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Review

PTS1-independent sorting of peroxisomal matrix proteins by Pex5p

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Abstract

Most peroxisomal matrix proteins contain a peroxisomal targeting signal 1 (PTS1) for sorting to the correct organelle. This signal is located at the extreme C-terminus and generally consists of only three amino acids. The PTS1 is recognized by the receptor protein Pex5p. Several examples have been reported of peroxisomal matrix proteins that are sorted to peroxisomes via Pex5p, but lack a typical PTS1 tripeptide. In this contribution we present an overview of these so-called non-PTS1 proteins and discuss the current knowledge of the molecular mechanisms involved in their sorting.

Keywords: Peroxisome; Pex5p; PTS1; PTS2; Acyl CoA oxidase; Alcohol oxidase

1. Introduction

The peroxisomal matrix is the site of the highest protein concentration within eukaryote cells. The matrix contains enzymes that are involved in various metabolic pathways. Generally a (short or longer) cascade of enzyme reactions is catalyzed by peroxisomal enzymes. A hydrogen peroxide producing oxidase together with catalase is an example of a short cascade, whereas the enzymes of the beta-oxidation pathway for fatty acid metabolism exemplify a longer one (for reviews see [1,2]).

Without exception, peroxisomal enzymes are nuclear encoded and synthesized on free ribosomes in the cytosol. A large group of specific proteins, peroxins, are involved in correct sorting of these proteins to the final subcellular destination [2].

Of all peroxins known to be involved in peroxisomal matrix protein import, Pex5p has been studied most extensively. Pex5p is one of the first peroxins identified and serves as the receptor for peroxisomal matrix proteins that are sorted via the Peroxisomal Targeting Signal 1 (PTS1) protein import pathway. The PTS1 is located at the extreme C-terminus of peroxisomal matrix proteins and consists of three amino acids: -SKL or conserved variants thereof. This signal is recognized by the C-terminal tetratricopeptide (TPR) repeat domain of Pex5p. The N-terminal part of Pex5p is important for docking at the peroxisomal membrane and subsequent translocation and recycling steps (recently reviewed in [3]).

Binding of the PTS1 tripeptide to the C-terminal TPR domain of Pex5p has been extensively studied by various genetic, biochemical and biophysical approaches. It is now generally accepted that one PTS1 signal binds to one Pex5p molecule. In addition to the three C-terminal PTS1 residues, the eight preceding residues may influence the strength of binding between receptor and cargo molecule [4]. A three-dimensional structure of the TPR domain with a bound PTS1 peptide, obtained by protein crystallization and roentgen diffraction, has been elucidated [5]. For details on the function of Pex5p, see other chapters within this issue of BBA.

Interestingly, several cases have been described of matrix proteins that are imported in a Pex5p dependent manner, despite the fact that these proteins lack a typical PTS1 [2]. Moreover, others do contain a PTS1, which however is redundant for peroxisomal targeting.

Several models have been proposed to explain these phenomena. The simplest one is that the protein contains a PTS2 as well (as e.g. for *Hansenula polymorpha* Pex8p [6]). Another possibility is that a PTS1-lacking matrix protein is

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sorted to peroxisomes in a hetero-oligomeric complex with a PTS containing matrix protein (designated as piggy-back import [7,8]). However, these models cannot explain sorting of all non-PTS1 proteins. Recent data indicated that some of these non-PTS1 proteins contain alternative targeting information, which is recognized by an alternative domain in the N-terminal half of Pex5p [9–11].

In this paper we present an overview on the proteins that have been suggested to follow the Pex5p dependent non-PTS1 import pathway. Possible mechanisms for this route are discussed.

2. Import of the intra-peroxisomal peroxin Pex8p

Pex8p is a peroxin that has so far only been identified in yeast. It is localized to the organelle lumen and most likely associated with the inner surface of the peroxisomal membrane, where it plays a role in cargo release from Pex5p [12] and also functions as central organizer of the peroxisomal protein import complex [13]. The absence of Pex8p affects both import of PTS1 and PTS1 proteins. Interestingly, Pex8p's generally contain a PTS1 but often also a second targeting signal at the N-terminus, which requires a functional PTS2 pathway for import.

H. polymorpha Pex8p contains a typical PTS2 consensus sequence at the extreme N-terminus, explaining why deletion of the PTS1 does not abrogate Pex8p import [6]. ScPex8p also contains a PTS1 and although a typical PTS2 consensus sequence is absent in this protein, the first N-terminal 112 residues of ScPex8p are sufficient to direct a reporter protein to peroxisomes [15,16]. Also *Pichia pastoris* Pex8p contains a PTS1 and lacks a PTS2 consensus sequence [17], but import of a C-terminal truncated PpPex8p variant that lacks a PTS1 depends on the PTS2 receptor proteins Pex7p/Pex20p, suggesting that also this protein has PTS2 information [14].

Summarizing, these data indicate that the above yeast Pex8p's can be targeted to peroxisomes both via the PTS1 and PTS2 protein import pathways.

Y. lipolytica Pex8p, however, represents an exception to this rule. YlPex8p lacks a typical PTS1, but does interact with YlPex5p in a two-hybrid assay [18]. In addition YlPex8p is still peroxisomal in cells of a *PEX20* disruption strain, indicating that its sorting occurs via the PTS1 pathway [18]. Hence, YlPex8p may contain an alternative Pex5p binding site that plays a role in YlPex8p sorting and thus represent the only non-PTS1 Pex8p that is imported in a Pex5p dependent manner.

Most yeast Pex8p's do interact with Pex5p, even when the PTS1 is removed from the protein. This has led to the assumption that in general Pex8p's contain an additional, non-PTS1 targeting signal for Pex5p dependent sorting. However, this alternative binding site most likely is not involved in Pex8p sorting, but required for the function of Pex8p in cargo release from Pex5p. This view is supported by *in vitro* binding experiments using fluorescence correlation spectroscopy (FCS) which indicated that *H. polymorpha* Pex5p and Pex8p still physically interact, when the PTS1 of Pex8p is destroyed by the addition of a His_6 tag to the extreme C-terminus [12]. These data showed that the association of HpPex8p and HpPex5p causes dissociation of the PTS1 cargo from HpPex5p.

A similar interaction most likely is responsible for the finding that ScPex8p directly interacts with ScPex5p in two-hybrid and co-immunoprecipitation experiments, an interaction that is not abolished upon deletion of the PTS1 of ScPex8p [15]. Also, C-terminal truncations in Pex5p, which fully blocked interaction of the protein with PTS1 cargo proteins, weakened but not disturbed the interaction between ScPex5p and ScPex8p. The second ScPex5p binding site in ScPex8p is most likely also involved in the function of Pex8p in cargo dissociation.

Interestingly, data in *P. pastoris* and *Y. lipolytica* also revealed physical interactions between Pex8p and Pex20p, which may be important for dissociation of the PTS2 cargo from Pex20p. In line with this assumption is the observation that the PpPex20p–PpPex8p interaction is independent of Pex7p [14,18].

3. Piggy-back import

Peroxisomal matrix proteins that lack a PTS1 can be sorted to peroxisomes in a Pex5p dependent manner, in complex with a PTS1 containing protein. This so-called piggy back import has been presented as possible explanation of Pex5p dependent import of proteins that lack a PTS1.

The first examples of piggy back protein import were reported in 1994. Glover et al. [8] showed that an N-terminal truncated variant of peroxisomal thiolase, which lacks the PTS2, was mislocalized to the cytosol unless full-length, PTS2 containing thiolase subunits were co-produced. McNew and Goodman [7] used the bacterial trimeric protein chloramphenicol acetyltransferase (CAT) as a tool to show that subunits lacking a PTS1 were co-transported with PTS1-containing subunits because CAT heterotrimers were formed in the cytosol prior to translocation. Piggy back import was also demonstrated for oilseed isocitrate lyases, which form homo-oligomers [19] and for *S. cerevisiae* Dci1p and Ecip1, two PTS1 proteins that form hetero-oligomers [20].

In all of the above examples piggy back import was dependent on artificial co-production of PTS lacking and PTS containing subunits of an oligomeric protein. However, no examples of piggy back import have been presented yet, where this mechanism is operative for import of true, non-modified peroxisomal matrix protein.

The conclusion therefore must be that piggy back import generally cannot be considered as an explanation for Pex5p dependent import of non-PTS1 proteins.

4. Yeast acyl-CoA oxidase

Acyl-CoA oxidase is an enzyme of the beta-oxidation pathway of fatty acids and a ubiquitous peroxisomal matrix enzyme. In rat, mouse, man and *P. pastoris* [21] this protein has a PTS1 signal and import depends of Pex5p. However *Y. lipolytica* [22] and *S. cerevisiae* [9] acyl-CoA oxidases lack a PTS1, but their import also requires the function of Pex5p.

In *Y. lipolytica* peroxisomal acyl-CoA oxidase is a pentamer that consists of 5 different subunits, termed Aox1 to Aox5 [22]. Neither of these subunits contains a PTS1 or PTS2. Detailed

studies on the assembly and import of this enzyme revealed that monomeric Aox subunits first bind FAD in the cytosol followed by assembly into heteropentamers. Only oligomeric acyl-CoA oxidase is imported, a process that is strictly dependent on Pex5p. How Pex5p recognizes Aox oligomers is not yet known [22,23].

Two of the *Y. lipolytica* Aox subunits, Aox2 and Aox3, play a key role in the assembly of the acyl-CoA oxidase heteropentamers. Deletion of both the *AOX2* and *AOX3* genes leads to cytosolic accumulation of monomers of Aox1p, Aox4p and Aox5p. This observation suggests that the peroxisomal targeting signal is formed upon formation of Aox oligomers. Therefore conformational epitopes most likely form the peroxisomal sorting information of *Y. lipolytica* acyl-CoA oxidase [22,23].

S. cerevisiae has a single gene encoding acyl-CoA oxidase, *POX1* (also designated *FOX1*). ScPox1p also lacks a PTS1 or PTS2, but its import does require Pex5p [9]. Detailed mutagenesis studies have been performed to dissect the region in ScPex5p, which is involved in Pox1p binding [9]. These studies revealed that mutations in the Pex5p TPR domains, which fully abolish binding of true PTS1 proteins, did not affect association of Pox1p to Pex5p. In line with these data is the remarkable finding by Schaefer et al. [10], who showed that a C-terminal truncated version of ScPex5p, which lacks the PTS1 binding TPR domains, is fully functional as import receptor for Pox1p.

Deletion and mutagenesis experiments revealed that the region in the N-terminal domain of ScPex5p consisting of amino acids 239–300 is sufficient for Pox1p binding [9]. These observations are a refinement of earlier data from Skoneczny and Lazarow [24], who mapped residues 136–292 of Pex5p to be responsible for the interaction with Pox1p. Point mutation Y253N specifically abolished the association of Pox1p with Pex5p [9].

Which region in Pox1p is required for interaction with ScPex5p is still unknown. It is tempting to speculate that also here a conformational epitope is important [9], like in YlAox.

Interestingly, *in vitro* import experiments using acyl-CoA oxidase from *Candida tropicalis* (Pox4p, a peroxisomal protein that also lacks a PTS1 or PTS2), revealed that this protein contains two large internal regions that have peroxisomal targeting information [25]. These regions may be sufficiently large to form the required conformational epitopes that are recognized by Pex5p.

5. Carnitine acetyltransferase

Carnitine acetyltransferase (Cat2p) is present in mitochondria and peroxisomes of *S. cerevisiae*. Cat2p contains a mitochondrial targeting signal at the N-terminus and a PTS1 at the C-terminus. The peroxisomal Cat2p results from alternative initiation of translation yielding Cat2p lacking the N-terminal mitochondrial targeting information [26]. Upon deletion of both the mitochondrial signal and the PTS2, Cat2p is still sorted to peroxisomes in a Pex5p dependent way [26]]. This Cat2p variant is also correctly targeted to peroxisomes when the C-terminal TPR motifs of ScPex5p are removed [9].

The domains in the N-terminus of Pex5p that are required for Cat2p and Pox2p import are overlapping but not identical [9]. Also the point mutation in ScPex5p (Y253N) that fully blocks Pox1p import, does not affect Cat2p sorting [9]. Hence most likely not a single domain/region exists in the N-terminus of ScPex5p that is responsible for the recognition of all non-PTS1 proteins.

6. Alcohol oxidase

In methylotrophic yeast species the first step in methanol metabolism is catalyzed by the peroxisomal enzyme alcohol oxidase (AO). Despite the fact that this protein does contains a functional PTS1, which is capable to sort a reporter protein to peroxisomes, this tripeptide is redundant for AO sorting [11,27] (Fig. 1A). However, targeting of AO to peroxisomes strictly depends on Pex5p, also when the PTS1 is artificially removed. The redundancy of the AO PTS1 is in line with the observation that import of AO can be mediated by an C-terminal truncated Pex5p, lacking the PTS1-binding TPR domains [11] (Fig. 1C, D), like observed for *S. cerevisiae* Pox1p and Cat2p.

Where for peroxisomal sorting of *Y. lipolytica* Aox assembly of pentamers is essential, AO sorting strictly depends on binding of the co-factor FAD [11]. Possibly, this also results in the formation or stabilization of a conformational epitope in the folded AO protein that can be recognized by HpPex5p.

A point mutation in the FAD binding site of AO fully abrogates peroxisomal import (Fig. 1B). This was an unexpected result, because this mutated variant of AO contains a fully functional PTS1 at the extreme C-terminus [11]. Similarly, import of AO – but not other peroxisomal matrix enzymes – is severely reduced when intracellular FAD is artificially depleted [28]. Therefore HpPex5p is apparently unable to bind to the AO PTS1 in the context of the AO protein, but not when it is fused to a reporter protein like GFP [29]. Possibly, residues upstream of the AO PTS1 may prevent binding to HpPex5p.

In a search for mutants specifically defective in AO assembly, a mutant was isolated that contained a mutation in the gene encoding pyruvate carboxylase (Pyc1p; [30]). This protein is important both for assembly of AO into enzymatically active, FAD containing octamers and for import into peroxisomes, because in a constructed *H. polymorpha PYC1* deletion strain FAD lacking AO monomers accumulate in the cytosol [31]. Exactly the same occurs when FAD binding to AO is prevented (e.g. by intracellular FAD depletion or a mutation in the FAD binding site of AO) [11,28]. Therefore, HpPyc1p most likely functions in FAD binding to AO. This was an unexpected novel function of HpPyc1p. Eukaryotic Pyc proteins are highly conserved and serve an anapleurotic function in replenishing the citric acid cycle with oxaloacetate generated from pyruvate. Pyc proteins consist of three domains, namely an N- terminal biotin carboxylation domain (BC), a central transcarboxylation domain (TC) and a C-terminal biotin carboxyl carrier domain (BCC). Mutagenesis studies revealed that only the TC domain of HpPyc1p is required for AO assembly and sorting. Because mutations could be introduced that specifically affected AO assembly, but not the enzyme activity of HpPyc1, HpPyc1p

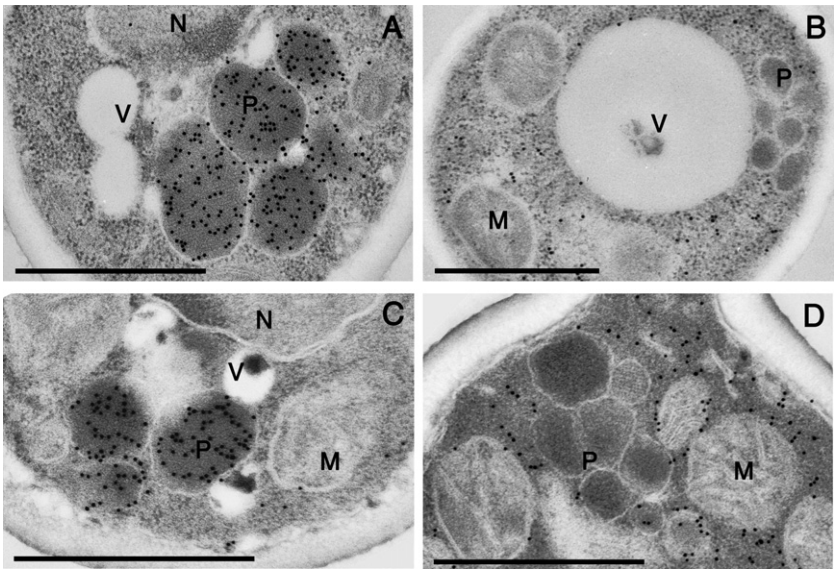

Fig. 1. Immunocytochemical demonstration of (A) alcohol oxidase (AO) protein in peroxisomes of a *H. polymorpha* WT strain producing a mutant AO protein that lacks the C-terminal 16 amino acids or (B) AO with a point mutation in the FAD binding site (G15A). (C–D) AO protein is localized in peroxisomes (C) of *PEX5*deletion cells that produce a C-terminal truncated Pex5p lacking the TPR domains. In these cells the PTS1 protein dihydroxyacetone synthase (DHAS) is not imported into peroxisomes (D). The immunocytochemical experiments were performed on ultrathin sections of unicryl embedded cells decorated with anti-AO or anti-DHAS antibodies. M—mitochondrion, N—nucleus, P—peroxisome, V—vacuole. The bar represents 0.5 μm.

apparently can fulfill a novel additional role, which is not related to its function as an enzyme [32]. The molecular principles of how HpPyc1p functions in mediating FAD binding to AO are still obscure.

The essential role of HpPyc1p in AO sorting is also evident from the analysis of the fate of *H. polymorpha* AO that is produced in the heterologous host *S. cerevisiae* [33, 34]. In *S. cerevisiae* AO does not bind FAD and only a minor portion of the protein is correctly sorted to peroxisomes. This sorting however is fully dependent of the AO PTS1, suggesting that ScPex5p (in contrast to HpPex5p) is capable to recognize this PTS in the context of AO protein [35]. Indeed, upon replacing the ScPex5p by HpPex5p in the heterologous host, AO was again mislocalized to the cytosol [35]. HpPex5p, however, was capable to sort AO to *S. cerevisiae* peroxisomes when HpPyc1p was co-produced. These data stress the essential role for HpPyc1p in AO sorting (see Fig. 2).

Summarizing, these data indicate that AO contains two independent peroxisomal sorting signals for Pex5p-dependent import: a PTS1 and a second PTS that is most likely a conformational epitope and dependent on FAD binding to AO subunits (see Fig. 2). The C-terminal PTS1 of AO cannot be bound by HpPex5p, however it can be recognized by ScPex5p. Remarkably, the alternative HpPyc1p dependent alternative PTS is only recognized by HpPex5p, but not by ScPex5p.

7. Human alanine:glyoxylate aminotransferase

Human alanine:glyoxylate aminotransferase (HsAGT) is a peroxisomal enzyme, which is imported into peroxisomes via Pex5p [36]. HsAGT is an important protein in human health: in various patients suffering from the hereditary kidney stone disease primary hyperoxaluria type 1, HsAGT is mistargeted to mitochondria. The cause of HsAGT mistargeting is related to point mutations that result in the formation of a weak mitochondrial targeting sequence together with inhibition of HsAGT dimerization [37].

Targeting of normal HsAGT to peroxisomes has also been studied in detail and revealed some unusual features [38]. The C-terminal tripeptide –KKL is necessary for import of HsAGT, despite the fact that it does not have typical features of a PTS1 and is not sufficient to target a reporter protein to peroxisomes. Using the two-hybrid system, HsAGT was shown to interact with human Pex5p in mammalian cells. This interaction

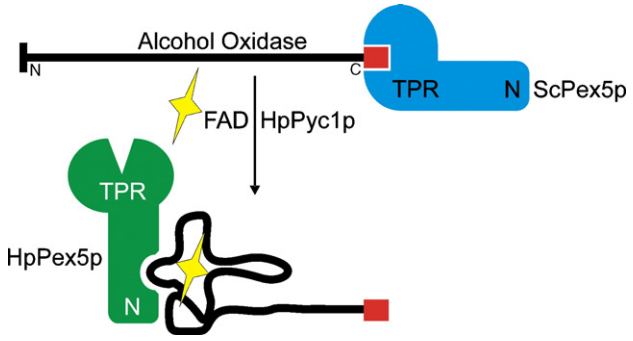

Fig. 2. Schematic representation of two independent PTS's in *H. polymorpha* alcohol oxidase. The C-terminal tripeptide (–AKL, red) can be recognized by the C-terminal TPR domains of ScPex5p (blue), but not by the TPR domains of HpPex5p (green). The N-terminal half of HpPex5p recognizes AO only upon HpPyc1p-mediated FAD binding (yellow). Most likely this results in the formation or stabilization of a conformational epitope in AO that is recognized by the N-terminal half of HpPex5p, but not by the corresponding region in ScPex5p.

requires the C-terminal –KKL of HsAGT [39]. However, when a similar two-hybrid assay is performed in yeast no interaction was detected [38]. Possibly, an accessory protein which is absent in yeast is required for HsPex5p-HsAGT interaction. This may be comparable to what was observed for *H. polymorpha* AO and HpPex5p, whose interaction depends on the function of HpPyc1p (Fig. 2; [35]). However, while HpPyc1p is required to modify the Pex5p cargo protein (i.e. FAD binding to AO), Huber et al. [39] speculated that the putative accessory protein for HsAGT may modify HsPex5p to allow binding of the unusual PTS1, –KKL.

Indeed it is not very likely that the accessory protein functions in co-factor binding to HsATG. Recently, the three-dimensional structure of HsAGT was solved [40]. HsAGT is a dimer composed of two identical subunits. Each subunit consists of an N-terminal extension of about 20 amino acids, a large N-terminal domain of about 260 amino acids containing most of the active site, the cofactor binding site and the dimerization interface, and a smaller C-terminal domain of about 110 amino acids of uncertain function. Analysis of the subcellular location of C-terminal fragments of HsAGT fused to GFP indicated that the smaller C-terminal domain harbors peroxisomal targeting information, especially the internal region between Val-324 and Ile-345. This part of the protein is exposed on the surface of the HsAGT dimer. It is located 47–68 residues upstream of the PTS1, but located very close to the PTS1 in the 3D structure. Therefore, this internal region most likely physically interacts together with the PTS1 to the TPR domains of HsPex5p. The putative accessory protein may reshape the HsPex5p TPR domains allosterically to allow it to accept KKL as a functional PTS1 [39].

8. Other non-PTS1 proteins

Several other non-PTS1 proteins are known to be imported in a Pex5p dependent manner. However, so far little is known of regions in these cargo-proteins and the corresponding Pex5p's that are involved in their recognition. The proteins that are included in this category of non-PTS1 proteins are discussed below.

S. cerevisiae catalase A contains an internal peroxisomal targeting signal [41] in addition to the C-terminal –SKF, which can function as a PTS1, but is dispensable for catalase A sorting. The internal PTS is located in the amino-terminal third catalase A and its function is highly context dependent [41].

The *H. polymorpha* malate synthase enzyme terminates with Ser–Leu–Lys, which is not conform to the canonical PTS1 sequence [42], but its import depends on Pex5p [43].

Removal of the C-terminal tripeptide –ARM from castor bean isocitrate lyase (ICL) does not affect import into plant peroxisomes [44]. However, in the heterologous host *S. cerevisiae*import of ICL into peroxisomes is lost upon deletion of the C-terminal PTS1 tripeptide. Import in yeast peroxisomes is however restored upon removal of 19 C-terminal residues of ICL [45]. Possibly, the accessory PTS of ICL is better recognized by ScPex5p when the 19 C-terminal residues are removed.

9. Concluding remarks

Several data suggest that proper folding/assembly of newly synthesized peroxisomal matrix proteins is a prerequisite for efficient import of peroxisomal matrix proteins, like in the twin arginine (Tat) [46] and cytosol-to-vacuole (CVT) [47] protein translocation pathways. For instance, in patients suffering from primary hyperoxaluria type I, a defect in the dimerization of peroxisomal glyoxylate aminotransferase I contributes to mistargeting of the protein to mitochondria [37]. *Y. lipolytica* acyl-CoA oxidase has to be properly assembled into oligomers to allow sorting to peroxisomes [22] and in the same yeast a defect in thiolase assembly leads to mislocalization of this in peroxisomal PTS2 protein to the cytosol [48]. For alcohol oxidase not oligomerisation, but FAD binding to AO monomers, was shown to be important for peroxisomal sorting [11,28,49].

The significance of protein folding and/or assembly into oligomers has so far obtained little attention in peroxisome biogenesis research. However, its importance may be overlooked because of the general use of non-peroxisomal reporter proteins in peroxisomal research (e.g. green fluorescent protein, chemically modified proteins).

Cytosolic folding of peroxisomal matrix proteins may be required to allow yet unknown protein–protein interactions between peroxisomal matrix proteins and PTS-receptors. As indicated in this contribution this is especially clear for non-PTS1 proteins that are imported via the function Pex5p. However, accessory conformational epitopes are most likely also important for binding of genuine PTS1 proteins to Pex5p. For instance *S. cerevisiae* peroxisomal malate dehydrogenase (MDH3) possesses the typical PTS1 sequence –SKL. As expected deletion of the last two residues resulted in a full block in import. However when the PTS1 is replaced by the non-PTS1 tripeptide –SEL, still a significant portion of the protein is correctly sorted to peroxisomes. Similarly, the tripeptide –KKL at the C-terminus of MDH3 (which is also present at the C-terminus of HsAGT) did not results in a complete block in MDH3 import [50]. None of the alternative C-terminal tripeptides however were sufficient to direct a reporter protein to peroxisomes. Hence, also in the PTS1 protein MDH3 accessory Pex5p binding sites are involved in targeting.

How did the non-PTS1 proteins evolve? It is tempting to speculate that the accessory conformational epitopes may have evolved to assure that solely proper folded/assembled (or co-factor containing proteins) are recognized for sorting peroxisomes (like in Tat and CVT). In those cases where the accessory PTS became sufficiently strong, the genuine PTS1 may have degenerated and in cases where the accessory PTS became dominant, the genuine PTS1 may even have been lost. Alternatively, the PTS1 may have been maintained but not accessible anymore for the TPR domains of Pex5p (like in HpAO and HpPex5p).

The accessory PTS may especially be important for sorting of complex, oligomeric enzymes to prevent accumulation of non-functional subunits inside peroxisomes.

The occurrence of non-PTS1 proteins is a problem in searches in genome sequencing data bases, because these will be overlooked. Therefore, current proteomics approaches which aim to elucidate complete peroxisomal proteomes are likely to yield novel peroxisomal proteins.

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