The EMBO Journal vol.7 no.4 pp.1167 - 1173, 1988

Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes

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Communicated by C.de Duve

Acyl-CoA oxidase is a major induced enzyme in peroxisomes of *Candida tropicalis* grown on fatty acids. The gene, *POX4*, encoding acyl-CoA oxidase was expressed *in vitro*, and the resulting polypeptide was imported into purified peroxisomes in a temperature-dependent fashion. Plasmids containing fragments of *POX4* were prepared, expressed and the polypeptides tested for import into peroxisomes. We identified two regions of acyl-CoA oxidase (amino acids 1–118 and 309–427) that contained information that specifically targeted fragments of acyl-CoA oxidase to peroxisomes. The corresponding regions of the gene were fused to cDNA encoding the cytosolic enzyme dihydrofolate reductase (DHFR), and the expressed fusion proteins were likewise imported into peroxisomes. DHFR itself neither bound to, nor was imported into peroxisomes. Thus, there are at least two regions of peroxisomal targeting information in the acyl-CoA oxidase gene.

Key words: acyl-CoA oxidase/*Candida tropicalis*/gene fusions/peroxisomal import/targeting signals

Introduction

The biogenesis of peroxisomes appears to be a unique process in that it does not conform to the mechanisms elucidated thus far for other cell organelles. Peroxisomal proteins, including integral membrane proteins, are synthesized on free polyribosomes and are imported post-translationally into preexisting peroxisomes (Lazarow and Fujiki, 1985). In this respect peroxisomal biogenesis resembles that of mitochondria and chloroplasts. However, in the case of mitochondrial and chloroplast (as well as secretory) proteins, the newly synthesized polypeptides generally contain an extra sequence of amino acid residues at their amino terminus. This sequence is essential for targeting and is removed enzymatically during or after translocation into the organelle (Walter *et al.*, 1984; Hurt and Van Loon, 1986; Schmidt and Mishkind, 1986). In contrast, the majority of peroxisomal proteins lack cleavable topogenic sequences and therefore are synthesized at their mature size (Lazarow and Fujiki, 1985; Borst, 1986). The nature and location of the targeting information within peroxisomal proteins is unknown.

In order to address the question of topogenic targeting information in peroxisomal proteins, we reconstituted *in vitro* the import of newly synthesized polypeptides into highly purified peroxisomes of the yeast *Candida tropicalis* (Small and Lazarow, 1987; Small *et al.*, 1987). Peroxisomes and mRNAs encoding peroxisomal proteins are markedly induced when this organism is grown on fatty acids (Osumi *et al.*, 1974; Kamiryo *et al.*, 1982; Fujiki *et al.*, 1986). Peroxisomes were incubated with ^{35}S-labeled products of the *in vitro* translation of total RNA from oleate-grown *C.tropicalis*. Ten polypeptides became associated with peroxisomes in a time- and temperature-dependent fashion. These were identified as peroxisomal proteins by immunoprecipitation (Small *et al.*, 1987). The most abundant of these proteins was acyl-CoA oxidase, the first enzyme in the fatty acid β-oxidation system which allows this yeast to grow on oleate.

We have previously isolated a partial cDNA clone (No. 1:18) for acyl-CoA oxidase (Rachubinski *et al.*, 1985). This cDNA was expressed *in vitro*, and the resulting 44-kd polypeptide, representing the carboxy-terminal 56% of acyl-CoA oxidase, was efficiently imported into peroxisomes (Small and Lazarow, 1987). This result demonstrated that there is sufficient information in the carboxy-terminal half of this peroxisomal protein for it to be directed to, and translocated inside its organelle. Thus, targeting of peroxisomal proteins may share some features with nuclear targeting, where the proteins studied have been found to contain internal topogenic information (Dingwall and Laskey, 1986).

In order to define further this targeting information in acyl-CoA oxidase, and to investigate whether or not there is additional information in the amino-terminal half of this protein, we have continued our experiments using *POX4*, the gene encoding acyl-CoA oxidase (Okazaki *et al.*, 1986). When *POX4* is expressed *in vitro* the acyl-CoA oxidase thus synthesized is imported into peroxisomes in our *in vitro* system. Plasmids encoding various portions of this protein were prepared and their expression products tested for the ability to become imported into peroxisomes. Furthermore, we tested whether targeting regions thus defined could direct a non-peroxisomal protein to peroxisomes. For this purpose, gene fusions were carried out between portions of *POX4* and the coding sequence for the mouse cytosolic enzyme, dihydrofolate reductase (DHFR).

Results

In vitro expression and import of POX4

Plasmid pPOX4w, containing the entire *POX4* gene in pGEM-3, was linearized wtih *Eco*RI and expressed *in vitro* using an SP6 transcription system as described previously (Small and Lazarow, 1987). The resulting mRNA was translated *in vitro* and produced a single polypeptide with an apparent mass of 76 kd (Figure 1, lane 1). This is identical with the apparent mass of the purified enzyme and is in reasonable agreement with the predicted mass of 78.7 kd. Its identity was confirmed by immunoprecipitation with antiserum against acyl-CoA oxidase (not shown).

The [^{35}S]acyl-CoA oxidase was tested for import into

purified peroxisomes at 26°C. Approximately 13% became associated with peroxisomes (Figure 2, lane 2). One-quarter of this was imported within the organelle in a protease-resistant fashion (Figure 2, lane 4, Table I). ^{35}S-Labeled protein in the supernatant was completely digested by protease (lane 3), and all [^{35}S]acyl-CoA oxidase was digested in the presence of detergent (Figure 2, lanes 5 and 6). When the import assay was carried out at 4°C, acyl-CoA oxidase bound to the peroxisomes, but no protease-resistant import was detected (Figure 2, lanes 7–12). The faint band seen in lane 10 is at a lower mol. wt than acyl-CoA oxidase, and is probably a residual digestion product, as are the lower bands in lanes 11 and 12. This incomplete digestion is sometimes noted when samples are not prewarmed at 26°C, as in a usual import assay, before carrying out the digestion.

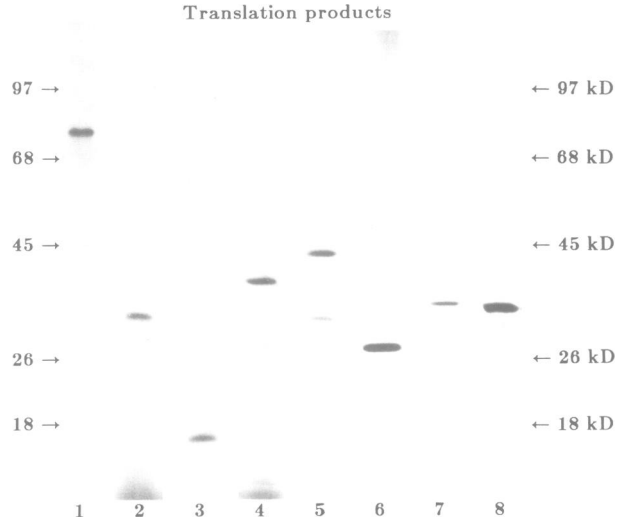

Fig. 1. *In vitro* expression of *POX4*, parts of *POX4* and fusion plasmids. Each plasmid was transcribed and translated *in vitro*, either in rabbit reticulocyte lysate (**lanes 2, 4, 5**) or in wheat germ extract (**lanes 1, 3, 6, 7,8**). 1 μl of translation product was subjected to SDS–PAGE and fluorography. The lanes contain expression products from the following plasmids: **1**, POX4w; **2**, POX4a.1; **3**, POX4a.2; **4**, POX4m.1; **5**, POX4c.1; **6**, POX4c.2; **7**, POX4a.3-DHFR; **8**, POX4m.2-DHFR. Each lane is from a separate experiment, the molecular masses indicated are approximate; masses mentioned in the text were accurately determined from standards in the individual experiments.

Preparation, expression and import of POX4 constructs

In order to define the region or regions containing topogenic sequence(s), constructs were prepared containing various segments of the *POX4* gene (Figure 3). These were transcribed and translated *in vitro*, and the resultant polypeptides were tested for import into peroxisomes.

The plasmid, pPOX4c.1 contained the 3′ 56% of the coding region for acyl-CoA oxidase and corresponded to the cDNA construct described in the Introduction (Small and Lazarow, 1987). This plasmid produced a polypeptide with a mass of 44 kd (Figure 1, lane 5) as expected, and the polypeptide was imported into peroxisomes (Figure 4a, Table I). As with the cDNA construct, we also observed a second polypeptide with a mass of 34 kd, which is consistent with secondary initiation of translation at the next downstream methionine codon (Figure 3, arrowhead), (Small and Lazarow, 1987). This polypeptide was also imported in these experiments (Figure 4a, asterisk).

We prepared a smaller carboxy-terminal fragment of *POX4, POX4c.2* (Figure 3). This was expressed from the first methionine downstream from the *Bal*I site and produced a polypeptide with a mass of 27 kd (Figure 1, lane 6), as predicted from the amino acid sequence. Only a very small proportion (~2%) of this polypeptide bound to peroxisomes and none of this was protease resistant, indicating that this polypeptide was not translocated inside the organelle (Figure 4b). These results suggested that the targeting information in *POX4c.1* is located in the region of amino acids 309–462, which was missing from *POX4c.2*.

We next investigated the amino-terminal portion of acyl-CoA oxidase. Plasmid pPOX4a.1 included the 5′ 33% of the gene and did not overlap with any of the constructs described above. The expression product of 28 kd from *POX4a.1* (Figure 1, lane 2) was consistent with initiation at the first methionine codon. This product was efficiently

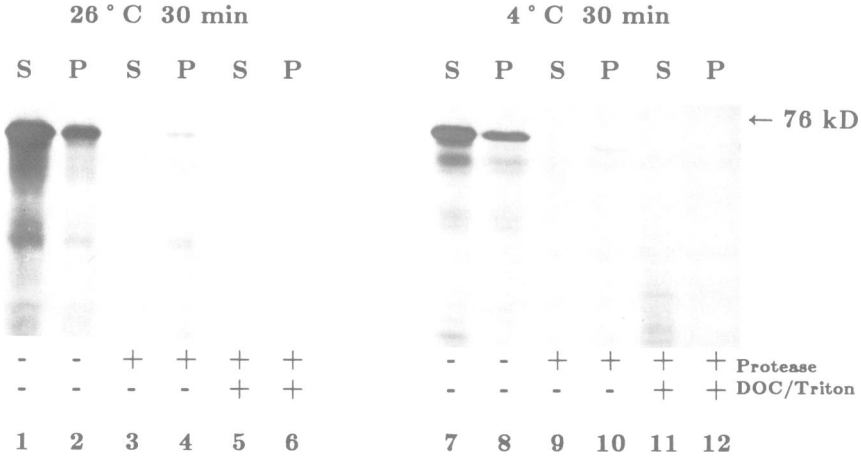

Fig. 2. Import of acyl-CoA oxidase into peroxisomes *in vitro*. Newly synthesized translation products in wheat germ extract (60 μl) were mixed with peroxisomes (336 μg protein) and incubated at 26°C (**left panel**) or 4°C (**right panel**) for 30 min. The mixtures were then divided into three equal samples which were (+) or were not (−) treated with 20 μg of Proteinase K at 4°C for 15 min, in the presence (**lanes 5, 6, 11,12**) or absence (**lanes 1–4** and **7–10**) of detergent. Supernatants and peroxisome pellets were then separated by centrifugation, and equal amounts of each were analyzed by SDS–PAGE and fluorography. S, supernatant; P, peroxisome pellet.

imported into peroxisomes, such that 37% of the added ^{35}S-labeled polypeptide was protected from protease digestion (Figure 4c, Table I). A smaller 5′ construct, *POX4a.2* (Figure 3), produced a polypeptide with the expected mass of 12 kd (Figure 1, lane 3). This smaller amino-terminal polypeptide of acyl-CoA oxidase was also imported into peroxisomes at 26°C (Figure 5, left). Moreover, when the peroxisomes were replaced by mitochondria no import was observed (Figure 5, right).

The results thus far indicated that acyl-CoA oxidase contains two non-overlapping sequences, one within the first 112 amino acids, and the second presumably between amino acids 309 and 462, that can act independently to target part of the protein to peroxisomes. Another large construct (pPOX4m.1), encoding the middle portion of the protein was prepared. Its expression product of 36 kd (Figure 1, lane 4) was consistent with initiation at the first downstream methionine codon after the *Kpn*I site (Figure 3). This polypeptide was efficiently imported (54%) into peroxisomes at 26°C (Figure 6, Table I).

Figure 6 shows that the import of the *POX4m.1* expression product is dependent on temperature whereas binding is not. All of the other constructs described in this paper showed the same temperature dependence (not shown).

Table I. Import efficiency of acyl-CoA oxidase, parts of this enzyme and fusion proteins

Construct	Translation system	Associated with peroxisomes %	Imported into peroxisomes %
POX4w	RL	16	3
	WG	13	3
POX4a.1	RL	57	37
POX4a.2	WG	30	10
POX4c.1	RL	36	13
	WG	33	19
POX4c.2	RL	2	0
POX4m.1	RL	70	54
POX4a.3-DHFR	WG	43	14
POX4m.2-DHFR	WG	31	5
DHFR	RL	0	0
	WG	0	0

The experiments were carried out as described in legends to Figures 2 and 4–8. Translations and import assays were carried out with reticulocyte lysate (RL) or wheat germ (WG). Association and import are expressed as percentages of the input radioactivity.

In vitro synthesis and import of chimeric proteins

We investigated whether the targeting regions identified above could act to target a foreign protein to peroxisomes. The protein we chose was mouse DHFR, a cytosolic protein that has previously been used as a passenger in chimeric proteins targeted to mitochondria (Hurt *et al.*, 1984; Horwich *et al.*, 1985). DHFR neither bound to, nor was translocated into, peroxisomes (Figure 7, Table I). We prepared two fusion proteins consisting of the putative targeting regions of *POX4* fused to DHFR (Figure 8). pPOX4a.3-DHFR contained the DNA encoding the first 118 amino acids of acyl-CoA oxidase fused to the coding region of DHFR (Figure 8a). This produced a protein of molecular mass 34 kd (Figure 1, lane 7), as predicted for the chimeric protein. The second plasmid, pPOX4m.2-DHFR, encoded amino acids 309 (methionine) to 427 of acyl-CoA oxidase and DHFR, and produced a protein of molecular mass 34 kd (Figure 1, lane 8), also consistent with that predicted. Both chimeric proteins were immunoprecipitated with an antiserum against DHFR, and with an antiserum against peroxisomal proteins of *C.tropicalis* (not shown). Both chimeric proteins were imported into peroxisomes in a protease-resistant fashion (Figure 8, Table I). No import was observed when the incubation was carried out at 4°C (Figure 8, lanes 9 and 10), even when the gel was exposed for five times longer than for Figure 8 (not shown).

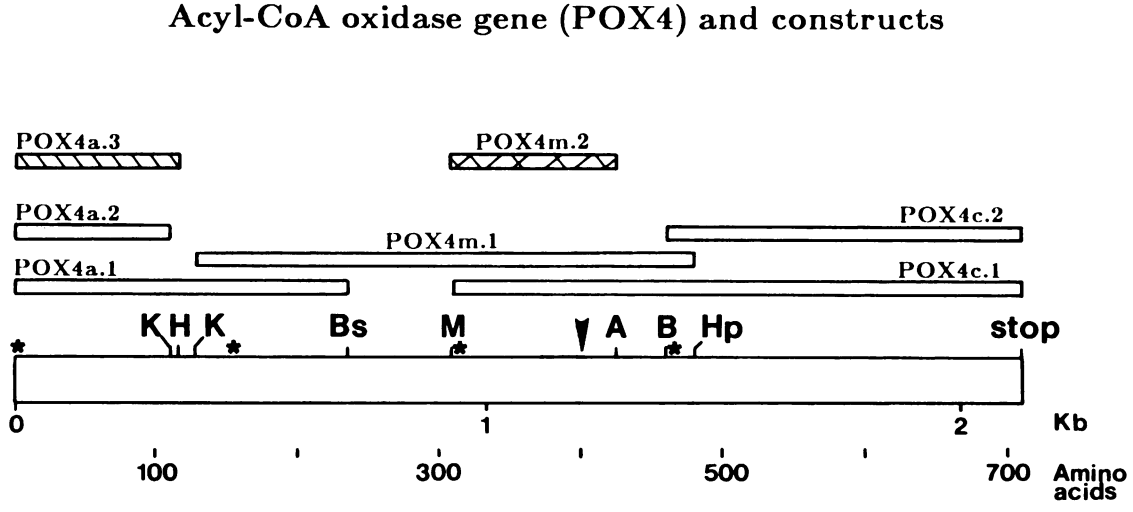

Fig. 3. *POX4* gene and prepared constructs. The 2.2-kb coding region of *POX4* is illustrated with the positions of restriction endonuclease sites that were used for cloning of gene fragments. Open bars indicate five fragments of *POX4* which were excised, subcloned into pGEM vectors and expressed *in vitro*. The position of ATGs that serve as initiators of translation are marked with an asterisk. Also shown are two parts of the gene that were fused to DHFR to encode chimeric proteins (see Figures 8 and 9), *POX4a.3* (diagonal shade) and *POX4m.1* (cross-hatched). K, *Kpn*I; H, *Hin*cII; Bs, *Bst*NI; M, *Mae*I; A, *Acc*I; B, *Bal*I; Hp, *Hpa*II. The arrowhead indicates the second ATG in *POX4c.1*.

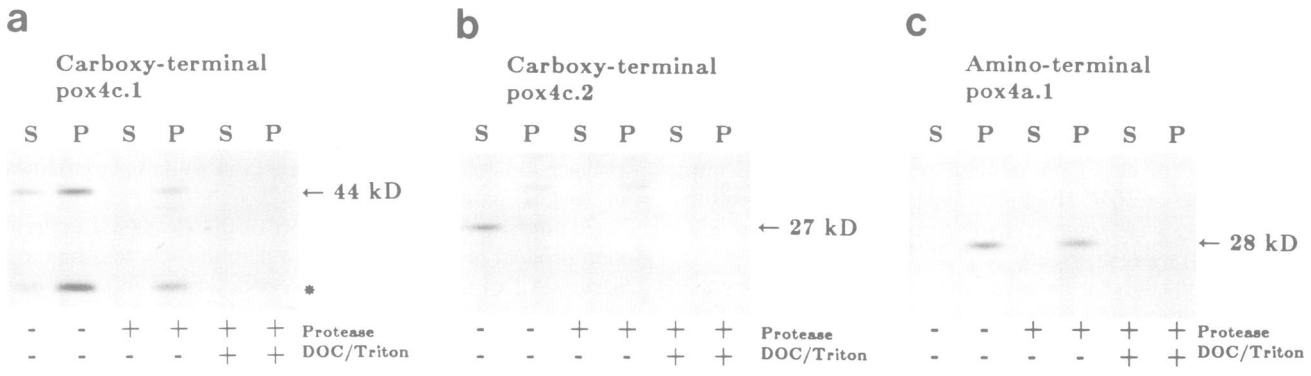

Fig. 4. Import of carboxy- and amino-terminal portions of *POX4*. Newly synthesized translation products in reticulocyte lysate were mixed with peroxisomes and incubated at 26°C for 30 min. Samples were then divided and analyzed as described in Figure 2, except that four times more of the pellet fraction than of the supernatant was loaded on the gel. (**a**) 44-kd carboxy-terminal fragment, *POX4c.1*; the 34-kb polypeptide expressed from this construct is indicated by an asterisk. (**b**) 27-kd carboxy-terminal fragment, *POX4c.2*. (**c**) 28-kd amino-terminal fragment, *POX4a.1*.

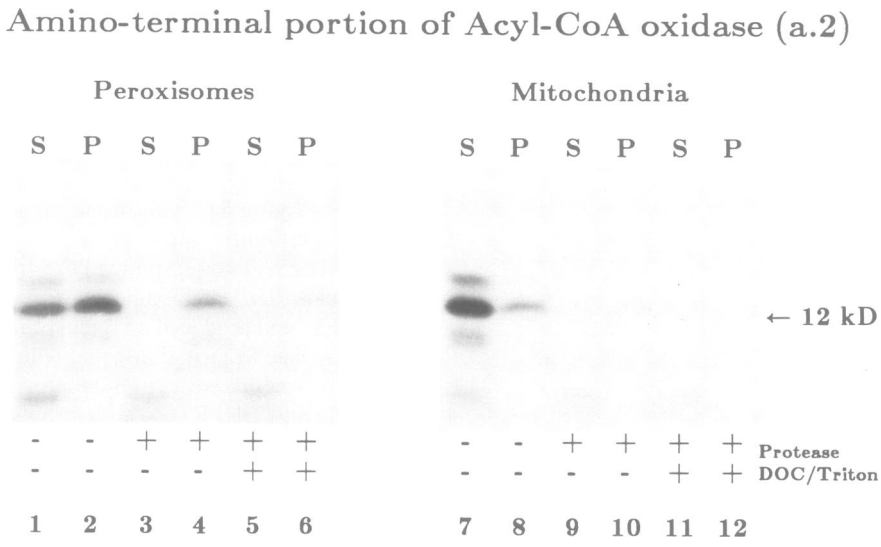

Fig. 5. Organelle specificity of import of short amino-terminal fragment of *POX4* (*POX4a.2*). Translation import and analysis were carried out as described in Figure 2 except that for the right-hand panel mitochondria (395 μg protein) were used in place of peroxisomes.

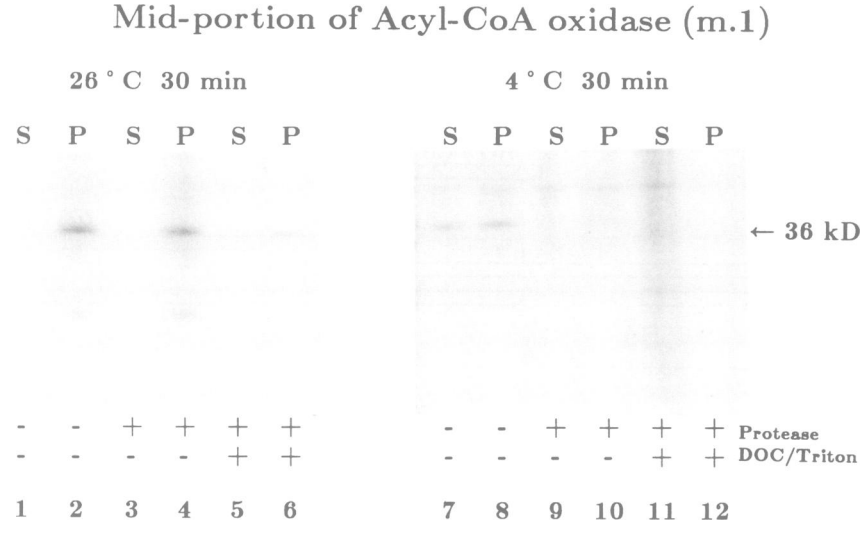

Fig. 6. Temperature dependence of import of the mid-portion of *POX4* (*POX4m.1*). Translation, import and subsequent analysis were carried out as described for Figure 4. Incubations were at 26°C (**left**) or 4°C (**right**).

Discussion

We have investigated the sites of topogenic information in the *POX4* gene that act to direct acyl-CoA oxidase to peroxisomes of *C.tropicalis*. *POX4*, and five fragments prepared from this gene (Figure 3), were expressed *in vitro* and the products were tested for their ability to be imported into peroxisomes. Translations and import assays were carried out initially in reticulocyte lysate, and later in wheat germ, where expression of *C.tropicalis* proteins was found to be much greater. The choice of translation system did not affect the import efficiency for three polypeptides tested (Table I). Therefore subsequent experiments were carried out in the wheat germ system.

The expression product of the entire gene, a 76-kd protein, was imported into the same temperature-dependent fashion as was its counterpart in rat liver (Imanaka *et al.*, 1987). However, in *C.tropicalis* the efficiency of import was considerably lower (~3% compared with 30% in rat liver). We are not sure why the import efficiency is low in these experiments; similar examples exist of mitochondrial proteins that are imported with low efficiency (Hurt *et al.*, 1985a). It is possible that unnatural folding of the *in vitro* synthesized protein could occur in such a manner that targeting sequences are not freely exposed for interaction with the peroxisome membrane and possible receptor. This could be due to the requirement for a factor which is not being supplied in the *in vitro* experiments [for example, an enzyme involved in unfolding proteins for translocation (Rothman and Kornberg, 1986)]. Another possible cause of unnatural folding could be the formation of intrachain disulfide bridges that could cross-link the protein and render it incapable of unfolding for translocation (Maher and Singer, 1986). There are eight cysteine residues in acyl-CoA oxidase, so disulfide cross-links may form in this protein. We carried out translation and import experiments in the presence of dithiothreitol (at final concentrations up to 100 mM). However, while this reduced the efficiency of protein synthesis, it did not enhance import (data not shown).

Some large fragments of acyl-CoA oxidase (expressed from pPOX4a.1 and pPOX4m.1) were imported into peroxisomes with high efficiency (37–54% of input radioactivity was protected from protease digestion). This indicates that at least two-thirds of the enzyme can readily pass through the peroxisome membrane *in vitro*, and supports the interpretation that folding of the complete protein may impede translocation.

The results on the import of acyl-CoA oxidase fragments and fusion proteins demonstrates that this enzyme contains two non-overlapping topogenic sequences. The first,

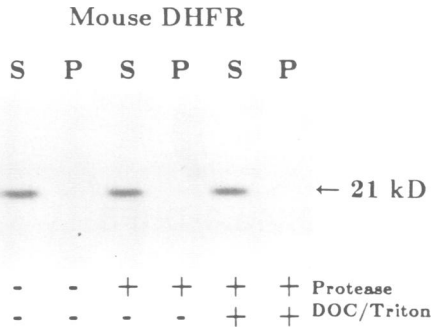

Fig. 7. Non-import of mouse DHFR. The import assay was carried out as described for Figure 4, except that equal amounts of supernatants and pellets were analyzed.

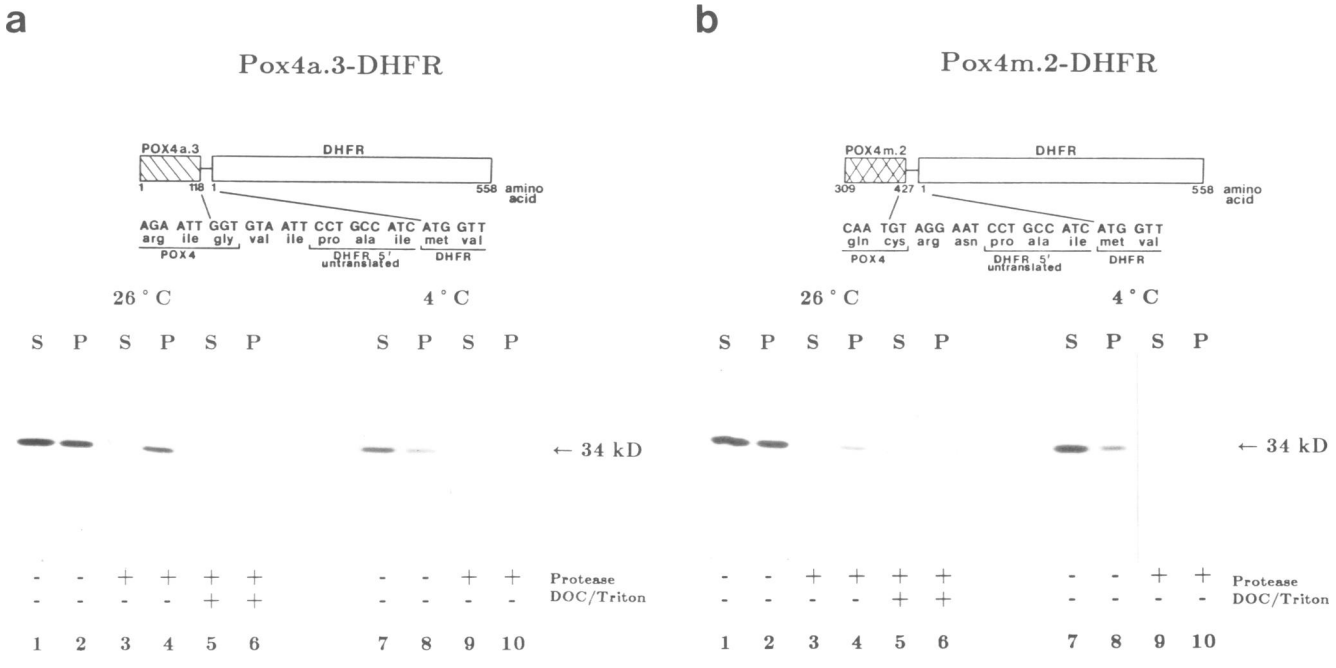

Fig. 8. Structure and import of chimeric proteins. (**a**) The DNA construct encoding the 118 N-terminal amino acids of acyl-CoA oxidase fused to DHFR (POX4a.3-DHFR) is diagrammed together with the observed DNA sequence in the juncture region and the inferred amino acid sequence. The fusion protein was expressed and used in an import assay as described for Figure 2. Equal amounts of supernatants and peroxisome pellets were analyzed. (**b**) The DNA construct encoding amino acids 309–427 of acyl-CoA oxidase fused to DHFR (POX4m.2-DHFR) is shown together with the relevant sequences and import data as above.

designated tops-1, is located within the first 112 amino acids (Figure 3). This N-terminal polypeptide is itself imported into peroxisomes (10% protease protection), and a slightly longer (118 amino acids) N-terminal polypeptide directs DHFR into peroxisomes (14% protease protection). The similar import efficiencies of the fusion protein and the N-terminal fragment suggest that DHFR readily passes through the peroxisomal membrane and is suitable as a 'passenger protein' in these targeting experiments.

The second topogenic sequence (tops-2) is located near the middle of acyl-CoA oxidase. The position of this sequence, between residues 309 and 464, was suggested by the import of the fragment expressed from pPOX4c.1 and the non-import of the smaller fragment expressed from pPOX4c.2. This was confirmed by the import of the fusion protein expressed from pPOX4m.2-DHFR, which contains residues 309–427 of POX4. If the smaller (34-kd) polypeptide produced from pPOX4c-1 (Figure 4a, asterisk) is due to secondary initiation (at the arrowhead in Figure 3), as discussed (Small and Lazarow, 1987), then this implies that the region from amino acids 402 to 464 is sufficient to direct efficient import (20%). This region was truncated at residue 427 in the preparation of the fusion construct POX4m.2-DHFR, which was imported with lower efficiency (5%) (Figure 8b). Therefore it is possible that this fusion protein does not contain the entire targeting sequence. However, as indicated by Hurt *et al.* (1985b), conclusions drawn from quantitation of gene fusion experiments must be cautious because joining DHFR to putative targeting regions may unpredictably increase, or interfere with, targeting function. The precise location of tops-2 within this central region of acyl-CoA oxidase will be determined in future experiments.

The efficient import of the large central polypeptide (Figure 6, Table I) demonstrates that neither end of acyl-CoA oxidase is essential for the targeting and translocation of the protein into peroxisomes. This situation is different from the targeting of luciferase to mammalian peroxisomes where *in vivo* experiments suggest that the topogenic information is located in the protein's carboxy terminus (Gould *et al.*, 1987). *POX4m.1* contains tops-2, and therefore no additional targeting information need be postulated between amino acid residues 119 and 313. A construct encoding this region is being prepared in order to test this experimentally. Residues 465–709 lack topogenic information as shown by the non-import of the polypeptide expressed from pPOX4c.2.

We have compared the amino acid sequences in the two putative targeting regions of acyl-CoA oxidase, and have found no significant homology. To date, there is no information on what constitutes a signal for targeting proteins to peroxisomes. It has been suggested that pairs of positively charged amino acids, situated 40 Å apart, are important in the import of proteins into glycosomes, a microbody of trypanosomes (Wierenga *et al.*, 1987). We do see some areas containing clusters of positive amino acids in the signal-containing regions of acyl-CoA oxidase; however, we cannot comment on their arrangement within the mature protein as we have no information on the three-dimensional structure.

The most common feature seen in mitochondrial targeting sequences is the ability to form positively charged amphipathic α-helices with large hydrophobic moments (von Heijne, 1986). Without exception, these structures are located at the amino-terminal end of the protein. Within the targeting regions that we have defined in acyl-CoA oxidase, there are some regions where the amino acid composition resembles that of mitochondrial targeting sequences. However, these regions do not form amphipathic α-helices according to helical wheel predictions, and our results demonstrate that they do not target acyl-CoA oxidase to mitochondria (see Figure 5).

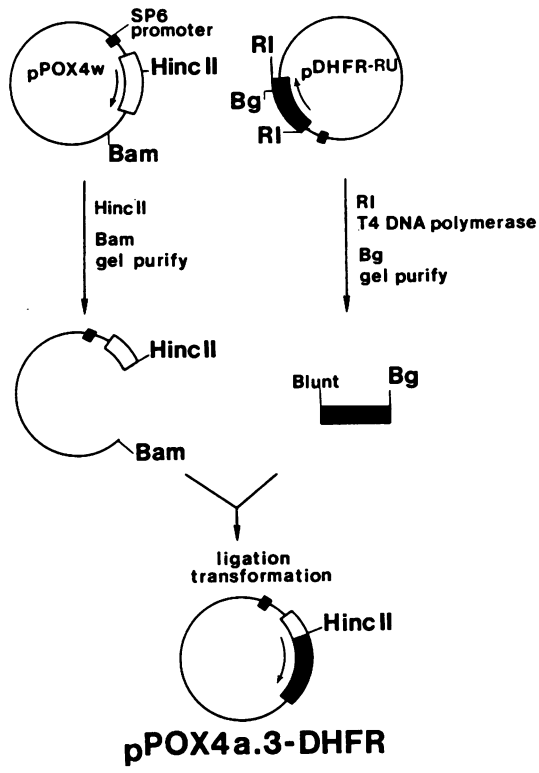

Fig. 9. Steps in the construction of pPOX4a.3-DHFR. Plasmid pPOX4w contains the gene *POX4* encoding acyl-CoA oxidase (shown by the open bar) in pGEM-3. This was cut with *Hin*cII and *Bam*HI, which removed the carboxy-terminal 83% of *POX4* and part of the pGEM polylinker. The linear plasmid was purified by agarose gel electrophoresis. Plasmid pDHFR-RU contains mouse DHFR cDNA (shown by the dark bar) inserted into the *Eco*RI site of pGEM-4. The DHFR cDNA was excised with *Eco*RI and incubated with T4 DNA polymerase to produce blunt ends. It was then digested with *Bgl*II to remove part of the 3′ untranslated region. The 653-bp fragment containing the coding region of DHFR was purified by agarose gel electrophoresis and ligated unidirectionally into the *Bam*HI and *Hin*cII sites of the prepared plasmid encoding the amino-terminal bases of *POX4*. RI, *Eco*RI; Bg, *Bgl*II; Bam, *Bam*HI.

In summary, we have demonstrated that there are two non-overlapping regions (tops) of acyl-CoA oxidase that can act independently to direct import into peroxisomes. How the regions function *in vivo* is not, as yet, known. We are currently in the process of preparing further constructs to define more clearly the two tops and their characteristics.

Materials and methods

Materials

Restriction enzymes and other DNA-modifying enzymes were obtained from Boehringer Mannheim. RNasin, RNA polymerases and pGEM vectors were from Promega Biotech. [^{35}S]Methionine was from New England Nuclear, and Tran^{35}S-label was from ICN Radiochemicals.

Construction of expression plasmid encoding acyl-CoA oxidase

We received the *POX4* gene encoding acyl-CoA oxidase (Okazaki *et al.*, 1986) in a 10-kb *Eco*RI fragment cloneed into pUC19 from Dr D.Hill (Genetics Institute, Cambridge, MA, clone CT102). A 2.6-kb *Nsi*I fragment, containing the entire coding region for acyl-CoA oxidase, was subcloned into pGEM-3 at the *Pst*I site and designated pPOX4w. There are ~390 bases between the SP6 promoter of pGEM-3 and the first ATG codon in *POX4*. We sequenced bases 310–609, 626–709 and 936–1260 of the gene (Figure 3); all 706 bases were identical with the *pox4-1* allele isolated by Okazaki *et al.* (1986). It is 97% homologous to our cDNA 1:18, which we designated *pox4-2* (Small and Lazarow, 1987). For the sake of clarity, throughout this paper we refer to *pox4-1* as *POX4*.

Construction of plasmids encoding parts of acyl-CoA oxidase

Plasmids were prepared to express various portions of the *POX4* gene shown in Figure 3. Where unique restriction sites in pPOX4w could be used, the plasmid was cut, the ends were filled using T4 DNA polymerase and the plasmid was recircularized with T4 DNA ligase. When the desired restriction endonucleases sites also existed in the vector, fragments of *POX4* were excised, purified by agarose gel electrophoresis and recloned into pGEM-4.

A construct containing the first 708 bases of *POX4* (pPOX4a.1) was prepared by digesting pPOX4w with *Bst*NI and *Hin*dIII (in the upstream polylinker), and subcloning the gel-purified frament into pGEM-4. pPOX4a.2, which encodes a shorter amino-terminal fragment of the gene, was prepared from pPOX4a.1 by digestion with *Kpn*I. A plasmid (pPOX4m.1) containing bases 393–1439 of *POX4* was prepared by digestion of pPOX4w with *Kpn*I and *Hpa*II; the ends of the 1046-bp fragment were filled in and the fragment was purified and recloned into the *Kpn*I site of pGEM-4.

A construct of the 3′ portion of *POX4* (pPOX4c.1) was prepared by digesting pPOX4w with *Mae*I and *Sal*I (in the downstream polylinker). The ends of the excised *POX4* fragment were made blunt, and the fragment was purified and recloned into pGEM-3 at the *Sal*I site, after treatment of the vector with T4 DNA polymerase to produce blunt ends. A shorter carboxy-terminal construct (*POX4c.2*) was prepared by digesting pPOX4w with *Bal*I and *Hin*dIII (in the upstream polylinker) to remove the amino-terminal portion of *POX4*; the ends of the plasmid were then made blunt and the plasmid was recircularized.

Construction of plasmids encoding chimeric proteins

Plasmids encoding two chimeric proteins (Figure 8) were constructed by fusing segments of *POX4* with DHFR cDNA. The fusion junctions were confirmed by DNA sequencing.

Plasmid PR26 (Horwich *et al.*, 1985) contains a mouse cDNA sequence encoding DHFR cloned into the *Eco*RI site of pBR328. This cDNA was excised with *Eco*RI and recloned into pGEM-4 at the *Eco*RI site to produce plasmid pDHFR-RU.

Plasmid pPOX4a.3-DHFR (Figure 8a) was constructed as shown in Figure 9. Plasmid pPOX4m.2-DHFR (Figure 8b) was constructed in a similar fashion. pPOX4c.1 containing the carboxy-terminal 1195 bases of the POX4 gene was cleaved with *Acc*I, the ends were made blunt with T4 DNA polymerase, and it was then cut with *Bam*HI (in the downstream polylinker). The 3223-bp fragment containing the vector pGEM-3 and bases 923–1279 of POX4 was gel-purified. This was ligated to the 653-bp fragment of DHFR cDNA (prepared as in the legend to Figure 9).

In vitro transcription, translation and import

Plasmids were linearized and transcribed as described previously using SP6 polymerase (Small and Lazarow, 1987), or, for DNA cloned into pGEM vectors in the T7 orientation, using T7 RNA polymerase. The RNA was extracted with phenol, ethanol precipitated and translated *in vitro*. The translations were carried out either in rabbit reticulocyte lysate that had previously been treated with S1 nuclease (Pelham and Jackson, 1976), in the presence of [^{35}S]methionine; or in wheat germ extract (Erickson and Blobel, 1983) in the presence of Tran^{35}S-label. The newly synthesized translation products were used in an *in vitro* import assay, with 300–400 μg of peroxisomal protein, as described previously (Small *et al.*, 1987).

Other procedures

DNA manipulations were done essentially as described by Maniatis *et al.* (1982). DNA sequencing was by the dideoxy method (Sanger *et al.*, 1977). SDS–PAGE was done in slab gels which were either 15% acrylamide or gradients of 7–15% acrylamide. Fluorography was as described (Laskey and Mills, 1975). Radioactivity was quantitated by densitometric scanning of the fluorograms (Small and Lazarow, 1987). Immunoprecipitations were carried out as described (Small *et al.*, 1987) with antisera against *C.tropicalis* peroxisomes (Small *et al.*, 1987), *C.tropicalis* acyl-CoA oxidase (anti-AOx_t, Fujiki *et al.*, 1986) and DHFR (Horwich *et al.*, 1985).

Acknowledgements

We thank Mr Gary Richo, Mr David Allman and Mr Bruce Fieldman for technical assistance, Dr David Hill (Genetics Institute, Cambridge, MA) for the isolation of the *POX4* gene, and Dr Arthur L.Horwich for the cDNA encoding DHFR and for antiserum against DHFR. This work was supported by National Science Foundation grant DCB83-09222.

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Received on January 4, 1988; revised on February 3, 1988