Receptor-mediated import of proteins into peroxisomes

Bottger, G.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 5

**Peroxisomal import of yeast *Saccharomyces cerevisiae* acyl-CoA oxidase is mediated by Pex5p, but is independent of the PTS1 binding site on Pex5p**

Gina Bottger, André T.J. Klein, Henk F. Tabak and Ben Distel
Peroxisomal import of yeast *Saccharomyces cerevisiae* acyl-CoA oxidase is mediated by the PTS1 receptor Pex5p, but is independent of the PTS1 binding site on Pex5p

Gina Bottger, André T. J. Klein, Henk F. Tabak and Ben Distel

Department of Biochemistry, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

ABSTRACT

Most peroxisomal matrix proteins contain either a peroxisomal targeting signal type I (PTS1) or type II (PTS2) that directs the protein to the organelle. Only a few peroxisomal matrix proteins do not contain a recognizable PTS. We investigated the targeting of *Saccharomyces cerevisiae* acyl-CoA oxidase, a peroxisomal matrix protein that does not contain either a recognizable PTS1 or a PTS2 sequence. Subcellular fractionation and protease protection assays showed that acyl-CoA oxidase is dependent on Pex5p for import into peroxisomes. However, the Pex5p(N393D) mutant that is specifically disturbed in binding of PTS1 proteins, still targets acyl-CoA oxidase to the peroxisome, whereas PTS1 proteins are mislocalized to the cytosol. These findings suggest that acyl-CoA oxidase is imported into the peroxisome via a novel interaction with Pex5p that is not dependent on its PTS1 binding site.
INTRODUCTION

Peroxisomal matrix proteins are synthesized on free polyribosomes in the cytosol and are posttranslationally imported into the peroxisome (Lazarow and Fujiki, 1985). Conserved targeting sequences, composed of the carboxyterminal tripeptide S-K-L or a derivative thereof (PTS1), or an N-terminal stretch of nine amino acids consisting of the consensus sequence R/K - L/V/I - X₅ - H/Q - L/A (PTS2) guarantee the peroxisomal localization of the protein (Hettema et al., 1999; Subramani, 1998). PTS1 and PTS2 sequences are recognized by their matching receptors Pex5p and Pex7p, respectively. Pex5p and Pex7p are proposed to function as mobile receptors that bind newly synthesized PTS-containing proteins in the cytoplasm and that direct them to the peroxisome (Erdmann et al., 1997; Hettema et al., 1999; Subramani, 1998). Pex5p can target proteins with a wide variability in PTS1 sequences to the peroxisome (Elgersma et al., 1996; Gould et al., 1989; Kragler et al., 1998). Mutagenesis studies of Pex5p and crystallographic analysis of a PTS1-containing pentapeptide bound to Pex5p revealed that the binding site for the PTS1 is formed by the seven tetratricopeptiderepeat (TPR) motifs of Pex5p (Gatto et al., 2000; Klein et al., 2001). However, some proteins of which the PTS has been deleted, can still be imported into the peroxisome. These proteins are able to oligomerize in the cytoplasm with a PTS-containing version of the protein, which allows the PTS-less protein to hitch a ride to the peroxisomal lumen (Elgersma et al., 1996; Glover et al., 1994; Lee et al., 1997; Leiper et al., 1996; McNew and Goodman, 1994; Smith et al., 2000). In addition, there are several peroxisomal matrix proteins that are, based upon their amino acid sequence, not equipped with a recognizable PTS1 or PTS2. Examples of such proteins are *Hansenula polymorpha* malate synthase (Bruinenberg et al., 1990), *Saccharomyces cerevisiae* Dci1p (Karpichev and Small, 2000) and acyl-CoA oxidases of the yeasts *Yarrowia lipolytica* (Wang et al., 1998), *Candida tropicalis* (Small et al., 1988) and *S. cerevisiae* (Dmochowska et al., 1990). Although alternative targeting sequences in these proteins have not been identified yet, the existence of an internal targeting sequence has been suggested for *C. tropicalis* acyl-CoA oxidase (Small et al., 1988). In human (Fournier et al., 1994), rat (Miyazawa et al., 1989) and mouse (Nohammer et al., 2000) and in the yeast *Pichia pastoris* (Koller et al., 1999) acyl-CoA oxidase is imported via the PTS1-mediated import route.

We show that *S. cerevisiae* acyl-CoA oxidase requires the PTS1 receptor Pex5p for its import into the peroxisome, although the carboxyterminal is composed of the non-PTS1 sequence I-N-K. A *S. cerevisiae* pex5 mutant that is specifically disturbed in the interaction and the import of PTS1 proteins, Pex5p(N393D), efficiently imports acyl-CoA oxidase, suggesting alternative Pex5p-mediated targeting of this protein to the peroxisome.
Chapter 5

MATERIALS AND METHODS

Yeast strains and culture conditions
Yeasts used in this study was Saccharomyces cerevisiae BJ1991 (Mata, leu2, trpl, ura3-251, prbl-1122, pep4-3, gal2). The BJ1991 pex3Δ and pex7Δ strains are described in Hettema et al. (2000) and the pex5Δ strain is described in Van der Leij et al. (1993).
Cell culture conditions: cells were pregrown overnight on minimal 0.3% glucose medium (0.3% glucose, 0.67% yeast nitrogen base (YNB; Difco) and amino acids (20-30 μg/ml) as required. These cultures were inoculated in fresh 0.3% glucose medium and further grown to log phase. For induction on oleate these cultures were inoculated 1:10 in fresh oleate medium (0.5% potassium phosphate buffer pH 6.0, 0.5% peptone and 0.3% yeast extract, 0.1% oleate, 2% Tween-40) and grown overnight at 28°C.

Plasmids
Plasmids for expression of PEX5 promoter controlled Pex5p(N393D) and for wild-type Pex5p are described in Klein et al. (2001).

Subcellular fractionation and protease protection assays
Subcellular fractionation experiments were performed as previously described (Bottger et al., 2000). Protease protection was performed on oleate-grown cells (200 OD units) that were spheroplasted and lysed in hypotonic buffer similar as described for the preparation of homogenates for subcellular fractionation. 20 μg of Proteinase K (Boehringer) was added to 50 μg of protein sample and incubated with or without Triton X-100 (final concentration 0.15%) at room temperature for 5, 10, 15 and 30 min. Protease activity was stopped by addition of an equal volume of 20% Trichloric acid (TCA) and proteins were precipitated on ice for minimal 1 h. Samples were centrifuged for 30 min at 20,000 x g, pellets were washed with acetone and resuspended in Laemmli sample buffer (Sambrook et al., 1989).

Miscellaneous
Procedures for SDS-PAGE, Western blotting and immunodetection analysis are described by Bottger et al. (2000). Catalase A enzyme activity was measured as described by Lücke et al. (1963). Antibodies used were: anti NH (a generous gift from Dr. P. van der Sluijs, Utrecht, The Netherlands); anti acyl-Co A oxidase (a generous gift from Dr. J.M. Goodman, Dallas); anti-GFP (a generous gift from Jack Fransen, Nijmegen, The Netherlands).

RESULTS

Peroxisomal targeting of acyl-CoA oxidase is dependent on Pex5p but is unaffected by a mutation in the PTS1 binding site of Pex5p
Since S. cerevisiae acyl-CoA oxidase does not contain a recognizable PTS1 or PTS2 in its amino acid sequence, it is unclear whether this protein follows either the Pex5p-dependent or the Pex7p-dependent pathway to peroxisomes. To investigate this, the subcellular distribution of acyl-CoA oxidase was examined in pex7Δ cells, in pex5Δ cells and in pex5Δ cells expressing wild-type Pex5p. To determine the subcellular distribution of a control PTS1 protein Mdh3p, cells were (co-)transformed with a plasmid expressing NH-tagged Mdh3pSKL. After differential centrifugation
Acyl-CoA oxidase import is dependent on Pex5p

Figure 1. Subcellular distribution of acyl-CoA oxidase in wild-type and pex5 mutant cells. Pex5Δ cells were transformed with a plasmid encoding NH-tagged Mdh3p and either with a plasmid encoding wild-type Pex5p (wild-type) or Pex5p(N393D) or were left untransformed (pex5Δ). Cells were grown on oleate, converted to spheroplasts and cleared homogenates (H) were subjected to differential centrifugation at 25,000 x g. The obtained pellet fractions (P) and supernatant fractions (S) were analyzed by immunoblotting using antibodies specific for the NH epitope, acyl-CoA oxidase and thiolase (A), or by measuring the enzyme activity of catalase A (B).

at 25,000 x g, the obtained organellar pellet fractions and supernatant fractions were analyzed by immunoblotting with antibodies specific for acyl-CoA oxidase, 3-ketoacyl-CoA thiolase and the NH epitope. In addition, catalase A (a PTS1 protein) enzyme activity was measured in the fractions. Figure 1A shows that Mdh3p and acyl-CoA oxidase are mislocalized to the 25,000 x g supernatant fractions of pex5Δ cells, although a significant amount of both proteins was recovered from the 25,000 x g pellet fractions. Catalase A predominantly appeared in the supernatant fraction of pex5Δ cells (Figure 1B). Acyl-CoA oxidase on the other hand, was exclusively localized in the 25,000 x g pellet fraction in pex5Δ cells expressing wild-type Pex5p and in pex7Δ cells (Figure 1A and data not shown). To further investigate the effect of Pex5p on the import of acyl-CoA oxidase, we analyzed the subcellular localization of the oxidase in pex5Δ cells expressing the Pex5p(N393D) mutant. Pex5p(N393D) has been characterized as a mutant that is specifically disturbed in the interaction with PTS1 sequences and has lost the ability to import PTS1-containing proteins (Klein et al., 2001). The subcellular distribution of acyl-CoA oxidase in cells expressing Pex5p(N393D) was comparable with the distribution in cells expressing wild-type Pex5p (Figure 1A). In contrast, the subcellular distribution of Mdh3p and catalase A was comparable with that in pex5Δ cells, indicating that both proteins are mislocalized to the supernatant fraction in the pex5 mutant cells (Figure 1A and 1B).
Figure 2. Membrane translocation of acyl-CoA oxidase requires Pex5p, but not the PTS1 binding-site of Pex5p. Wild-type, pex5Δ, pex3Δ and pex7Δ strains (A) and pex5Δ cells co-expressing NH-Mdh3p and Pex5p(N393D) (B) were grown on oleate and converted to spheroplasts. Cleared homogenates were exposed to proteinase K for the time indicated in either the presence (+) or in the absence (-) of 0.15% Triton X-100 (TX100). Samples were analyzed by immunoblotting with antibodies specific for acyl-CoA oxidase, thiolase and the NH epitope.

The subcellular distribution of the PTS2 protein 3-ketoacyl-CoA thiolase was not affected in either pex5Δ cells or Pex5p(N393D) mutant cells (Figure 1A). Together these results suggest that peroxisomal targeting of acyl-CoA oxidase is mediated by Pex5p, but appears to be independent of the PTS1 binding site of Pex5p.
To prove that the acyl-CoA oxidase recovered in the 25,000 x g pellet of pex5Δ cells and the Pex5p(N393D) mutant represents true import into peroxisomes, a protease protection experiment was carried out. Cells from wild-type, pex3Δ, pex5Δ and pex7Δ strains were spheroplasted and lysed in hypotonic buffer. Equal amounts of cleared homogenates were exposed to proteinase K in the absence or presence of detergent (Figure 2A). The PTS2 matrix protein thiolase was used as an internal control for peroxisomal membrane integrity in the pex5Δ and wild-type strains. The protease protection experiment in the pex3Δ strain served as a control for protein degradation in the absence of detectable peroxisomal membrane remnants (Baerends et al., 1996; Hettema et al., 2000; Wiemer et al., 1996). In wild-type cells acyl-CoA oxidase is protected from protease degradation in the absence of detergent, but is completely degraded in the presence of detergent, indicating that the protein has been imported into peroxisomes (Figure 2A). Similar results were found in pex7Δ cells. These results show that acyl-CoA oxidase does not use the PTS2 targeting pathway for its import into peroxisomes. By contrast, in pex5Δ cells acyl-CoA oxidase, was rapidly broken down in the absence of detergent, whereas thiolase was not affected by Proteinase K activity. These results reveal that import of acyl-CoA oxidase into peroxisomes is dependent on Pex5p. To further analyze Pex5p-dependent import of acyl-CoA oxidase, we performed a protease protection experiment in pex5Δ cells expressing the mutant Pex5p(N393D). Figure 2B shows that the PTS1 protein Mdh3p is rapidly degraded in the absence of detergent, which indicates that Mdh3p is not protected by a membrane in the Pex5p(N393D) mutant. This finding suggests that the presence of Mdh3p in the organellar pellet of the Pex5p(N393D) mutant in the subcellular fractionation experiment (Figure 1A) is likely the result of aspecific association with membranes or protein aggregates. Acyl-CoA oxidase on the other hand, showed complete protection from the protease in the Pex5p(N393D) mutant, indicating that the oxidase has been translocated across the peroxisomal membrane (Figure 2B). Taken together, the results show that peroxisomal import of acyl-CoA oxidase requires the PTS1 receptor Pex5p, although import of acyl-CoA oxidase may not be mediated via classical recognition and binding of a PTS1 sequence by Pex5p.

**DISCUSSION**

Here we report that in the yeast *S. cerevisiae*, the peroxisomal matrix protein acyl-CoA oxidase depends on the PTS1 receptor Pex5p for correct targeting and import into peroxisomes. Import of acyl-CoA oxidase does, however, not require the recently characterized PTS1 binding site in Pex5p (Gatto et al., 2000; Klein et al., 2001). We show by differential centrifugation and protease protection assays that the Pex5p(N393D) mutant, which is selectively disturbed in the interaction with PTS1 proteins, specifically affects the subcellular localization of PTS1 proteins, but not of
acyl-CoA oxidase. Further analysis of peroxisomal protein import using protease protection assays revealed that acyl-CoA oxidase is mistargeted to the cytosol in pex5Δ cells, whereas it is still imported in peroxisomes of the Pex5p(N393D) mutant. In line with these findings, the Pex5p(N393D) mutant still bound to acyl-CoA oxidase in the two-hybrid assay (data not shown), whereas interaction with PTS1 proteins Mdh3p and GFP-PTS1 was abolished (Klein et al., 2001). Similarly, truncation of the C-terminal TPR domains 5-7 of Pex5p, which are essential for the interaction with PTS1 sequences (Brocard et al., 1994; de Walque et al., 1999; Dodt et al., 1995; Gatto et al., 2000; Klein et al., 2001; Szilard and Rachubinski, 2000; Terlecky et al., 1995), did not inhibit the two-hybrid interaction with acyl-CoA oxidase (data not shown). Together, these findings show that Pex5p binds acyl-CoA oxidase in a way that is different from the interaction with PTS1-containing proteins.

The carboxyterminal sequence I-N-K of S. cerevisiae acyl-CoA oxidase does not match the PTS1 consensus, just like the homologous acyl-CoA oxidases in Y. lipolytica and C. tropicalis, that contain the carboxyterminal sequence D-E-E and L-K-S, respectively. Since these oxidases do not possess a PTS2, it is likely that peroxisomal targeting and import is facilitated by an alternative targeting signal. Two regions bearing internal targeting signals have been identified in C. tropicalis acyl-CoA oxidase (Small et al., 1988). However, the important residues within these targeting regions were not identified. The existence of an alternative targeting sequence has also been suggested for S. cerevisiae carnitine acetyl transferase (CAT). A PTS1-deleted version of CAT interacts with Pex5p in the two-hybrid assay and, in addition, can be imported into the peroxisome independent of its PTS1 sequence (Elgersma et al., 1995). It may not be surprising that, just like S. cerevisiae acyl-CoA oxidase, CAT efficiently binds Pex5p(N393D) in the two-hybrid assay (data not shown). H. polymorpha malate synthase and Y. lipolytica acyl-CoA oxidase have previously shown to be imported in a Pex5p-dependent manner (Szilard et al., 1995; Van der Klei et al., 1995), although a PTS1 sequence is absent in these proteins. It would be of interest to investigate which sequences target these proteins to the peroxisome and whether these sequences are conserved and are involved in the interaction with Pex5p. The existence of additional binding sites on Pex5p that do not contact the PTS1 tripeptide is supported by interaction studies with synthetic PTS1-containing peptides and Pex5p. These studies have shown that amino acids adjacent to the PTS1 sequence play an essential role in determining the affinity of binding to Pex5p and the specificity of interaction with Pex5 proteins from different species (Lametschwandtner et al., 1998). For several PTS1-containing proteins it has been shown that the PTS1 sequence is necessary but not sufficient for peroxisomal import, suggesting that specific protein sequences other than the PTS1 support targeting or import of these proteins (Bongcam et al., 2000; Motley et al., 1995; Mullen et al., 1997; Purdue and Lazarow, 1996).

The different binding sites on Pex5p for PTS1-containing proteins and for proteins such as acyl-CoA oxidase and CAT could also explain the findings of Zhang et al. (1993), who described a S. cerevisiae mutant (peb5-I) that mislocalized catalase
Acyl-CoA oxidase import is dependent on Pex5p

A to the cytoplasm, whereas 3-ketoacyl-CoA thiolase and acyl-CoA oxidase were imported. Which gene is mutated in the peb5-1 cells is however not known, although it is tempting to speculate that peb5-1 cells contain a mutation in the PEX5 gene that affects PTS1 binding. It is however not known whether the peb5-1 mutant is analogous to a pex5 mutant. Despite of the differential import of PTS1 proteins and acyl-CoA oxidase, it cannot be excluded yet that the carboxyterminal sequence I-N-K of S. cerevisiae acyl-CoA oxidase is a functional PTS1 sequence. To provide further evidence that this sequence is not a functional PTS1, a set of additional experiments are required. The subcellular localization of the carboxyterminal deleted version of acyl-CoA oxidase(ΔI-N-K) in wild-type cells and of a reporter protein containing the tripeptide I-N-K at its carboxyterminus could indicate whether the carboxyterminal tripeptide contains targeting information. Additional binding studies with acyl-CoA oxidase(ΔI-N-K) and Pex5p could show whether the I-N-K tripeptide is involved in the interaction with Pex5p. It should be noted that Mdh3p with a C-terminal extension ending in the sequence S-N-K (indicated as MDH3ΔPTS1 in Elgersma et al., 1996) is mislocalized to the cytoplasm of wild-type S. cerevisiae cells, indicating that this sequence is not functional in peroxisomal targeting.

ACKNOWLEDGEMENTS

We thank Ewald Hettema and Wim de Jonge for stimulating discussions and helpful suggestions. We also thank Dr. Joel Goodman (Dallas) for the acyl-CoA oxidase antibody.

REFERENCES


Acyl-CoA oxidase import is dependent on Pex5p