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PEROXISOMAL ACTIVATION OF LONG- AND VERY LONG-CHAIN FATTY ACIDS IN THE YEAST *PICHIA PASTORIS*

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SUMMARY: In mammals, ß-oxidation of very long-chain fatty acids (VLCFA) takes place in peroxisomes. This process is impaired in X-linked adrenoleukodystrophy (XALD) patients as a result of decreased activity of peroxisomal very long-chain acyl-CoA synthetase (VLCS). We investigated VLCFA and long chain fatty acid (LCFA) activation in the yeast *Pichia pastoris*. Both VLCFA and LCFA were activated to their CoA derivatives in an organelle fraction. When organelles were fractionated on a sucrose gradient, VLCS activity co-localized with peroxisomes while long chain acyl-CoA synthetase activity associated primarily with mitochondria. Consistent with these findings, only VLCS activity was reduced in organelle fractions from peroxisome assembly (*pas*) mutants. Furthermore, no VLCS activity was detected in *pas* mutants at the density of normal peroxisomes. Thus, we conclude that VLCS is a peroxisomal enzyme in *P. pastoris* and this organism may serve as an excellent model system to investigate the molecular basis of XALD.

In humans and other mammals, ß-oxidation of very long-chain fatty acids (VLCFA; ≥C22:0) is exclusively a peroxisomal process (1,2). Prior to ß-oxidation, a VLCFA must be activated to its coenzyme A (CoA) derivative, VLCFA-CoA. This reaction is catalyzed by very long-chain acyl-CoA synthetase (VLCS), an enzyme found associated with peroxisomes and microsomes, but not with mitochondria (3,4). This enzyme is distinct from long-chain acyl-CoA synthetase (LCS) which is found in peroxisomes, mitochondria, and microsomes (5).

In the human genetic disorder X-linked adrenoleukodystrophy (XALD), peroxisomal VLCS activity is decreased, resulting in impaired ß-oxidation and elevated tissue levels of VLCFA (reviewed in (6)). Because microsomal VLCS activity is normal in XALD patients, it has been hypothesized that VLCFA-CoA produced by microsomes is inaccessible to peroxisomes. Recently, it was reported that the gene defective in XALD encodes a peroxisomal protein (ALDP) that is not related to LCS (7,8). Rather, ALDP is a member of the ATP-binding cassette membrane transporter protein family (7). Thus, it appears likely that both VLCS and ALDP are required for peroxisomal activation of VLCFA.

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Abbreviations: VLCS, very long-chain acyl-CoA synthetase; LCS, long-chain acyl-CoA synthetase; VLCFA, very long-chain fatty acid; XALD, X-linked adrenoleukodystrophy; SDH, succinate dehydrogenase.

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While significant progress toward understanding the molecular basis of XALD has been made, availability of an easily manipulable model system would facilitate investigation of this complex process. Yeast contain an active fatty acid ß-oxidation pathway that is found exclusively in peroxisomes (9). Furthermore, in the methylotrophic yeast *Pichia pastoris*, peroxisomes are induced when grown on oleic acid-containing medium (10). These properties suggested that *P. pastoris* might be an excellent model organism in which to study the subcellular distribution of VLCS activity. We report here that *P. pastoris* VLCS is a peripheral peroxisomal membrane protein; in addition, its activity is markedly reduced in yeast strains defective in peroxisome assembly.

MATERIALS AND METHODS

Yeast strains, media, and subcellular fractionation: The three *Pichia pastoris* strains utilized in this report are: *arg4-1, his4Δ::ARG4*, (SGY55); *arg4-1, pas7Δ::LEU2, his4Δ::ARG4*, (SGY131); and *arg4-1, pas10Δ::ARG4, his4Δ ::ARG4*, (SGY443) (11, Kalish *et al.*, submitted). Media and growth conditions have been described previously (10,12). For subcellular fractionation studies, wild-type and mutant yeast strains were pre-cultivated in dextrose medium and then induced in oleic acid medium as described (13). Following induction of peroxisomes, cells were converted to spheroplasts, homogenized, and subjected to differential centrifugation to yield a 25,000 x *g* supernatant fraction consisting of cytosol and low density organelles and a pellet fraction containing predominantly peroxisomes and mitochondria as previously described (13). Peroxisomes and mitochondria were separated on a 32-60% (w/w) sucrose gradient as described (13). Fractions of 1 ml were collected and assayed for catalase (peroxisomal marker) (14) and succinate dehydrogenase (SDH; mitochondrial marker) (15). Density of fractions was determined by refractometry. Microsomes were sedimented by centrifugation of the 25,000 x *g* supernatant fraction at 150,000 x *g* for 30 min at 4°C.

Assay of fatty acyl-CoA synthetase activity: VLCS and LCS activities were assayed as described previously using [1-^{14}C]lignoceric acid (C24:0; Research Products International, Mount Prospect, IL) or [1-^{14}C]palmitic acid (C16:0; Dupont/New England Nuclear, Boston, MA) as substrates, respectively (16).

RESULTS

Activation of VLCFA in *P. pastoris*. The initial step in peroxisomal ß-oxidation is the activation of fatty acids by acyl-CoA synthetase. We sought to determine whether peroxisomes in the yeast *Pichia pastoris* contained acyl-CoA synthetase activity, particularly VLCS activity. Wild-type yeast cells were grown in oleic acid-containing medium, converted to spheroplasts, and subjected to differential centrifugation to obtain a supernatant consisting primarily of cytosol and microsomes and an organelle pellet containing peroxisomes and mitochondria. Both fractions contained acyl-CoA synthetase activity when either palmitic acid, a long-chain fatty acid, or lignoceric acid, a VLCFA, was used as substrate (Table 1). In *P. pastoris* cells, VLCS activity was more than 100-fold lower than LCS activity and the distribution of both activities between the organelle pellet and supernatant was similar; approximately half of both VLCS and LCS activities was detected in the pellet (Table 1). When the supernatant was separated into cytosolic and microsomal fractions, the latter contained more than 80% of the VLCS activity and more than 90% of the LCS activity (data not shown).

VLCS is a peroxisomal protein in *P. pastoris*. To determine the subcellular location of the non-microsomal VLCS and LCS activities more precisely, peroxisomes and mitochondria from an organelle pellet were separated by sucrose density-gradient centrifugation. Nearly complete

Table 1. VLCS and LCS activities in wild-type *P. pastoris* and in *pas* mutants

	S	P	%P
	(nmol/hr/mg protein)		
VLCS			
Wild-type	0.40	1.9	46
pas7Δ	0.39	0.41	16
pas10Δ	0.29	0.35	18
LCS			
Wild-type	48	335	56
pas7Δ	131	470	41
pas10Δ	40	214	57

Yeast cells induced in oleic acid-containing medium were converted to spheroplasts, homogenized, and separated into a supernatant fraction (S) and an organellar pellet (P) as described in Methods. VLCS and LCS activities were measured as in Methods. %P is the percent of total activity found in the organelle pellet.

resolution of these organelles was achieved as illustrated by the positions of the marker enzymes catalase (peroxisomes; $density_{avg}$ = 1.22 g/cm^3) and succinate dehydrogenase (SDH; mitochondria; $density_{avg}$ = 1.17 g/cm^3) (Fig. 1). VLCS activity co-localized exclusively with the peroxisomal protein catalase (Fig. 1). In contrast, LCS activity was associated primarily with mitochondria and, to a lesser extent, with peroxisomes (Fig. 1).

VLCS is a peripheral peroxisomal membrane protein. In mammals, VLCS has been shown to be associated with peroxisomal membranes (17,18). To determine whether peroxisomal VLCS in *P. pastoris* was a matrix or membrane protein, an organelle pellet was resuspended in 100 mM Tris, pH 7.5, homogenized in a Dounce tissue grinder, and centrifuged for 30 min at 25,000 x *g*. While more than 90% of the matrix enzyme catalase was released to the supernatant, more than 85% of VLCS activity remained associated with the membrane pellet. In contrast, when purified *P. pastoris* peroxisomes were incubated with 1M NaCl, more than 85% of VLCS activity was released into a 25,000 x *g* supernatant. These findings suggest that VLCS is a peripheral peroxisomal membrane protein.

Decreased peroxisomal VLCS activity in *pas* mutants. To ensure that VLCS activity was truly associated with peroxisomes, we determined whether this activity was altered in yeast strains unable to assemble normal peroxisomes (peroxisome assembly or *pas* mutants) (10). These *pas* mutants contain peroxisome-like structures which sediment in the organellar pellet fraction; furthermore, these vesicles are composed of membrane proteins but lack all or most matrix proteins (10,19,20, and Kalish *et al.*, submitted). We examined LCS and VLCS activity in both the supernatant and organellar pellet fractions of two *pas* mutants: *pas7Δ* and *pas10Δ*. LCS activity was detected in both fractions from these *pas* mutants and specific activities were comparable to those values found in the wild-type strain (Table 1). Although wild-type levels of VLCS activity were found in the supernatant fractions of both *pas* mutants as well, the specific activity of VLCS in the organelle pellet was only 18-21% of values observed for the wild-type strain (Table 1). These findings provide further evidence that VLCS is a peroxisomal protein.

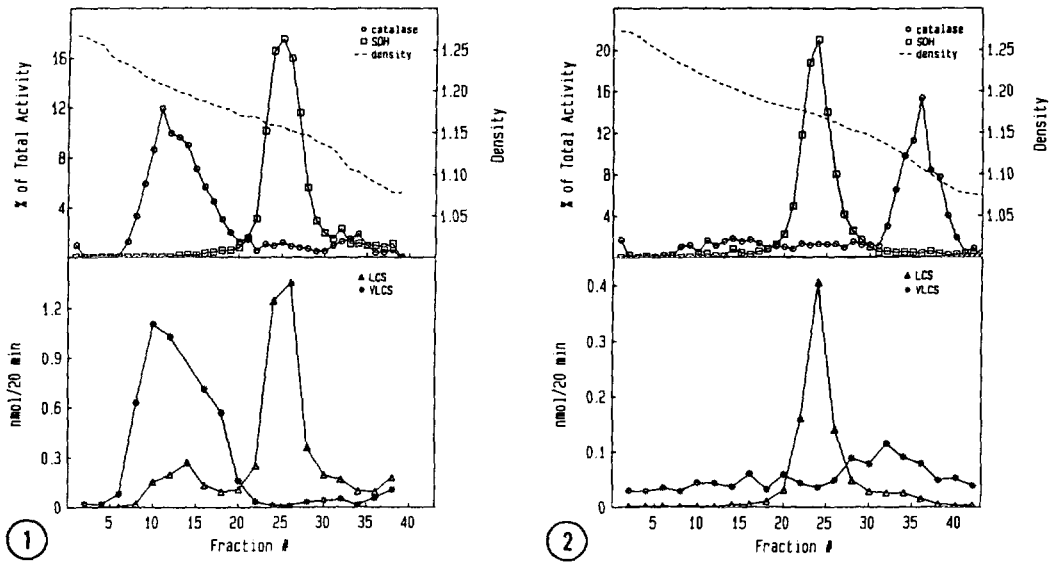

Figure 1. VLCS and LCS activities in subcellular fractions of wild-type *P. pastoris*. A 25,000 x g organellar pellet from a wild-type yeast strain was fractionated by sucrose density-gradient centrifugation as described in Methods. *Top:* Fractions of 1 ml were collected and assayed for catalase activity (peroxisomal marker; o), SDH activity (mitochondrial marker; □), and density (--). Activities are presented as % of total activity across the gradient. Density was measured by refractometry. *Bottom:* Equal volumes of each fraction were assayed for VLCS (*) and LCS (Δ) activity. The amount of each fraction assayed for LCS activity was one hundred-fold less than that assayed for VLCS activity. Results are expressed as nmol of fatty acyl-CoA synthesized in 20 min.

Figure 2. VLCS and LCS activities in subcellular fractions of the *pas7*Δ mutant. An organellar pellet isolated from *pas7*Δ was fractionated by sucrose density-gradient centrifugation. Marker enzymes (*top*) and activities of VLCS and LCS (*bottom*) were assayed as described in Figure 1.

To determine whether the small amount of VLCS activity was associated with peroxisome-like structures in these *pas* mutants, organelle pellets were fractionated on sucrose gradients. While mitochondria from all three *pas* mutants sedimented at a density comparable to that observed in a wild-type yeast strain (Fig. 2, and data not shown), the distribution of catalase differed greatly. No catalase activity was detected at the density typical of normal peroxisomes (1.22 g/cm^3) in either the *pas7*Δ or *pas10*Δ mutant (Fig. 2, and data not shown). Instead, most of the catalase activity was found near the top of the gradient at a density of 1.11-1.12 g/cm^3 (Fig. 2, and data not shown). When VLCS was assayed in gradient fractions from these *pas* mutants, no significant activity was detected at the density of normal peroxisomes; however, a small amount of VLCS activity was found at a density of about 1.15 g/cm^3, intermediate between the SDH and catalase peaks (Fig. 2, and data not shown). Interestingly, a membrane protein that associates with the cytoplasmic face of the peroxisome, Pas4p, co-localized with VLCS activity in the *pas10*Δ mutant (Kalish *et al.*, submitted). As expected, LCS activity was associated primarily with mitochondrial fractions of the *pas* mutants and no LCS activity was found at a density of 1.22 g/cm^3 (Fig. 2, and data not shown).

DISCUSSION

The importance of peroxisomes in metabolic processes has been underscored by the recognition of human diseases that result when these processes are impaired. For example, several human disorders are associated with defective peroxisomal ß-oxidation of fatty acids, particularly VLCFA (reviewed in (6)). Defective ß-oxidation can result either from deficiency of an enzyme in the pathway or from failure of the entire organelle to assemble properly (6). While considerable efforts have been directed toward understanding human disease using skin fibroblasts from affected patients, progress has been limited. In contrast, several genes involved in peroxisome assembly have been identified in the yeast *P. pastoris* (13,19,20; Kalish *et al.*, submitted). Furthermore, the recent discoveries that *P. pastoris PAS10* and *PAS8* are the yeast homologs of the human PAF-1 gene and *PXR1*, respectively, provide direct evidence that there are human counterparts to yeast genes (Kalish *et al.*, submitted; Dodt *et al.*, submitted). Based on these findings and the facts that 1) ß-oxidation is exclusively a peroxisomal process in yeast (9), 2) peroxisomes can be induced when yeast strains are grown in oleic acid (10), 3) highly purified peroxisomes can be isolated easily (13), and 4) techniques for genetic and biochemical manipulation of yeast are readily available, we have examined the utility of *P. pastoris* as a system for studying the activation and oxidation of both LCFA and VLCFA. Our data suggest that the subcellular distribution of LCS and VLCS activities in *P. pastoris* paralleled the distribution in mammals: LCS activity was observed in peroxisomes, mitochondria, and microsomes, and VLCS activity was detected in peroxisomes and microsomes (3,4). In addition, peroxisomal VLCS activity was significantly reduced in yeast strains unable to assemble functional peroxisomes.

To date, four distinct mammalian acyl-CoA synthetases have been reported, and at least three of these, including VLCS, are peroxisomal membrane proteins (3-5,21). While the other acyl-CoA synthetases are oriented in the membrane with their active sites facing the cytoplasm, the topography of VLCS is controversial. Singh and coworkers postulate that the active site of VLCS is oriented toward the peroxisome matrix (17), whereas Wanders and coworkers suggest that it is present on the outer face of the peroxisome (18). Our data suggest that *P. pastoris* peroxisomal VLCS is a peripheral membrane protein; studies to determine its topography are underway. The yeast *Saccharomyces cerevisiae* has at least four acyl-CoA synthetases, Faa1p, Faa2p, Faa3p, and Faa4p, but the subcellular localization and topography of these enzymes have not been determined (22). Gordon and coworkers reported that only Faa3p was active toward VLCFA, but its overall activity was significantly lower than either Faa1p or Faa2p (23). Our current study with *P. pastoris* suggests that at least two acyl-CoA synthetases exist in this yeast, one with VLCS activity. The *P. pastoris* VLCS, like *S. cerevisiae* Faa3p, has less than 1% of the activity of LCS.

Several inherited disorders of peroxisomal metabolism, including XALD, present with impaired VLCFA ß-oxidation (6). Because the biochemical defect in XALD is decreased peroxisomal VLCS activity, it was initially hypothesized that the gene coding for this enzyme was defective in XALD. However, the recent identification of the ALD gene by positional cloning revealed that this was not the case. The product of the ALD gene, ALDP, is an 75 kDa peroxisomal membrane protein that shares significant sequence similarities and topological characteristics with a 70 kDa peroxisomal membrane protein, PMP70 (7,8). Both ALDP and

PMP70 are integral peroxisomal membrane proteins and are members of the ATP binding cassette (ABC) superfamily of membrane transporters (7,24). Based on these findings, several groups suggested two possible functions for ALDP: it may function to transport VLCS into peroxisomal membranes or alternatively, aid in the association of VLCS with peroxisomal membranes (7,8). Consistent with either of these hypotheses is our finding that purified peroxisomes from *P. pastoris* can oxidize VLCFAs (P.A. Watkins and C.I. Chen, unpublished observation). Purification of VLCS from peroxisomes of *P. pastoris* is presently underway. Recently, Valle and coworkers identified and characterized a protein (Pxa1p) in *S. cerevisiae* thought to be the yeast homolog of human ALDP (25). Currently, we are trying to identify a similar protein in *P. pastoris*. Once ALDP and VLCS are identified and characterized in *P. pastoris*, genetic manipulation should provide valuable insight into the roles of these proteins in normal VLCFA oxidation and in XALD.

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