hsp90-dependent fashion. Adding geldanamycin, an Hsp90 ATPase inhibitor, to living cells inhibited nuclear import of GR-GFP and DR-GFP fusion proteins. This mechanism appears to rely on immunophilins or immunophilin-like proteins, present in late Hsp90 complexes: FKBP52 and XAP1 for GR and DR, respectively (Silverstein et al. 1999; Kazlauskas et al. 2002). Upon ligand binding to GR, FKBP52 is recruited to the Hsp90-GR complex, and mediates transport of this complex along the cytoskeleton towards the nucleus (Davies et al. 2002). This process is probably mediated via the interaction of FKBP52 with dynein (Galigniana et al. 2002).

As previously mentioned, lamp2a-mediated lysosomal import involves cytosolic Hsp70. Hsp90 is also involved in this import process. Several components of the Hsp90 machinery can associate with import substrates and the lamp2a translocon. Antibodies directed against Hsp70, Hsp40, Hip, or Hop inhibit import into isolated lysosomes. The Hsp90 chaperone machinery might act as a targeting factor for KEFRQ-containing proteins, and/or assist in their translocation (Agarraberes and Dice 2001).

4.4 Hsp90 and peroxisomal protein import

The first report implicating Hsp90 in peroxisomal protein import showed that addition of anti-Hsp90 antibodies to an *in vitro* plant peroxisomal import assay resulted in retarded import of isocitrate lyase (Crookes and Olsen 1998). The role of Hsp70 was different from that of Hsp90: the effect of anti-Hsp90 antibody was pronounced when added both during synthesis and after synthesis, whereas the major effect of anti-Hsp70 antibodies was during synthesis, suggesting a post-translational role for Hsp90. We recently found retarded import of both PTS1 and PTS2 containing proteins in the absence of functional Hsp90 in yeast (our unpublished observations). This general import defect could be informative regarding the function of Hsp90 in peroxisomal protein import. Three possibilities come to mind: 1) Hsp90 acts on the PTS-containing protein, 2) Hsp90 acts at the level of import receptors, 3) Hsp90 acts indirectly through signal transduction.

Arguing against the first scenario is the lack of involvement in *de novo* folding by Hsp90. Hsp90 is found in complex with some nuclear, mitochondrial, and lysosomal proteins and has an active role in their transport. In contrast, in the aforementioned *in vitro* import assay, antibodies directed to Hsp70 precipitated isocitrate lyase, but an interaction between this import substrate and Hsp90 was not observed. This finding suggests that Hsp90 does not act on peroxisomal protein import via interaction with the peroxisomal proteins prior to import, but the same conclusion need not necessarily apply to all peroxisomal proteins.

The second scenario would imply that both the PTS1 and the PTS2 receptor are chaperoned by Hsp90, resulting in a more efficient function of these receptors in the import process. In line with this notion is the observation that Pex5p can be co-immunoprecipitated with Hsp90 using *in vitro* translated proteins (Pratt et al. 2001). Perhaps Hsp90 has a direct role in keeping Pex5p "receptive" for PTS1 recognition. Despite several attempts, we did not find an interaction between Pex5p and Hsp90 in yeast lysates, and therefore, could not confirm this hypothesis (our unpublished observations).

In the final scenario, Hsp90 functions at a later step in peroxisomal protein import, where the PTS1 and PTS2 pathways converge. In this context, it is interesting to note that some peroxins are phosphorylated *in vivo* (Elgersma et al. 1997; Komori et al. 1999). By regulating the activity of kinases, Hsp90 could regulate activity of peroxins.

5 Concluding remarks

Despite the considerable efforts to delineate the contribution of protein folding and chaperones in the process of peroxisomal protein import our insights remain rather limited and fragmentary. This is due to a number of drawbacks:

i. The difficulty to establish the degree of folding *in vivo* of a protein before, during, or after its itinerary from cytosol into the organelle.

ii. The lack of success in establishing a reliable *in vitro* system to study the import of proteins into peroxisomes. This limits experimentation to intact or semi-permeabilised cells, which makes it difficult to distinguish between direct and indirect effects of manipulation. Also, it limits the ability to determine at which step along the import pathway and effect is exerted.

iii. The complexity and redundancy of chaperone families and their regulators makes it difficult to trace the contribution of individual chaperone proteins, even in a genetically tractable organism as *S. cerevisiae*. Most experiments performed in mammalian cells use antibodies, which remove a complete subclass of chaperones, and it is difficult to assess where the chaperone function acts in the import process.

At this moment it is almost impossible to extract a coherent picture about the role of folding and chaperones from the mosaic of various reported observations. Improvement of this impasse awaits the development of *in vitro* techniques to reconstitute partial steps in the peroxisomal protein import pathway.

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Braakman, Ineke
Department of Cellular Protein Chemistry, Utrecht University, Padualaan 8, 3584 CH, Utrecht, The Netherlands
i.braakman@chem.uu.nl

de Jonge, Wim
Department of Cellular Protein Chemistry, Utrecht University, Padualaan 8, 3584 CH, Utrecht, The Netherlands. Current adress: Institute of Information and Computing Sciences, Utrecht University, Padualaan 14, 3584CH Utrecht, The Netherlands.

Tabak, Henk F.
Department of Cellular Protein Chemistry, Utrecht University, Padualaan 8, 3584 CH, Utrecht, The Netherlands, and Laboratory of Cell Biology, University Medical Center Utrecht and Center for Biomedical Genetics, 3584 CX Utrecht, The Netherlands