A novel peroxisomal nonspecific lipid transfer protein from *Candida tropicalis* - Gene structure, purification and possible role in β-oxidation

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Abbreviations. nsLTP, nonspecific lipid transfer protein; PXP, peroxisomal polypeptide; SCP-2, sterol carrier protein 2; YPBG, medium composed of 0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1% Brij 58 and 2% glucose; YPBO, medium similar to YPBG, but contains 1% oleic acid instead of glucose.

Enzymes. Acyl-CoA oxidase (EC 1.3.3.6); catalase (EC 1.11.1.6); DNA ligase (EC 6.5.1.1); DNA polymerase I (EC 2.7.7.7); 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34); lysozyme (EC 3.2.1.17); peroxidase (EC 1.11.1.7); restriction endonucleases (EC 3.1.21.4).

SUMMARY

We have sequenced the nucleotides of the gene POX18 that encodes PXP-18, a major peroxisomal polypeptide inducible by oleic acid in the yeast Candida tropicalis. POX18 had a single open reading frame of 127 amino acids. Some 33% of the amino acid sequence of the predicted basic polypeptide (13 805 Da), was identical to that of the nonspecific lipid transfer protein (sterol carrier protein 2) from rat liver. PXP-18, purified to near homogeneity from isolated peroxisomes, had an amino-terminal sequence identical to that of the predicted polypeptide except for the initiator methionine, and had nonspecific lipid transfer activity comparable to that of its mammalian equivalents. Unexpectedly, PXP-18 lacked the cysteine residue thought to be essential for the activity of this protein in mammals. RNA blot analysis showed that the POX18 gene was expressed exclusively in cells grown on oleic acid, suggesting that PXP-18 has a role in the β-oxidation of long-chain fatty acids. PXP-18 modulated acyl-coenzyme A oxidase activity at low pH.

INTRODUCTION

Peroxisomal proteins are synthesized on free polysomes and imported post-translationally into the organelle, but most of them have no cleavable targeting (topogenic) sequence at the amino-terminus (for review, see [1]). The targeting sequences of five peroxisomal proteins from higher eucaryotes have been found at the carboxy-terminus and shown to contain a tripeptide, Ser-Lys/His-Leu, at or near the end of the sequence [2-4]. We have cloned genes encoding peroxisomal proteins of the yeast Candida tropicalis [5] and sequenced three genes for the subunits (PXP-2, PXP-4 and PXP-5) of acyl-CoA oxidase isozymes [6, 7] and the gene for catalase (PXP-9) [8]. None of the polypeptides encoded by these genes has the Ser-Lys/His-Leu sequence in their carboxy-terminal regions. The targeting information of PXP-4, which has the molecular mass of 78 554 Da, was found in various locations by both in vitro [9] and in vivo [10] experiments. Thus, systematic analysis of an entire protein molecule must be done if the yeast peroxisomal targeting sequence is to be identified. Since a small protein is more convenient for this purpose, we

chose a 16-kDa protein, PXP-18, which is encoded by the gene POX18 and induced markedly by oleic acid [5]. Its function is not known.

3-Hydroxy-3-methylglutaryl-CoA reductase has been found not only in microsomes but also in peroxisomes of normal rat liver cells [11]. Moreover, rat hepatic peroxisomes can convert mevalonic acid to cholesterol in the presence of cytosolic fraction from rat hepatocytes [12]. Furthermore, sterol carrier protein 2 (SCP-2) was found to be concentrated in rat liver peroxisomes [13, 14]. SCP-2 was originally purified as a cytosolic factor that activates the enzymatic conversion of lanosterol to cholesterol by the microsomal fraction of rat liver [15]; it is identical to nonspecific lipid transfer protein (nsLTP) [15, 16]. The primary structure of nsLTPs from rat [17, 18] and bovine [19] livers and plant sources [20-23] have been determined. We describe here the similarity of PXP-18 to mammalian nsLTPs and report that purified PXP-18 has nonspecific lipid transfer activity comparable to that of its mammalian equivalents, despite the lack of the cysteine residue thought to

be essential for the activity of this protein in mammals.

MATERIALS AND METHODS

Biological materials

A 4.2-kb BglII-HindIII fragment of genomic DNA carrying the POX18 gene of C. tropicalis pK233 (ATCC 20336) was obtained from plasmid pC7 [5] and subcloned in pBR322, giving pC7-5. The 1.4-kb BglII-SpeI fragment of pC7 was subcloned in pGEM3 (Promega, Madison, WI, USA), giving pG18. Peroxisomes and mitochondria were isolated from oleic-acid-grown cells of C. tropicalis by the method of Kamiryo et al. [24] with sucrose density gradient centrifugation, except that the spheroplasts were prepared with 2-mg Zymolyase 100T (Kirin Brewery, Tokyo, Japan) per gram (wet mass) of cells. Poly(A)-rich RNA from cells grown in YPBO medium composed of 0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1% Brij 58 and 1% oleic acid or in YPBG medium, which is similar to YPBO but contains 2% glucose instead of oleic acid, was prepared as described previously [5]. Acyl-CoA oxidase was obtained as the major fraction from Mono Q column

chromatography during the isolation of PXP-4 [6]. This fraction was about 20% PXP-5, but it contained no PXP-18. The fraction containing β-oxidation activity was the unadsorbed material of CM-Toyopearl column chromatography in the purification of PXP-18 (see below). This fraction was free of PXP-18. Rat liver nsLTP [13] and bovine heart mitochondria [25] were the kind gifts of Drs M. Tsuneoka and Y. Tashiro, Kansai Medical University, Osaka, Japan. Egg yolk phosphatidylethanolamine and phosphatidylcholine were purchased from Green Cross Corp., Osaka, and acyl-CoAs from Sigma, St. Louis, MO, USA. The sources of the other enzymes and biological materials have been listed elsewhere [5, 6, 24].

General methods

Procedures for the manipulation [5] and sequencing [6] of DNA, DNA [5] and RNA [6] blot hybridization experiments, SDS/PAGE and the staining of proteins [24], and the assay of the protein [10] with bovine serum albumin as the standard were as described previously. Acyl-CoA oxidase activity was assayed at 25°C as described by Shimizu et al. [26]. One unit (U) of acyl-CoA

oxidase activity is defined as the amount that catalyzes the production of 1 µmol of H_2O_2 per minute. The β-oxidation activity was assayed at 25°C by monitoring of the reduction of NAD dependent on long-chain acyl-CoA by the method of Cooper and Beevers [27]; the reaction mixture contained 50 µmol of potassium phosphate, pH 7.2, 20 nmol of long-chain acyl-CoA, 0.8 µmol of CoA, 1 µmol of NAD, 10 µmol of dithiothreitol and enzyme, in a total volume of 1 ml. The activity of fatty-acid-binding protein was assayed according to Scallen et al. [28] by the method of Glatz and Veerkamp [29].

Purification and characterization of PXP-18

All procedures were at 0-4°C and PXP-18 was assessed by SDS/PAGE. The peroxisomes recovered from the sucrose density gradient were mixed with 4 volumes of 50 mM Tris/HCl, pH 7.4, containing 0.5 M NaCl and stirred for 30 min. The solubilized materials obtained by centrifugation at 126 000 x g for 1 h were dialyzed against a buffer of 10 mM potassium phosphate, pH 7.4, 50 mM KCl, 1 mM EDTA and 20% (mass/vol.) glycerol for 19 h. The

dialyzate was put on a CM-Toyopearl 650M column (Tosoh, Tokyo, Japan) equilibrated with the same buffer and eluted with a linear concentration gradient of KCl (50 to 300 mM). The fraction enriched with PXP-18 was dialyzed against 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 M ammonium sulfate for 15 h. The dialyzate was then put on a Phenyl Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer and eluted with a linear concentration gradient of both ammonium sulfate (1 to 0 M) and ethylene glycol [0% to 50% (mass/vol.)]. The fraction enriched with PXP-18 was dialyzed against a buffer of 10 mM potassium phosphate, pH 7.4, 1 mM EDTA and 20% glycerol and stored at -80°C.

To estimate the molecular mass of the native form of PXP-18, the fraction obtained from the Phenyl Sepharose chromatography was dialyzed against 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl, concentrated with Centriprep-10 (Amicon, Danvers, MA, USA) and treated by gel filtration on a TSK G3000SW column (Tosoh) with the same buffer. The amino-terminal sequence of PXP-18 was identified as described previously [6].

Assay of nonspecific lipid transfer activity

The activity causing nonspecific lipid transfer was assayed by the method of Poorthuis and Wirtz [30]. Labelled liposomes were prepared as follows: 0.2 µCi of 1, 2-di[1-^{14}C]palmitoyl phosphatidylethanolamine (110 mCi/mmol; Amersham, Amersham, UK), 10 µCi of [9, 10-^{3}H(N)]triolein (26.8 Ci/mmol; Du Pont/New England Nuclear, Boston, MA, USA), 125 µg of phosphatidylethanolamine (average, 761 Da) and 125 µg of phosphatidylcholine were put in a glass vial, dried under reduced pressure and mixed with 50 µl of 10 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 0.25 M sucrose (S buffer). After the suspension was mixed, it was sonicated with an ultrasonic processor (model W-225, Heatsystems-Ultrasonic, New York, NY, USA) at 0-4°C under an argon stream for 15 min. The lipid transfer reaction was carried out at 30°C for 90 min with mixing every 15 min in 0.5 ml of S buffer containing 3.4 µl of the labelled liposome suspension, 0.5 mg (as protein) of bovine heart mitochondria or other organelles, 2.1 mg of bovine serum albumin (essentially fatty acid free, Sigma) and the protein fraction to be assayed. The reaction was stopped by

centrifugation at 10 000 x g for 10 min, and the resulting pellet was resuspended in 0.5 ml of S buffer and sedimented (at 10 000 x g for 10 min) through a cushion of 0.5 ml of 14% (mass/vol.) sucrose. The mitochondrial pellet was dissolved in 0.1 ml of 10% (mass/vol.) SDS by being heated at 80°C and the radioactivity was counted with a liquid scintillation spectrometer. Blanks without a protein fraction were incubated to correct for noncatalyzed transfer. [^{3}H]triolein in the mitochondrial pellet served as a measure for liposomal contamination. One unit (U) of nonspecific lipid transfer activity is defined as the amount that catalyzes the specific transfer of 1 nmol of phosphatidylethanolamine per hour.

RESULTS AND DISCUSSION

Nucleotide sequence of POX18

We subcloned a 4.2-kb BglII-HindIII fragment (Fig. 1, top) carrying the POX18 gene in plasmid pC7-5 to examine the transcriptional coding region in detail, because Southern analysis of plasmid pC7 [5] showed a coding region of about 3 kb,

longer than that expected from the molecular mass of PXP-18 (16 kDa). Two coding regions were found. Preliminary sequencing of the 4.0-kb BglII-SalI region showed there were two open reading frames: one of nucleotides +1 to +384 and the other of nucleotides +2305 to +3141. The first open reading frame, of 127 amino acid residues, seemed more likely to encode PXP-18 than the other, of 278 residues, so the 1.4-kb BglII-SpeI fragment was subcloned in plasmid pG18 and the nucleotides of both strands were sequenced completely, as shown in Fig. 1. After the end of the experiment, we learned that Szabo et al. [31] have also sequenced a POX18 gene cloned in plasmid pLSP18C1-1. Our sequence (nucleotides -728 to +685) was essentially the same as that in pLSP18C1-1 (-583 to +668), but there were some differences between the two in the 5' upstream region (Fig. 1, bottom). The sequence in pLSP18C1-1 has two extra nucleotides, A and C, at -532 and -510, respectively; it has an oligo(dG) stretch of 17-mer instead of 18-mer at -471; it lacks the sequence ACATGA at -309; it has a CG sequence instead of a GC sequence at -244; and it contains the tandem repeat of a

tridecanucleotide, GATTACGTAAGCA, at -224 (alternatively, ATTACGTAAGCAG at -223). The POX18 gene in pG18 could be allelic to that in pLSP18C1-1, although the two sequences are indistinguishable from each other between -224 (or -223) and +668, even at the third nucleotide of any of the 127 codons used. We have cloned another allele of POX18 that lacks restriction sites for SfiI (+63) and KpnI (+178) (N. Mito and T. Kamiryo, unpublished data).

The basic polypeptide deduced from the open reading frame had a molecular mass (13 805 Da) reasonable for that of PXP-18 and an amino-terminal sequence (see Fig. 2) identical to that of the purified PXP-18 (see below) found by Edman degradation: Ser-Val-Glu-Val-Asp-Gly-Phe-Asn-Ala-Ser-Pro-Leu-Phe-Lys-(Glu/Gln)-. The initiator methionine had been removed from the mature protein as in PXP-2 [7], PXP-4 [6], PXP-5 [6] and PXP-9 [8]. The deduced amino acid sequence of PXP-18 had considerable similarity with that of nsLTP (SCP-2) purified from rat [17] and bovine [19] livers. In Figure 2, we compare the sequences of PXP-18 and rat nsLTP, the carboxy-terminal sequence of which was found to be

-Lys-Ala-Lys-Leu by Morris et al. [18]. Forty-two of the 127 amino acid residues in PXP-18 were identical to the rat nsLTP. Thus, there was 33% amino acid identity between the two proteins. If conservative amino acid changes were included, similarity was 59%. The amino acid compositions were similar, as well, except that PXP-18 lacked a cysteine residue found in the animal nsLTP (Cys-71), which has been thought to be essential for the activity of mammalian nsLTPs [30, 32]. Little similarity in the amino acid sequences of PXP-18 and plant nsLTPs [20-23] was found.

Purification of PXP-18 and its nonspecific lipid transfer activity

We purified PXP-18 as a 16-kDa protein to near homogeneity from the peroxisomes of cells grown on oleic acid by three steps of purification: solubilization of the organelles with salt, CM-Toyopearl chromatography of the resulting supernatant and Phenyl Sepharose chromatography. Figure 3 shows the progress of the purification, and that the final preparation (lane 4) looked homogeneous. The amino-terminal sequence of this fraction was

identified as described above. The results obtained from gel filtration suggested that the PXP-18 protein was a monomer in its native form, unlike most peroxisomal proteins (see [1]).

The nonspecific lipid transfer activity of the PXP-18 fractions obtained during purification is summarized in Table 1. The increase in the specific activity agreed well with that in the purity of PXP-18 (Fig. 3), confirming that PXP-18 has nonspecific lipid transfer activity. The activity shown in Table 1 was assayed at 30°C with bovine heart mitochondria as the acceptor of phosphatidylethanolamine. Under these assay conditions, purified nsLTP from rat liver had the specific activity of about 200 U/mg. The activities of PXP-18 and rat nsLTP assayed with the mitochondria and peroxisomes of C. tropicalis as the acceptor were all similar to this activity. As expected from the absence of the cysteine residue, the activity of PXP-18 was inhibited neither by 10 mM N-ethylmaleimide nor by 1 mM p-chloromercuribenzoate, unlike mammalian nsLTPs [30, 32]. The activity of PXP-18 in the fraction from Phenyl Sepharose chromatography was stable at -80°C for at least 6 months, but

about 90% of the activity was lost when the fraction was concentrated by more than 10-fold. The highly purified nsLTP from an animal source tends to become dimers because of the formation of intermolecular disulfide bridges, and to lose its activity [33]. However, the highly concentrated inactive PXP-18 was monomeric, to judge from results of gel filtration. These findings and the similarity in the primary structures of PXP-18 and rat nsLTP suggest that the cysteine residue in mammalian nsLTPs is not in fact essential for their activities. Consistent with this view is that all eight cysteine residues (six in spinach [21]) found in plant nsLTPs are involved in intramolecular disulfide bridges [22].

Possible role of PXP-18

The results described above indicate that PXP-18 is a yeast homologue of nsLTP, but its physiological function in cells is not known. If PXP-18 functions like SCP-2 and is essential for the synthesis of yeast sterols, it must be constitutively expressed, because yeast membranes require much ergosterol

regardless of the carbon source on which the cells are growing. To examine this possibility, poly(A)-rich RNA from cells grown on glucose or on oleic acid was analyzed by Northern blotting (Fig. 4). Plasmid pC2001, which carries a gene expressed constitutively (K. Okazaki, unpublished data), was used as an internal marker. The POX18 mRNA (about 0.8 kb) was found to be expressed exclusively in the oleate-grown cells; the mRNA from the gene in pC2001 (about 1.3 kb) was expressed to an equal extent in cells grown on glucose or on oleic acid. These results indicate that PXP-18 was not constitutively produced, but that it functions solely in cells grown on oleic acid.

Alternatively, if PXP-18 works like nsLTP, which carries phospholipids and sterols from the site of their synthesis to the site where they are needed, it must be present on or between donor and acceptor [34] organelles, including endoplasmic reticulum. However, PXP-18 is probably mainly to be found in the peroxisomal matrix, because it was almost all in the peroxisomal fraction; its nonspecific lipid transfer activity was undetectable without disruption of the organelle; and it was not

an integral membrane protein, according to the criterion of Fujiki et al. [35]. Rat liver nsLTP was immunocytologically located mainly in the matrix of peroxisomes [13, 14], although the location of this protein in rat hepatocyte is still controversial [36] and the nature of a cross-reactive 58-kDa protein [37] has not been known. In addition, the largest concentration of castor bean nsLTP was found to be in the matrix of germinated seed glyoxysomes (M. Yamada, personal communication). Unlike almost all microbody proteins (see [38]), nsLTPs of the mouse (see [14]), maize [23] and castor beans (M. Yamada, personal communication) are synthesized with extra peptides of about 20 amino acid residues at their amino-terminus; the role of the extra peptides is unknown. POX18 encoded no extra peptides (Fig. 1), and therefore, we expect PXP-18 to function in no other organelles than peroxisomes.

The major role of the peroxisomes of oleate-grown cells could be the β-oxidation of long-chain fatty acids, and thus, PXP-18 might modulate the rate of β-oxidation. Like rat nsLTP [28], PXP-18 did not act as a fatty-acid-binding protein when

assayed with up to 20 μM palmitic acid as the ligand, but bovine serum albumin showed saturable binding under the same assay conditions. Therefore, it is unlikely that PXP-18 is involved in the activation of fatty acids. We examined the effects of PXP-18 when added to an assay mixture of acyl-CoA oxidase, because this enzymatic reaction is the rate-limiting step of peroxisomal β-oxidation in rat liver [39]. Various conditions were tried, and PXP-18 stimulated the acyl-CoA oxidase activity when assayed with lauroyl-CoA as the substrate at pH 5.5 (Fig. 5). This stimulation was dose-dependent and specific to PXP-18. No effect was found by the addition of egg white lysozyme, which was a monomeric basic protein with a low molecular mass, like PXP-18. Stimulation was not found, however, when the oxidase was assayed with oleoyl-CoA or palmitoyl-CoA as the substrate at a pH of from 5.0 to 8.0, or with lauroyl-CoA at a pH of 6.0 or above. Therefore, it is uncertain whether PXP-18 stimulates acyl-CoA oxidase activity *in vivo*, but it is of interest that the internal pH of yeast peroxisomes has been reported to be low [40]. Further studies are necessary to elucidate the physiological

function of not only PXP-18 but also nsLTPs from plant and animal sources.

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TABLE

Table 1. Increase in nonspecific lipid transfer activity during the purification of PXP-18

Details for the purification steps are in Materials and Methods.

n.d., not detected

Step	Total protein	Total activity	Specific activity	Purification	Yield
	mg	U	U/mg	-fold	%
Peroxisome	257	n.d.	-	-	-
Supernatant	188	677	3.60	1	100
CM-Toyopearl	1.49	345	232	64	51
Phenyl Sepharose	0.41	184	449	125	27

LEGENDS TO FIGURES

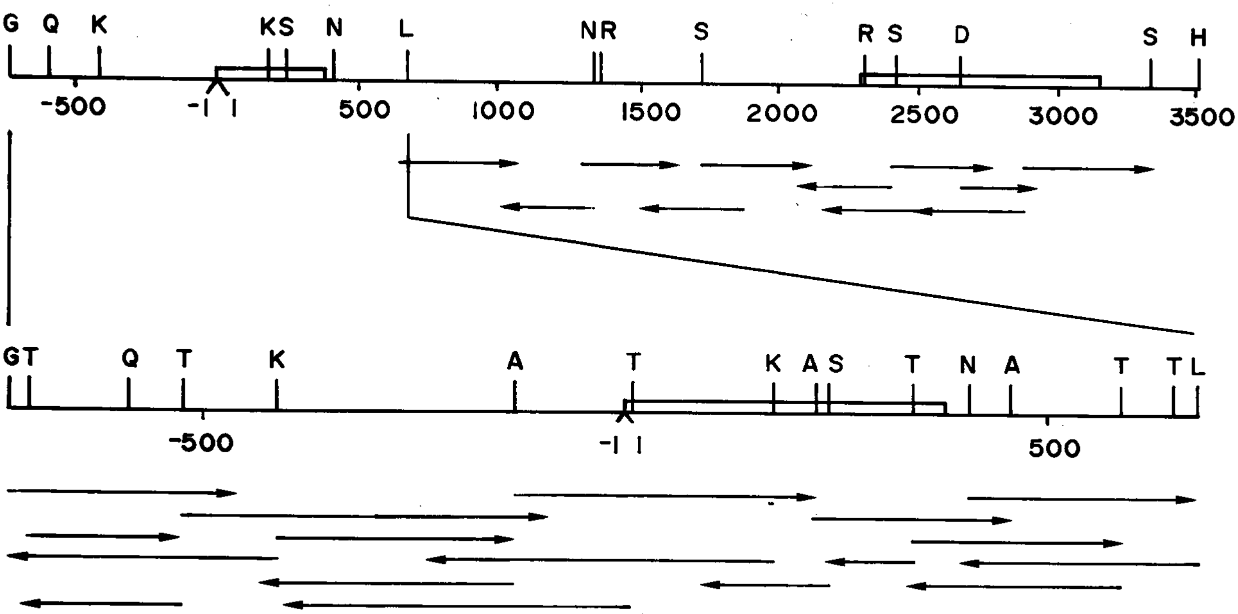
Fig. 1. Restriction map (top) and nucleotide sequence (bottom) of POX18 and its flanking regions. Restriction maps of the insert in pC7-5 (upper line) and that in pG18 (lower line) are shown with the sequence strategy. Restriction sites used for M13 cloning (not all for pC7-5) are indicated above the lines: A, Sau3AI; D, DraI; G, BglII; H, HindIII; K, KpnI; L, SpeI; N, NsiI; Q, SacI; R, EcoRV S, SalI; T, TaqI. Arrows represent the direction and extent of sequences identified with sufficient accuracy and double lines show the main open reading frames. The nucleotides on the mRNA-like strand are numbered in the 5' to 3' direction: position 1 is the first nucleotide of the ATG initiation codon of POX18 (the left open reading frame). The "TATA box" sequence is boxed and the initiation and termination codons are underscored. Below the sequence, the nucleotides derived from pLSP18C1-1 [31] are indicated if they differ from those in pG18. Arrowheads mark the termini of the sequence from pLSP18C1-1 and dashes denote nucleotides missing in either sequence

Fig. 2. Comparison of the primary structures of PXP-18 and rat liver nsLTP. The amino acid sequences of PXP-18 deduced from POX18 (the initiator methionine is omitted) and rat liver nsLTP (SCP-2) [17, 18] are represented by the single-letter code for amino acids. Identical amino acids are boxed: dashes indicate gaps introduced to obtain higher similarity. The amino-terminal sequence of PXP-18 confirmed by Edman degradation is overscored. The numbers of the amino acid residues are shown on the right

Fig. 3. Proteins from steps in the purification of PXP-18. The preparations were treated by SDS/PAGE with a 15% polyacrylamide gel, stained with Coomassie brilliant blue. Lanes 1 and 5, 5.0 µg (as proteins) of intact purified peroxisomes; lane 2, 2.9 µg of supernatant obtained at 126 000 x g from solubilized peroxisomes; lane 3, 0.9 µg of eluate from CM-Toyopearl chromatography; and lane 4, 2.1 µg of dialyzed eluate from Phenyl Sepharose chromatography. The position of PXP-18 is indicated on the left

Fig. 4. Northern blotting of POX18 transcript. About 3 μg of poly(A)-rich RNA from cells grown on glucose (lane 1) or on oleic acid (lane 2), separated on a 1.1% agarose gel, was probed with the 0.4-kb TaqI-NsiI fragment (+9 to +415) of POX18 and 14.0-kb pC2001 (a derivative of pBR322 containing a C. tropicalis gene expressed constitutively). The transcripts from POX18 and from the gene in pC2001 are marked with an arrow and an arrowhead, respectively. The positions and lengths of markers, which are mixtures of denatured pBR322 fragments obtained by digestions with AluI or with HincII, are shown on the left

Fig. 5. Stimulation of acyl-CoA oxidase activity by PXP-18. Acyl-CoA oxidase (a mixture of PXP-4 and PXP-5, 1 μg as protein) was assayed with lauroyl-CoA as the substrate in 50 mM sodium citrate, pH 5.5. The amounts of PXP-18 (O) or egg white lysozyme (●) added to the reaction mixture (1.0 ml) is indicated on the abscissa. Similar results were obtained in reactions with 50 mM potassium phosphate, pH 5.5, instead of sodium citrate



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A C

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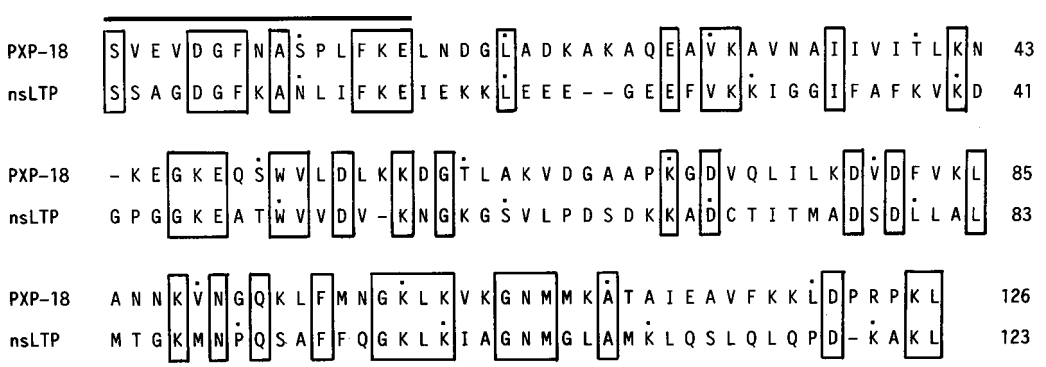
Fig. 2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nsLTP G P G G K E A T W V V D V - K N G K G S V L P D S D K K A D C T I T M A D S D L L A L 83
PXP-18 A N N K V N G Q K L F M N G K L K V K G N M M K A T A I E A V F K K L D P R P K L 126
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Fig. 3

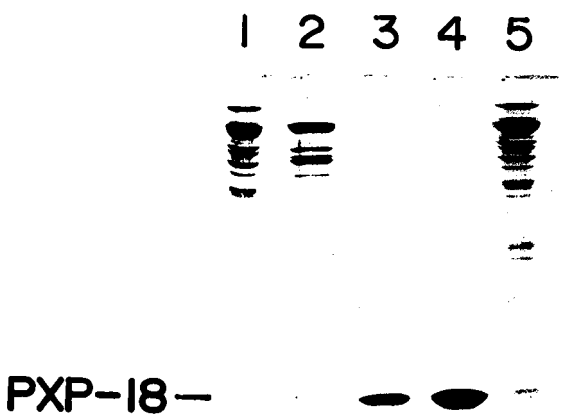

Fig. 4

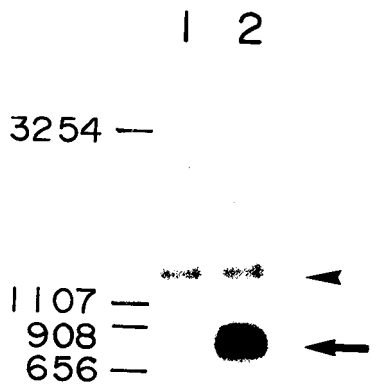

Fig. 5