Import of the Carboxy-Terminal Portion of Acyl-CoA Oxidase into Peroxisomes of *Candida tropicalis*

Gillian M. Small and Paul B. Lazarow

The Rockefeller University, New York

Abstract. We report the sequence of a cDNA clone that codes for the carboxy-terminal portion of the peroxisomal protein, acyl-CoA oxidase, from the yeast, *Candida tropicalis*. This is a newly identified acyl-CoA oxidase sequence, most likely a second allele of *POX4*.

The cDNA clone was expressed by in vitro transcription followed by translation. The major product, a 43-kD protein, associated with isolated peroxisomes in an in vitro import assay. More than half of the peroxisome-associated protein was protected from added protease, implying that it was internalized within the organelle. These findings indicate that there is sufficient information in the carboxy-terminal portion of the protein to target it to peroxisomes.

OUR current understanding of peroxisome biogenesis is that all of the organelle's proteins are synthesized in the cell cytosol on free polysomes. Most are synthesized at their mature size and all are transported into the organelle posttranslationally (1, 8). The information for the targeting of proteins to peroxisomes is presumed to reside in the mature amino acid sequence but the details are unknown.

Candida tropicalis is a useful organism in which to investigate the nature and location of this topogenic information. Peroxisomes are markedly induced when this yeast is grown on alkanes or long-chain fatty acids (12, 20) and repressed when it is grown on glucose (2). Growth on fatty acids induces approximately nine abundant mRNAs that code for peroxisomal proteins; when translated in vitro the most abundant product is acyl-CoA oxidase, the first enzyme in the peroxisomal β-oxidation system (5, 6). We isolated a cDNA clone encoding acyl-CoA oxidase (14) and raised an antiserum against this protein (5).

Recently, we established an in vitro import system in which peroxisomal cell-free translation products associate specifically with isolated *C. tropicalis* peroxisomes in a time- and temperature-dependent fashion (19). Prominent among these translation products was acyl-CoA oxidase. We report here the first of our studies on the targeting of this enzyme to peroxisomes. The cDNA encoding the carboxy-terminal part of acyl-CoA oxidase was expressed in vitro and the ability of the resultant polypeptide to enter peroxisomes was tested. Some of these data have been presented in abstract form (18).

Materials and Methods

The cDNA clone 1:18 encoding acyl-CoA oxidase (14) was excised from PBR322 and cut with Mae I. The 1,161-bp Mae I-Pst I fragment (Fig. 1) was subcloned in pGEM3 (Promega Biotec, Madison, WI). This construct, designated pGSP4-2a, was linearized and transcribed, using the SP6 promoter, with simultaneous capping of the mRNA as described by Promega Biotec based on the method of Melton et al. (10). After transcription, protein was extracted with phenol and the mRNA was precipitated with ethanol and translated in a nuclease-treated, rabbit-reticulocyte lysate (13). The newly synthesized translation products were used in an in vitro import assay with freshly isolated *C. tropicalis* peroxisomes as described (19).

Immunoprecipitation (4) was carried out with an antiserum against *C. tropicalis* acyl-CoA oxidase (rabbit 10-297, characterized as anti-AOx_t in reference 5). SDS PAGE (3) and fluorography (7) were as described. Radioactivity was quantitated by densitometric scanning of the fluorograms with a densitometer (model 1650; Bio-Rad Laboratories, Richmond, CA).

An overlapping series of DNA fragments, obtained by Bal 31 digestion from both ends of clone 1:18 (Fig. 1) were cloned into M13 vectors, and sequenced by the dideoxy method (15). Portions of clone 1:18 were also sequenced according to Maxam and Gilbert (9).

Results

Sequence

The sequence of cDNA clone 1:18 for acyl-CoA oxidase and the predicted amino acid sequence are shown in Fig. 2.[1] This cDNA sequence was compared with the two acyl-CoA oxidase genes, *POX4* and *POX5*, described by Okazaki et al. (11). It is 97.2% homologous to bases 622–2,130 of *POX4* and 55.6% homologous to *POX5* (bases 577–1,989). There are 42 differences in DNA sequence between *POX4* and our cDNA, of which 34 (81%) are third base changes. These result in 11 amino acid differences, of which at least four are conservative replacements. *POX4*, *POX5*, and our cDNA sequence are all found in *C. tropicalis* genomic DNA by Southern analyses (not shown). *C. tropicalis* is a diploid organism; we infer that the cDNA is the second allele of *POX4*, or less likely a third acyl-CoA oxidase gene.[2] We provisionally

1. This sequence data have been submitted to the EMBL/Gen Bank Data Libraries under the accession number Y00623.

2. *POX2*, another gene with some homology to *POX4* (6), differs from all three sequences by having two internal ECO RI sites.

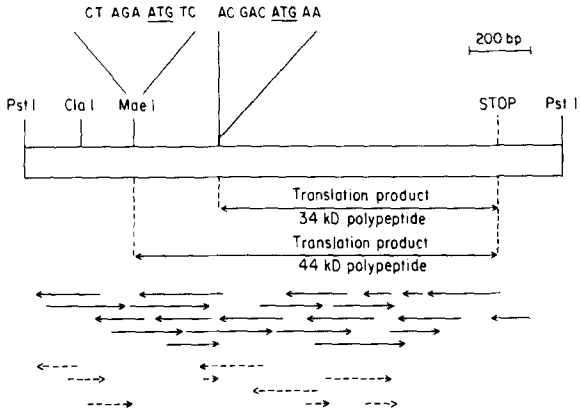

Figure 1. Sequencing and expression strategies for the acyl-CoA oxidase cDNA. Arrows indicate the Bal 31 fragments that were sequenced by the dideoxy method. Dotted arrows indicate sequences determined by the Maxam and Gilbert procedure (9). The 1,161-bp Mae I-Pst I fragment was cloned into the expression vector pGEM3 to form pGSP4-2a. The position of two internal ATGs that could serve as initiators of translation are indicated, together with the predicted sizes of the translation products.

designate our cDNA allele pox4-2 and that of Okazaki et al. pox4-1 in accordance with convention (17).

In vitro expression

The cDNA clone was expressed in pGEM3 (plasmid pGSP4-2a) from an internal methionine codon just downstream from a Mae I site (Fig. 1). The in vitro expression product had a mass of ~43 kD (Fig. 3 *a*), in agreement with the expected mass of 43,702 D. The predicted mass of the pox4-1 gene product is 78,554 D (11) and the apparent mass by SDS-PAGE is 76 kD (5). A second polypeptide with a mass of ~32 kD was also synthesized; this is consistent with initiation at the next downstream AUG (Figs. 1 and 2), in which case the predicted mass would be 33,928 D.

Import

The translation products were used in a posttranslational import assay with *C. tropicalis* peroxisomes. After a 30-min incubation, there was association of both the 43- and the 32-kD polypeptides with the peroxisomes (Fig. 3 *b* and Fig. 4 *b*). Some of each of the proteins in the pellet were resistant to protease digestion whereas the supernatant proteins were completely digested. In the experiment of Fig. 4 *b*, 37% of the added 43-kD expression product was associated with the peroxisomes; of this, 59% was protease resistant. 54% of the smaller expression product was associated with the peroxisomes, of which 63% was protease resistant. The addition of 1% deoxycholate and 1% Triton X-100 abolished the protease resistance in all cases. Globin did not associate with the peroxisomes (Fig. 3 *b*). These data imply that some of each of the 43- and 32-kD polypeptides were inside the peroxisomes, protected from proteolysis by the organelle's membrane.

Immunoprecipitation

Both the 43- and the 32-kD cell-free translation products were immunoprecipitated with an antiserum against acyl-CoA oxidase (Fig. 3 *c*). This confirms that plasmid pGSP4-2a encodes acyl-CoA oxidase and indicates that antigenic sites are present in the carboxy-terminal portion of this protein.

Figure 2. Nucleotide and predicted amino acid sequences of the acyl-CoA oxidase cDNA clone 1:18. Bases and amino acids of *POX4* (11) differing from 1:18 are indicated in italics above and below 1:18.

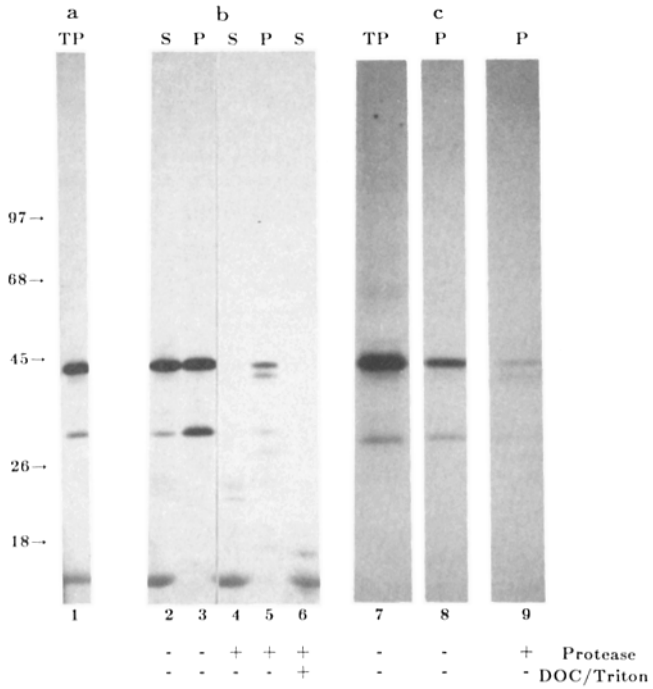

Figure 3. In vitro expression and import into peroxisomes of the carboxy-terminal portion of acyl-CoA oxidase. (*a*) Expression. pGSP4-2a was transcribed and translated in vitro. 1.3 μl of translation products, in the presence of 5 μg of peroxisomal protein, was subjected to SDS-PAGE and fluorography. (*b*) Import. Newly synthesized translation products (180 μl) were mixed with peroxisomes (700 μg of protein) and divided into three equal samples. After incubation at 26°C for 30 min, aliquots were (+) or were not (−) digested with 2.5 μg of thermolysin at 4°C for 30 min in the presence (lane *6*) or absence (lanes *2–5*) of 1% sodium deoxycholate and 1% Triton X-100. Supernatants and peroxisome pellets were then separated by centrifugation; 20% of the pellets and 5% of the supernatants were analyzed by SDS-PAGE and fluorography. The pellet after detergent treatment was omitted here because in preliminary experiments it was found to contain negligible protein or radioactivity (see Fig. 4). Globin is not digested under these conditions. (*c*) Immunoprecipitation. Samples from *a* and *b* (each equivalent to 5 μl of cell-free translation products) were subjected to immunoprecipitation with antiserum against acyl-CoA oxidase. Lanes *7*, *8*, and *9* contain the immunoprecipitated products from lanes *1*, *3*, and *5*, respectively.

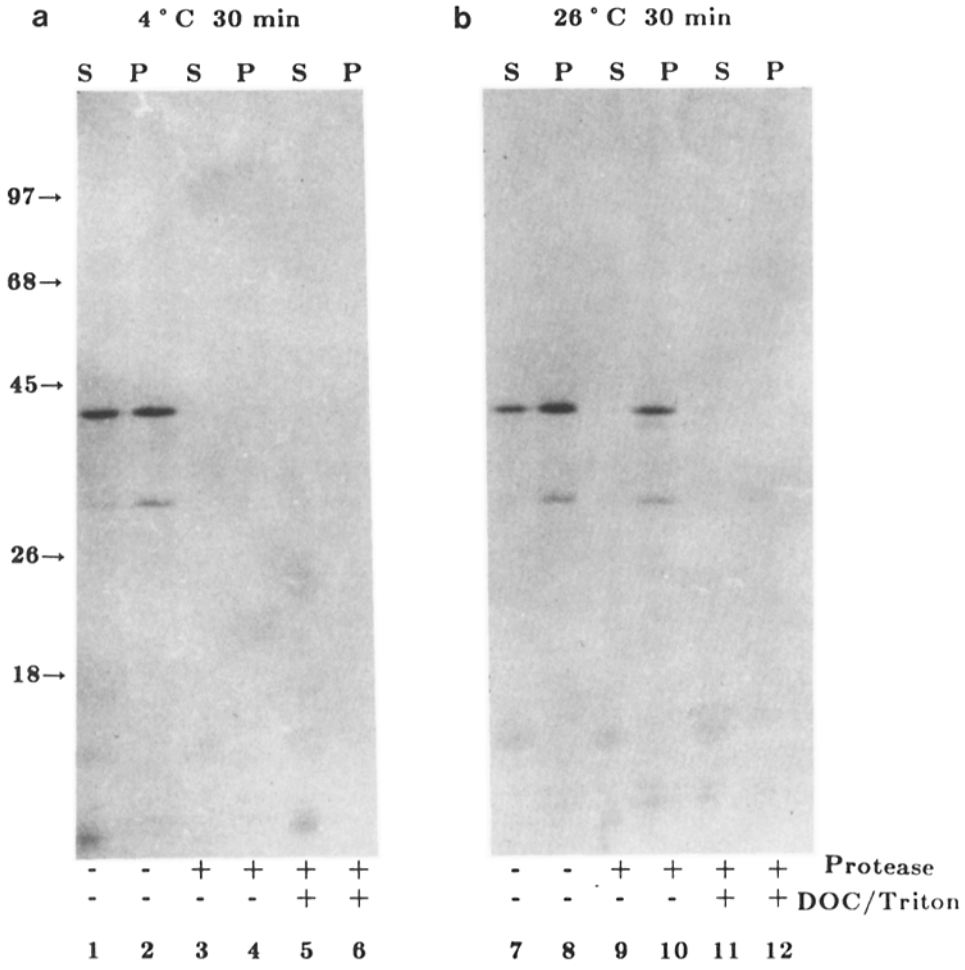

Figure 4. Temperature dependence of import. Newly synthesized translation products (300 μl) were mixed with peroxisomes (2.2 mg of protein) and divided into two equal samples. Incubation was at 4°C (*a*) or 26°C (*b*) for 30 min. After this each sample was divided in three and binding and import were assessed as in Fig. 3 *b* except that only 1 μg of thermolysin was used.

Temperature Dependence of Import

When the import assay was carried out on ice (Fig. 4 *a*), 24% of the 43-kD translation product and 40% of the 32-kD product bound to the peroxisomes, but neither product was protected from proteolysis. Therefore import but not binding requires incubation at 26°C.

Discussion

In the first of a planned series of experiments designed to study the information that targets a peroxisomal protein to peroxisomes, we have expressed the DNA encoding the carboxy-terminal portion of acyl-CoA oxidase by in vitro transcription and translation, and have followed the import of the resultant polypeptide into peroxisomes of *C. tropicalis*.

Our results indicate that sufficient information is present in the carboxy-terminal 40% of acyl-CoA oxidase to target it to peroxisomes, resulting in partial import (protease protection). Under optimal conditions (Fig. 4), 59–63% of the peroxisome-associated translation products were imported (protease resistant). Two different-sized carboxy-terminal polypeptides were expressed from the plasmid pGSP4-2a. It is interesting to note that both binding and import of the 32-kD polypeptide was greater than that of the 43-kD protein. This may reflect differences in the transient folding of these molecules. Although the specificity of binding and import was not tested in these experiments, we know that the full-length acyl-CoA oxidase associates in vitro only with peroxisomes (not with mitochondria), and does so in a time- and temperature-dependent fashion (19). These data suggest that targeting of peroxisomal proteins may differ from that of mitochondrial and chloroplast proteins, most of which possess a cleavable amino-terminal topogenic sequence (16, 21).

Our results do not exclude the possibility that targeting information may also be located within the amino-terminal 60% of acyl-CoA oxidase. Experiments are in progress to test this possibility.

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