YEAST
Yeast **15**, 1035–1044 (1999)

Analysis of the Peroxisomal Acyl-CoA Oxidase Gene Product from *Pichia pastoris* and Determination of its Targeting Signal

A. KOLLER[1], A. P. SPONG[1], G. H. LÜERS[1]† AND S. SUBRAMANI[1]*

[1]*Department of Biology, University of California at San Diego, La Jolla, CA 92093-0322, U.S.A.*

Acyl-CoA oxidase (Pox1p) is involved in the β-oxidation of fatty acids and is targeted to the peroxisomal matrix via the use of different signals in various organisms. In rat, mouse and human, Pox1p contains a canonical peroxisomal targeting signal 1 (PTS1), whereas in the yeasts *Candida tropicalis, Saccharomyces cerevisiae, C. maltosa* and *Yarrowia lipolytica* neither a PTS1 nor a PTS2 sequence is present, suggesting that Pox1p might be targeted to the peroxisomes via a third unknown pathway. Alternatively, since proteins lacking a PTS sequence can enter peroxisomes in association with other polypeptides containing a PTS, Pox1p might 'piggy-back' its way into the peroxisomal matrix together with other proteins. To understand the mechanism of peroxisomal targeting of a yeast Pox1p, we cloned the *Pichia pastoris POX1* gene to study the pathway of import of PpPox1p into peroxisomes. The gene was cloned by PCR, hybridization and plasmid rescue. The 2157 bp gene encodes a protein with a predicted molecular weight of 80 kDa. Antisera against PpPox1p detected a protein specifically induced on oleate with an apparent molecular weight of 72 kDa. Immunolocalization studies confirmed the peroxisomal localization of PpPox1p. The carboxy-terminus of PpPox1p ends with a PTS1-like sequence, APKI. The sequence PKI was necessary for transport of PpPox1p into peroxisomes and interacted with the PTS1 receptor, Pex5p. Furthermore, addition of the sequence APKI to the C-terminus of the green fluorescent protein directed this fusion protein to the peroxisome. Therefore, PpPox1p uses the PTS1 pathway for its import into peroxisomes. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — peroxisomal targeting signal; PTS1; fatty acid β-oxidation; protein import

INTRODUCTION

Several unique features characterize peroxisomal biogenesis. One of these is the rapid induction of this organelle and its contents in response to specific carbon sources in yeast. In *Pichia pastoris*, one of the more potent inducers of peroxisomes is the fatty acid oleate. A series of peroxisomal enzymes are required for β-oxidation of activated fatty acids into acetyl-CoA. The first enzyme in this pathway, acyl-CoA oxidase (Pox1p), is localized in peroxisomes in a diverse range of organisms from yeast to man.

For proteins to be correctly routed to their final destination, specific transport mechanisms are required that make use of targeting signals within the primary amino acid sequence of those proteins. Two different peroxisomal targeting signals (PTS) have already been elucidated. The first, PTS1, consists of a carboxy-terminal tripeptide, typically consisting of serine–lysine–leucine. A number of conserved substitutions within this amino acid sequence function with varying degrees of efficiency in different organisms (for review, see Subramani, 1998). The second signal, PTS2, is located at the amino-terminus and has a consensus sequence of R/K–L/V/I–X_5–H/Q–L/A (Subramani, 1998).

*Correspondence to: S. Subramani, Department of Biology, Bonner Hall 3230, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0322, U.S.A. Tel: (619) 534-2327; fax: (619) 534-0053; e-mail: ssubramani@ucsd.edu.
†Present address: University of Bonn, Institute of Anatomy, Nussallee 10, 53115 Bonn, Germany.
Contract/grant sponsor: Swiss National Fonds; Contract/grant number: 8230-046677.
Contract/grant sponsor: Deutsche Forschungsgemeinschaft; Contract/grant number: Lu 571/1-1.
Contract/grant sponsor: National Institutes of Health, U.S.A.; Contract/grant number: DK41737.

CCC 0749–503X/99/111035–10 $17.50

Received 4 February 1999
Accepted 24 February 1999

Although the acyl-CoA oxidase proteins from rat, mouse and human have PTS1 sequences (Miyazawa *et al.*, 1989; Fournier *et al.*, 1994; El-Shabrawi *et al.*, 1997), the same is not true for their yeast counterparts. Small *et al.* (1988) defined two regions, an amino-terminal portion (amino acids 1–118) and an internal region (amino acids 309–427), that were responsible for targeting *Candida tropicalis* Pox1p to the peroxisomes. They reasoned, therefore, that there are two independent targeting signals in CtPox1p, neither of which corresponds to the PTS1 or PTS2 sequences. Information from Zhang *et al.* (1993) lends further support for an alternative targeting signal for acyl-CoA oxidase. In characterizing mutants isolated from *Saccharomyces cerevisiae*, they discovered that a mutant in the PTS1 receptor (*Scpex5*) could not import catalase, but could import acyl-CoA oxidase and thiolase. A mutant in the PTS2 receptor (*Scpex7*) was unable to import thiolase but could import catalase and acyl-CoA oxidase (Zhang *et al.*, 1993). Taken together, this is genetic evidence to support the idea that these three proteins utilize three independent pathways for import, especially as the *S. cerevisiae* Pox1p seems to lack both PTS1 and PTS2 sequences. In other yeasts, *Candida maltosa* (Hill *et al.*, 1988) and *Yarrowia lipolytica* (Wang *et al.*, 1998), the C-terminus of the acyl-CoA oxidases does not resemble a PTS1 sequence and it is therefore possible that these proteins might take an alternative pathway into the peroxisome.

In the course of our work with *P. pastoris*, we found a 72 kDa protein greatly induced by oleic acid. We suspected that this protein might be acyl-CoA oxidase. The gene for this protein has not been cloned yet and since there was a question as to what import pathway was being used, we cloned the *PpPOX1* gene and investigated the targeting signals in the encoded protein.

MATERIALS AND METHODS

Bacterial strains

The *Escherichia coli* strains DH5α, JM109 and TOP10 (Invitrogen, Carlsbad, CA) were used in cloning experiments except for plasmid rescue for which the *E. coli* strain JA226 was used.

Yeast strains and growth conditions

The wild-type strain of *P. pastoris* is the arginine/histidine-requiring (PPY12) strain (Gould *et al.*, 1992). STK10 is PPY12 *Δpox1::Zeocin*, STK11 is PPY12 *Δpox1::Zeocin* transformed with plasmid pTK61, STK12 is PPY12 *Δpox1::Zeocin* transformed with plasmid pTK62. The *ΔPpex5* strain is PPY12 *Δpex5::ARG4* (McCollum *et al.*, 1993). The *S. cerevisiae* strain used for two-hybrid analysis was L40 (*MAT***a**, *his3Δ200, trp1-901, leu2-3,112, ade2, LYS2*::$(lexAop)_4$-*HIS3, URA3*::$(lexAop)_8$-lacZ). Yeast media were as described (Faber *et al.*, 1998).

Molecular biology methods

Recombinant DNA techniques were performed essentially as described (Sambrook *et al.*, 1989). In most cases, polymerase chain reaction (PCR) products were first cloned into either pCRII or pCRblunt vectors (Invitrogen, Carlsbad, CA) from which they were excised for further cloning. All oligonucleotide primers used are shown in Table 1.

Sequencing of the gene and computer analysis of the sequence

Sequencing of the *PpPOX1*-containing plasmid was performed by subcloning fragments into pBluescriptIIKS (Stratagene, La Jolla, CA). The Sequenase II system (United States Biochemical, Cleveland, OH) which is based on the dideoxynucleotide chain-termination method was used for nucleotide sequencing. T3 or T7 primers, as well as custom-ordered primers, were utilized together with denatured, double-stranded DNA templates. Both strands were entirely sequenced. Sequences were analysed using the MacVector software (IBI, New Haven, CT) and the FASTA program (Pearson and Lipman, 1988).

Cloning of PpPOX1 and plasmid rescue

PCR was performed on *P. pastoris* genomic DNA with the two degenerate oligos, POX5PRIME and POX3PRIME, designed from the sequences of previously published acyl-CoA oxidase genes. A fragment of the appropriate size (210 bp) was observed. This fragment was cloned into the pCRII vector (Invitrogen, Carlsbad, CA) and inserts were sequenced. Several plasmids were found to contain sequences encoding the presumed *P. pastoris POX1* gene, in the region expected from the oligos. The insert was then excised from the plasmid, labelled using a random-primed DNA labelling kit (Boehringer–Mannheim, Indianapolis, IN) and used to screen a *P. pastoris*

Table 1. Oligonucleotides used in this study.

Primer	5′-Sequence-3′
POX5PRIME	GCNACNAARTGGTGGATHGG*
POX3PRIME	AAYTGDATCCANCCRTTRTC*
TW14	CTCCACATTAATGGCGGCCGCTCTAGAACTAG
TW26	CTGCAGATCTTTCAGTTGTTAGACTG
GLO50	TAGCGGCCGCTCTCAAGATGAGCACGCG
TK56	AGATCTATGTTCAAAATTGAATCGATCAAAAGTCAGTCGCCGC
TK57	CTAAGCCAACTTCTTCAATACTTTGTCACTC
TK78	GGGGATCCGTCGACCTGCAGCGTACCATGTTGTGATTTTTCAGTTGTT AGACTGG
TK79	AACGAGCTCGAATTCATCGATGATATAGGGTTATTGATTACTAGTTTA ATGC
TK125	GGGATCCATGAGTAAAGGAGAAGAACTTTTC
TK126	GCTAAATCTTAGGAGCTTTGTATAGTTCATCCATGCC
pPAS8-1	AGATCTACCATGTCGCTTATTGGCGG

*N=A, C, G, T; Y=C, T; B=G, T, C; R=A, G; H=A, T, C; D=G, A, T.

genomic DNA library by hybridization. Positive colonies were screened by PCR, using the original oligos, and the fragments generated were checked by sequencing. Plasmid DNA from several colonies containing the correct fragment were further characterized. All contained an insert of about 4 kb. This insert was sequenced and found to encode the first 660 amino acids of the PpPox1p.

To clone the missing part of the *PpPOX1* gene, a null allele was generated by the insertion of the *ARG4* gene into the *PpPOX1* gene. In order to clone part of the gene, a disruption was made that introduced plasmid sequences into the genome along with the auxotrophic marker while at the same time deleting a 1·2 kb region of the gene. This was accomplished by first cloning a 3′ 800 bp *Xba*I–*Bam*HI fragment of *PpPOX1* into pBluescriptIIKS digested with *Xba*I and *Bam*HI. Then a 5′ 1·7 kb *Bam*HI–*Eco*RV fragment was placed into this plasmid (using the *Bam*HI site and a blunted *Pst*I site). Finally, the *P. pastoris ARG4* gene (2 kb) was inserted into this construct using the *Eco*RI and *Xho*I sites. This plasmid was then linearized by cutting with *Bam*HI and electroporated into PPY12 cells. The transformants that grew were tested for their ability to grow on oleic acid. Next, the oleate non-utilizing strains were examined by Western blot to see if they expressed any PpPox1p protein. Finally, to check that the plasmid had integrated correctly, a Southern blot was performed. The genomic DNA was digested with *Bam*HI and probed with a PCR-generated *PpPOX1* probe. The Southern blot revealed a single band of 10 kb, compared to the 7 kb band observed in wild-type cells with the same probe. This conclusively demonstrated that the null strain was indeed correctly constructed. Genomic DNA of a correctly-disrupted strain was digested with *Bam*HI, ligated, transformed into *E. coli* and the resulting rescued plasmid was sequenced to obtain the missing 3′ part of the *PpPOX1* gene.

Null allele construction

To disrupt the complete open reading frame of *PpPOX1*, the 5′ and 3′ regions of the gene were amplified with PCR (TW14 and TK78 for the 5′ region and TK79 and GLO50 for the 3′ region). The 5′ fragment was cloned as a *Hin*dIII–*Pst*I fragment into pBluescriptIIKS, which was cut with *Hin*dIII–*Pst*I. The 3′ fragment was then ligated as an *Not*I–*Cla*I (blunt-ended) fragment into the vector which was cut with *Not*I–*Spe*I (blunt-ended). The resulting plasmid was cut with *Bam*HI, blunt-ended with Klenow DNA polymerase and a blunt-ended, *Bam*HI–*Eco*RV Zeocin fragment (cut from plasmid pPICZA; Invitrogen, Carlsbad, CA) was inserted. The resulting plasmid, pTK60, was cut with *Hin*dIII and *Not*I and transformed into PPY12. Zeocin-resistant colonies were picked and checked for their ability to grow on oleate. The oleate non-utilizing colonies were picked and disruptions were confirmed by PCR.

Construction of plasmids

Plasmid pTW70 replaces the AOX promoter in pHILD2 (Invitrogen, Carlsbad, CA) with the *PpPOX1* promoter. The *PpPOX1* promoter was amplified with primers TW14 and TW26 from the original isolated *PpPOX1* plasmid, pAOX4. Plasmid pTK61, containing the full-length *PpPOX1* gene downstream of the *PpPOX1* promoter, was constructed as follows: the gene was amplified by PCR with primers TK56 and GLO50. The resulting fragment was cloned into the pCRblunt vector (Invitrogen, Carlsbad, CA). The fragment containing full-length *PpPOX1* was cut out with *Bgl*II–*Eco*RI and ligated into plasmid pTW70, which was cut with *Bgl*II and *Eco*RI. Plasmid pTK62, expressing a PpPox1p missing the last three amino acids from the *PpPOX1* promoter, was constructed as follows. The gene was amplified with primers TK56 and TK57. The resulting fragment was subcloned into pCRblunt. The fragment containing *Pppox1ΔPKI* was cut out with *Bgl*II–*Eco*RI and ligated into plasmid pTW70. Yeast two-hybrid plasmids were made by fusing appropriate gene fragments downstream of the DNA-binding (DB) domain of LexA. Plasmids pTK63 and pTK64 were made by ligating the full-length *PpPOX1* and *Pppox1ΔPKI* from plasmids pTK61 and pTK62 (excised with *Bgl*II and *Eco*RI) into the plasmid pBTM116 (Faber *et al*., 1998), which was cut with *Bam*HI and *Eco*RI. Plasmid pTK63, expressing GFP with the C-terminal sequence APKI, was made by PCR with the primers TK125 and TK126 and the resulting fragment was cloned into a *Bam*HI–*Eco*RI cut pPIC3K (Invitrogen, Carlsbad, CA). Plasmid pKNSD99, expressing PpPex5p in the pVP16 vector, was made as follows. PCR was performed with primers pPAS8-1 and the T7 primer on a template with *PpPEX5* in pBluescriptSKII (McCollum *et al*., 1993). The *PpPEX5* fragment was cut with *Bgl*II and *Eco*RI and cloned into a *Bam*HI–*Eco*RI-cut pVP16. The plasmid expressing PpPex7p in pVP16 was pM34 (Elgersma *et al*., 1998).

Generation of anti-PpPox1p antibody

A 1700 bp *Sst*I–*Hin*dIII fragment (*Sst*I site is located in the *PpPOX1* gene) was isolated from the original *PpPOX1*-containing plasmid, pAOX4, and cloned in-frame into the GST-fusion plasmid pGEX-KG (Mitchell *et al*., 1993), which was cut with *Sst*I and *Hin*dIII. Colonies containing the plasmid with insert were then examined for expression of the fusion protein after the addition of isopropoyl β-D-thiogalactopyranoside (IPTG). The fusion protein was isolated by electroelution of the appropriate band from a sodium dodecyl sulphate (SDS)–polyacrylamide gel. It was then dialysed against thrombin cleavage buffer, cleaved with thrombin and reisolated from another SDS–polyacrylamide gel by electroelution. The purified protein was then injected into a rabbit.

Whole cell lysates

Lysates were made with trichloroacetic acid (TCA) as follows: two OD_{600} units of cells were taken, spun down, resuspended in 10% TCA and incubated on ice for >30 min. The suspension was centrifuged and the pellet washed with acetone three times. The pellet was resuspended in sample buffer and glass beads were added. The tube was vortexed for 1 min and boiled for 1 min. This procedure was repeated four times. The sample was separated from the glass beads and loaded on SDS–polyacrylamide gels. Western blotting was performed according to standard procedures. Antibody dilutions were as follows:* α-PpPox1p, 1:10 000; α-ScZwf1p, 1:3000; α-Ala-Lys-Ile, 1:1000.

Immunoprecipitations

Immunoprecipitations were done according to Faber *et al*. (1998).

Immunofluoresence

Indirect immunofluorescence was performed as described (Gaynor *et al*., 1997). A 1:5000 dilution of the α-PpPox1p antibody and a 1:1000 dilution of α-Cta1p (catalase) antibody was used. The immune complexes were detected with a Cy3-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA). Fluorescence images were acquired using a CCD camera (Model 4995; COHU, San Diego, CA) and a CG-7 Frame Grabber (Scion Corp., Frederick, MD). The software used was Scion Image 1.6, Adobe Photoshop 3.0 and Adobe Freehand 5.5.

RESULTS

Cloning of the PpPOX1 gene and analysis of the gene product

Comparison of Pox1p proteins from several different species revealed several regions of

*ScZwf1p=*S. cerevisiae* glucose-6-phosphate dehydrogenase.

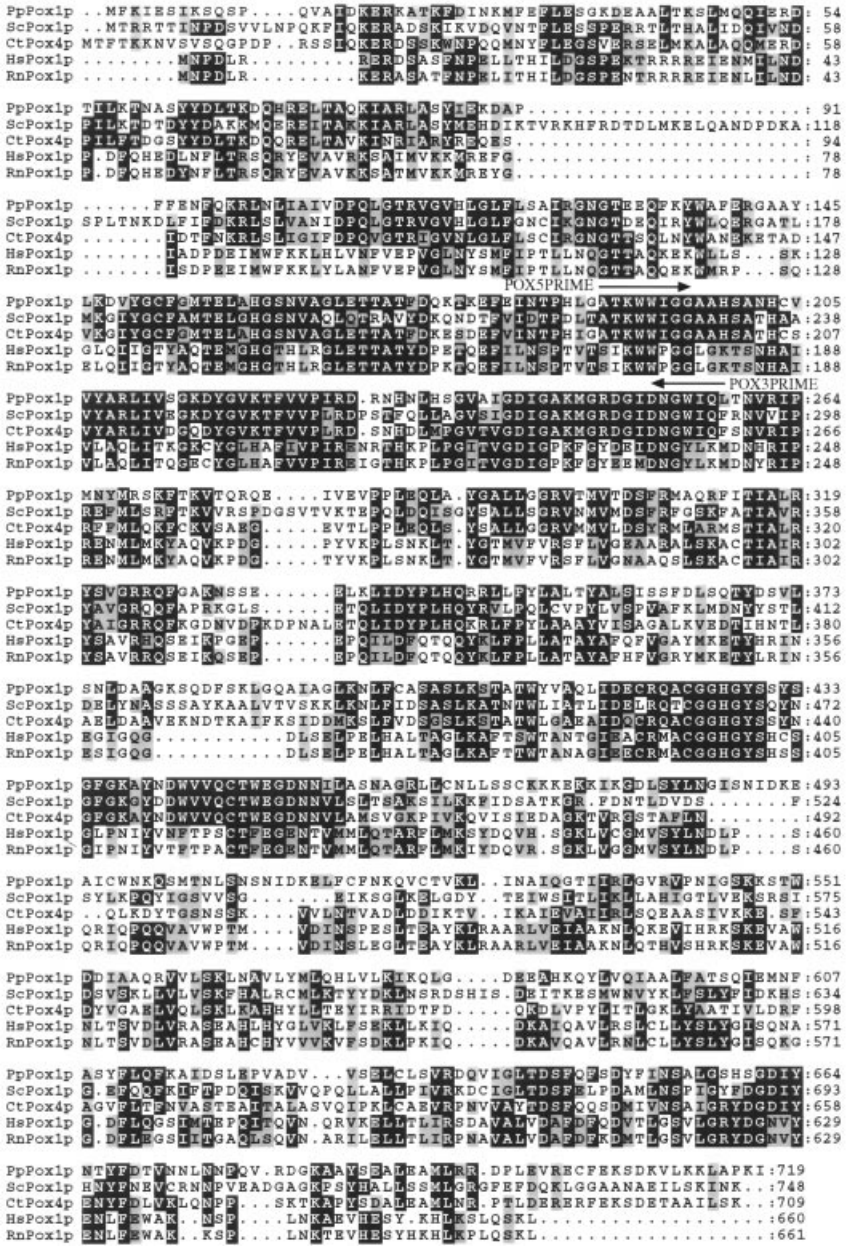

Figure 1. Amino acid sequence alignment of acyl-CoA oxidases from different organisms. Sequences were from *P. pastoris, S. cerevisiae, C. tropicalis*, rat and human. The black boxes represent conserved or identical amino acids, the grey boxes represent similar amino acids. Locations of the PCR primers (POX5PRIME and POX3PRIME) used for cloning a fragment of the gene are indicated by arrows.

homology. Two of these regions were picked (Figure 1), and degenerate oligos were designed for PCR. These were then used on genomic *P. pastoris* DNA to clone a gene (see Material and Methods) which consists of an open reading frame of 2157 bp (Genbank Accession No. AF133102).

The cloned gene encodes a protein of 719 amino acids with a predicted molecular weight of 80 kDa,

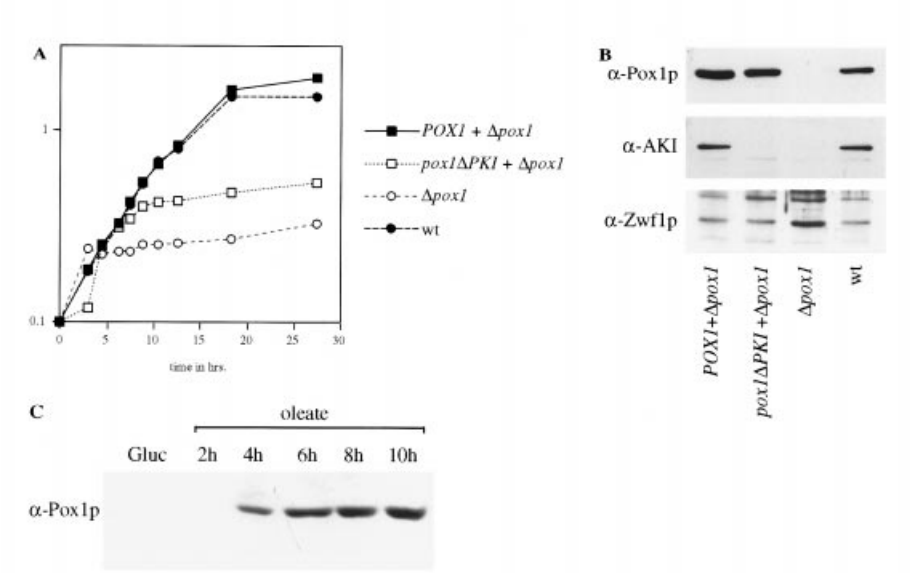

Figure 2. (A) Growth curves for different strains. Strains were grown in oleate and the OD_{600} was measured at specified times. *POX1+Δpox1* is STK11, *pox1ΔPKI+Δpox1* is STK12, *Δpox1* is STK10 and wild-type is PPY12. (B) Expression of PpPox1p protein in different strains. TCA lysates of oleate-grown strains were immunoblotted with different antibodies. (C) Induction of PpPox1p after shifting wild-type cells (PPY12) from dextrose to oleate-containing media. Time course of PpPox1p induction.

with a pI of 8·8 and the carboxy-terminal tripeptide PKI (see Figure 1). Comparison of this protein to homologues from *C. tropicalis* (CtPox4p; Okazaki *et al.*, 1986), *S. cerevisiae* (ScPox1p; Dmochowska *et al.*, 1990), rat (RnPox1p; Miyazawa *et al.*, 1989) and human (HsPox1p; Varanasi *et al.*, 1994) showed that it is highly homologous to these acyl-CoA oxidases and that Pox1p is a highly conserved protein among yeast and vertebrates (Figure 1). We therefore reasoned that we had in fact cloned the *P. pastoris* acyl-CoA oxidase gene and named it *PpPOX1*.

The null allele of PpPOX1 is unable to grow on oleate-containing media

A null allele of *PpPOX1* was generated by replacing the whole open reading frame of *PpPOX1* with the Zeocin-resistance gene (see Materials and Methods). This *ΔPppox1* strain (STK10) was unable to grow in oleate-containing media (see Figure 2A). Its growth on dextrose and methanol was not affected (data not shown).

An antibody to PpPox1p recognizes a protein of 72 kDa specifically induced in oleate-containing media

An antibody to PpPox1p (Materials and Methods) detected a band of 72 kDa in wild-type cells grown on oleate (Figure 2B), but not in cells grown on dextrose or methanol (Figure 2C and data not shown). In the *ΔPppox1* strain (STK10), no PpPox1p could be detected (Figure 2B). PpPox1p was induced rapidly after shifting cells from dextrose to oleate-containing media (Figure 2C).

PpPox1p is the protein recognized by the AKI antibody

When we tested an antibody raised to a PTS1 sequence, AKI, from *C. tropicalis* (Aitchison *et al.*, 1992) on lysates from oleate-grown *P. pastoris* cells, a 72 kDa protein was always detected. As this protein and the PpPox1p were the same size, and were induced on oleate, we checked if these two proteins were the same. Immunoprecipitations were carried out using oleate-induced, wild-type cell lysates, and either anti-AKI (Figure 3, lane 3

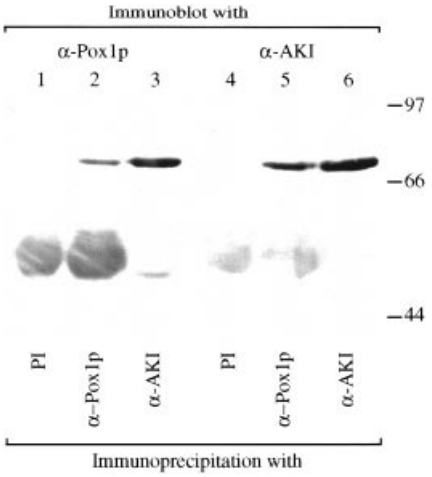

Figure 3. PpPox1p is recognized by antisera to the PTS1 sequence, AKI. Extracts from oleate-grown cells were immunoprecipitated with either pre-immune sera (PI), α-Pox1p or α-AKI. Samples were run on a SDS–polyacrylamide gel and immunoblotted with either α-Pox1p or α-AKI.

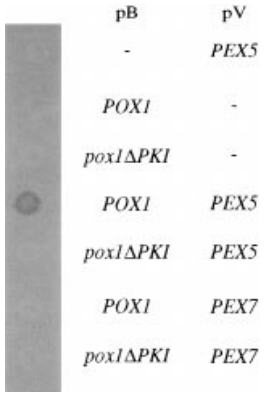

Figure 4. PpPox1p interacts with PpPex5p in a yeast two-hybrid assay. The *S. cerevisiae* strain L40 was transformed with a pB(DNA-binding domain plasmid)-based and a pV(activation-domain plasmid)-based plasmid with specified inserts (see Materials and Methods). Strains were grown in liquid medium. A sample of the cells was placed on a nylon filter and a β-galactosidase assay was performed to detect interacting proteins.

and 6) or anti-PpPox1p (Figure 3, lane 2 and 5), or pre-immune serum (PI) as a control (Figure 3, lanes 1 and 4). Precipitated proteins were analysed by Western blot with both anti-AKI (Figure 3, lanes 4–6) and anti-PpPox1p (Figure 3, lanes 1–3). In all cases, the protein immunoprecipitated by this treatment was recognized by both antibodies (Figure 3, lanes 2, 3, 5 and 6), indicating that PpPox1p is indeed recognized by the anti-AKI antibody.

The C-terminal tripeptide PKI is required for the import of PpPox1p into the peroxisomes

To address how PpPox1p is targeted to peroxisomes, several experiments were performed to determine the targeting signal. A construct was made which expressed PpPox1p missing the last three amino acids, PKI (*Pppox1ΔPKI*). This construct, as well as the full-length *PpPOX1*, was cloned into the two-hybrid vector pBTM116 (see Materials and Methods) and checked for interaction with *PpPEX5*, the gene encoding the PTS1 receptor, or *PpPEX7*, encoding the PTS2 receptor. We could only detect a two-hybrid interaction between the full-length PpPox1p and PpPex5p, and not with any other combination (Figure 4). This shows that PpPox1p is recognized by PpPex5p and that the last three amino acids PKI are required for this interaction.

Full-length *PpPOX1* and *Pppox1ΔPKI* were cloned downstream of the *PpPOX1* promoter in plasmid pTW70 (see Materials and Methods) and integrated into the *HIS4* locus in the *ΔPppox1* strain (resulting in strains STK11 and STK12). TCA lysates were made after induction in oleate-containing medium and checked for the presence of PpPox1p. Both strains (STK11 and STK12) expressed a protein that was recognized by the anti-PpPox1p antibody, but this protein was absent in the *ΔPppox1* strain (STK10) (Figure 2B). The anti-AKI antibody only recognized the full-length PpPox1p, whereas the PpPox1ΔPKIp was not recognized by this antibody. This again proves that PpPox1p is the protein recognized by the anti-AKI antibody and that the epitope of the antibody contains the C-terminal three amino acids.

Growth curves were done on the different strains growing in oleate medium. As shown in Figure 2A, the *ΔPppox1* strain expressing full-length *PpPOX1* (STK11) grew as well as the wild-type strain, whereas a *ΔPppox1* strain (STK10) did not. The *ΔPppox1* strain transformed with the *Pppox1ΔPKI* construct (STK12), however, grew less than wild-type, but did grow better than the *ΔPppox1* strain. This result suggests that either a small amount of PpPox1ΔPKIp is transported into the peroxisomes, or that PpPox1ΔPKIp is able to perform its function to a certain degree in the cytosol.

The localization of PpPox1p in peroxisomes and the requirement of the C-terminus PKI as well as the PTS1 receptor (PpPex5p) for import into the

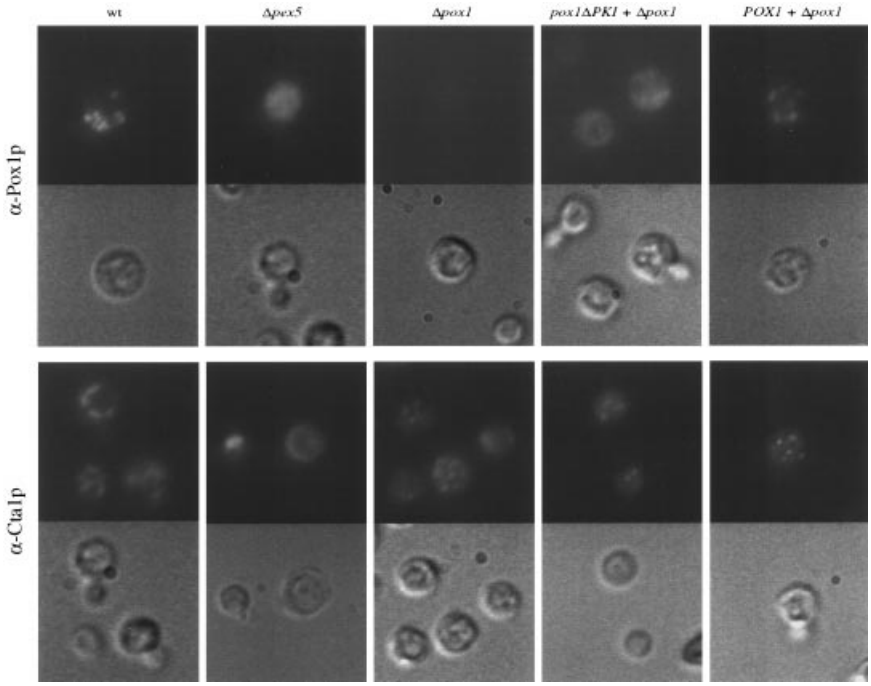

Figure 5. Wild-type (PPY12), *Δpex5* (PPY12 *Δpex5::ARG4*), *Δpox1* (STK10), *pox1ΔPKI+Δpox1* (STK12), and *POX1+Δpox1* (STK11) were grown in oleate and prepared for immunofluorescence, as described in Materials and Methods. The fixed cells were incubated with α-Pox1p or α-Cta1p.

peroxisomes was further analysed by immunofluorescence. Strains were grown in oleate, fixed and checked with either anti-PpPox1p or anti-Cta1p as the primary antibody. As shown in Figure 5, wild-type strains (wt and STK11), when analysed with either antibody, contained several small peroxisomes. In the *ΔPppex5* strain, the staining was cytosolic, suggesting that this strain was unable to transport either of the two proteins (PpPox1p and Cta1p) into the peroxisomes. In the *ΔPppox1* strain expressing *Pppox1ΔPKI* (STK12), Cta1p was targeted to the peroxisomes, whereas PpPox1ΔPKIp was localized in the cytosol.

These experiments suggest that PpPox1p was targeted into peroxisomes in a Pex5p-dependent manner and that the PKI sequence was necessary for peroxisomal import.

The C-terminal APKI is sufficient for import into peroxisomes

A green fluorescent protein (GFP) fusion was constructed which expressed APKI at the C-terminus (pTK63). This construct was transformed into PPY12 and checked for the localization of the GFP in methanol-induced cells. The GFP–APKI fusion protein was transported into peroxisomes, as was GFP–SKL (Figure 6). Therefore, the APKI sequence at the C-terminus of PpPox1p is both necessary and sufficient for import into the peroxisomal matrix.

DISCUSSION

In this paper we describe the cloning of acyl-CoA oxidase (*PpPOX1*) from *P. pastoris*, preliminary characterization of its expression and the findings that the carboxy-terminal sequence is both necessary and sufficient for import into peroxisomes via the PTS1 pathway.

The targeting signal that directs the β-oxidation enzyme Pox1p to peroxisomes in rats and human cells is the classical PTS1 sequence, SKL. However, there is no obvious PTS1 sequence in any of the yeast *POX1* genes so far sequenced; in addition, none of the amino termini show any degree of similarity with the PTS2 consensus sequence. Studies in *C. tropicalis* have suggested that an internal signal might be used (Small *et al.*, 1987,

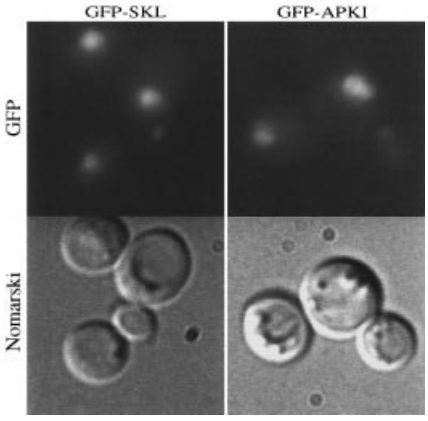

Figure 6. PPY12 was transformed with GFP–SKL (pTK51) or GFP–APKI (pTK63), grown on methanol and checked for the localization of GFP under a fluorescence microscope.

1988). Genetic studies performed in *S. cerevisiae* support the idea that ScPox1p does not use either the PTS1 or the PTS2 import pathways, but instead uses an independent pathway for protein import into peroxisomes (Zhang *et al*., 1993).

The *POX1* gene of *P. pastoris* was found to contain an open reading frame encoding a protein with a predicted molecular weight of 80 kDa. The amino acid sequence is highly homologous to acyl-CoA oxidase sequences from *C. tropicalis, S. cerevisiae*, rat and human. Consistent with the other yeast acyl-CoA oxidases already found, the *P. pastoris* Pox1p did not end in an obvious PTS1 sequence, but had instead the C-terminal sequence, PKI (Figure 1).

PpPox1p behaved in a manner consistent with that of a peroxisomal matrix protein. It was specifically induced by growth in oleate-containing media, and repressed in dextrose- or methanol-containing media (Figure 2C). The induction of PpPox1p was detectable 4 h after shifting from rich media containing dextrose to media containing oleate. It was also shown to be present in purified peroxisomes (data not shown).

One of the reasons for examining PpPox1p in *P. pastoris* was also its cross-reactivity with an antibody raised against the PTS1 signal, AKI (Aitchison *et al*., 1992). Immunoprecipitation experiments revealed that the 72 kDa protein recognized by the anti-AKI antibody is PpPox1p (Figure 3). The null allele of *PpPOX1* lacked both the anti-AKI- and the anti-PpPox1p-reactive proteins, confirming that PpPox1p and the AKI-reactive protein are one and the same (Figure 2B). This result shows that the APKI of PpPox1p is recognized as a PTSI. Confirmation of this fact is shown by the observation that PpPox1p missing the last three amino acids is not recognized by the anti-AKI antibody (Figure 2B).

The C-terminal sequence of PpPox1p is close to the AKI sequence that acts as a PTS1 in *C. tropicalis* (Aitchison *et al*., 1991). Since proline is a small, hydrophobic amino acid much like alanine, and PpPox1p clearly cross-reacts with the anti-AKI antibody, it seemed possible that PKI could after all be a PTS1 sequence.

Evidence that the PKI is recognized by the PTS1 receptor, PpPex5p, comes from yeast two-hybrid experiments (Figure 4) and localization studies (Figure 5). Full-length PpPox1p interacts with PpPex5p, whereas PpPox1ΔPKIp does not. In addition, PpPox1p resides in the cytosol in a *P. pastoris* strain lacking the PTS1 receptor (Figure 5). Furthermore, a PpPox1ΔPKIp is localized to the cytosol in a wild-type strain, supporting the fact that the C-terminal amino acids PKI are important for the targeting of PpPox1p (Figure 5). The truncated protein PpPox1ΔPKIp is unable to complement a *ΔPppox1* strain for its ability to grow on oleate-containing medium. Taken together, these data suggest that the last three amino acids are needed for proper localization of PpPox1p to peroxisomes in *P. pastoris* and that mislocalization of the protein is combined with impaired function. The sufficiency of the C-terminal sequence, APKI, to function as a PTS was demonstrated by the peroxisomal localization of the GFP–APKI fusion (Figure 6). These experiments prove that PpPox1p is targeted to the peroxisomes in a PTS1 receptor-dependent fashion and that the C-terminal amino acids act as the PTS1.

Although the C-terminal amino acids of PpPox1p do not fit the PTS1 consensus, many C-terminal tripeptides have been found that do not conform to the PTS1 consensus sequence and still function as one (Lamentschwandtner *et al*., 1998). This is the first yeast acyl-CoA oxidase that has been shown to take this route.

ACKNOWLEDGEMENTS

We thank Dr W. Snyder for critically reading the manuscript. A. Koller was supported by a fellowship of the Swiss National Fonds (No. 8230-046677) and G. H. Lüers was supported by the Deutsche Forschungsgemeinschaft (No. Lu

571/1-1). This work was supported by a grant from NIH to S. Subramani (No. DK41737).

REFERENCES

Aitchison, J. D., Murray, W. W. and Rachubinski, R. A. (1991). The carboxyl-terminal tripeptide Ala–Lys–Ile is essential for targeting *Candida tropicalis* trifunctional enzyme to yeast peroxisomes. *J. Biol. Chem.* **266,** 23 197–23 203.

Aitchison, J. D., Szilard, R. K., Nuttley, W. M. and Rachubinski, R. A. (1992). Antibodies directed against a yeast carboxyl-terminal peroxisomal targeting signal specifically recognize peroxisomal proteins from various yeasts. *Yeast* **8,** 721–734.

Dmochoska, A., Dignard, D., Maleszka, R. and Thomas, D. Y. (1990). Structure and transcriptional control of the *Saccharomyces cerevisiae POX1* gene encoding acyl-coenzyme A oxidase. *Gene* **88,** 247–252.

El-Shabrawi, Y., Noehammer, C. H., Schauer, S., Winter, E. and Hoefler, G. (1997). cDNA cloning and expression of mouse peroxisomal acyl-CoA oxidase. Submission to GENBANK.

Elgersma, Y., Elgersma, H. M., Wenzel, T., McCaffery, J. M., Farquhar, M. G. and Subramani, S. (1998). A mobile PTS2 receptor for peroxisomal protein import in *Pichia pastoris. J. Cell Biol.* **140,** 807–820.

Faber, K. N., Heyman, J. A. and Subramani, S. (1998). Two AAA family peroxins, PpPex1p and PpPex6p, interact with each other in an ATP-dependent manner and are associated with different subcellular membranous structures distinct from peroxisomes. *Mol. Cell Biol.* **18,** 936–943.

Fournier, B., Saudubray, J. M., Benichou, B., Lyonnet, S., Munnich, A., Clevers, H. and Poll-The, B. T. (1994). Large deletion of the peroxisomal acyl-CoA oxidase gene in pseudoneonatal adrenoleukodystrophy. *J. Clin. Invest.* **94,** 526–531.

Gaynor, E. C. and Emr, S. D. (1997). COPI-independent anterograde transport: cargo-selective ER-to-Golgi protein transport in yeast COPI mutants. *J. Cell Biol.* **136,** 789–802.

Gould, S. J., McCollum, D., Spong, A. P., Heyman, J. A. and Subramani, S. (1992). Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. *Yeast* **8,** 613–628.

Hill, D. E., Boulay, R. and Rogers, D. (1988). Complete nucleotide sequence of the peroxisomal acyl CoA oxidase from the alkane-utilizing yeast *Candida maltosa. Nucleic Acids Res.* **16,** 365–366.

Lamentschwandtner, G., Brocard, C., Fransen, M., Van Veldhoven, P., Berger, J. and Hartig, A. (1998). The difference in recognition of terminal tripeptides as peroxisomal targeting signal 1 between yeast and human is due to different affinities of their receptor Pex5p to the cognate signal and to residues adjacent to it. *J. Biol. Chem.* **273,** 33 635–33 643.

McCollum, D., Monosov, E. and Subramani, S. (1993). The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells—the PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family. *J. Cell Biol.* **121,** 761–774.

Mitchell, D. A., Marshall, T. K. and Deschenes, R. J. (1993). Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. *Yeast* **9,** 715–723.

Miyazawa, S., Osumi, T., Hashimoto, T., Ohno, K., Miura, S. and Fujiki, Y. (1989). Peroxisome targeting signal of rat liver acyl-coenzyme A oxidase residues at the carboxy terminus. *Mol. Cell. Biol.* **9,** 83–91.

Okazaki, K., Takechi, T., Kambara, N., Fukui, S., Kubota, I. and Kamiryo, T. (1986). Two acyl-coenzyme A oxidases in peroxisomes of the yeast *Candida tropicalis*: primary structures deduced from genomic DNA sequence. *Proc. Natl Acad. Sci. U S A* **83,** 1232–1236.

Pearson, W. R. and Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc. Natl Acad. Sci. U S A* **85,** 2444–2448.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Small, G. M. and Lazarow, P. B. (1987). Import of the carboxy-terminal portion of acyl-CoA oxidase into peroxisomes of *Candida tropicalis. J. Cell Biol.* **105,** 247–250.

Small, G. M., Szabo, L. J. and Lazarow, P. B. (1988). Acyl-CoA oxidase contains two targeting sequences, each of which can mediate protein import into peroxisomes. *EMBO J.* **7,** 1167–1173.

Subramani, S. (1998). Components involved in peroxisome import, biogenesis, proliferation, turnover, and movement. *Physiol. Rev.* **78,** 171–188.

Varanasi, U., Chu, S., Espinosa, R., LeBeau, M. M. and Reddy, J. K. (1994). Isolation of the human peroxisomal acyl-CoA oxidase gene: organization, promoter analysis, and chromosomal localization. *Proc. Natl Acad. Sci. U S A* **91,** 3107–3111.

Wang, H., Le Clainche, A., Le Dall, M.-T., Wache, Y., Pagot, Y., Belin, J.-M., Gaillardin, C. and Nicaud, J.-M. (1998). Cloning and characterization of the peroxisomal acyl CoA oxidase *ACO3* gene from the alkane-utilizing yeast *Yarrowia lipolytica. Yeast* **14,** 1373–1386.

Zhang, J. W., Han, Y. and Lazarow, P. B. (1993). Novel peroxisome clustering mutants and peroxisome biogenesis mutants of *Saccharomyces cerevisiae. J. Cell Biol.* **123,** 1133–1147.

