

Purification and Characterization of a Novel Pumpkin Short-Chain Acyl-Coenzyme A Oxidase with Structural Similarity to Acyl-Coenzyme A Dehydrogenases

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A novel pumpkin (*Cucurbita pepo*) short-chain acyl-coenzyme A (CoA) oxidase (ACOX) was purified to homogeneity by hydrophobic-interaction, hydroxyapatite, affinity, and anion-exchange chromatography. The purified enzyme is a tetrameric protein, consisting of apparently identical 47-kD subunits. The protein structure of this oxidase differs from other plant and mammalian ACOXs, but is similar to the protein structure of mammalian mitochondrial acyl-CoA dehydrogenase (ACDH) and the recently identified plant mitochondrial ACDH. Subcellular organelle separation by sucrose density gradient centrifugation revealed that the enzyme is localized in glyoxysomes, whereas no immunoreactive bands of similar molecular weight were detected in mitochondrial fractions. The enzyme selectively catalyzes the oxidation of CoA esters of fatty acids with 4 to 10 carbon atoms, and exhibits the highest activity on C-6 fatty acids. Apparently, the enzyme has no activity on CoA esters of branched-chain or dicarboxylic fatty acids. The enzyme is slightly inhibited by high concentrations of substrate and it is not inhibited by Triton X-100 at concentrations up to 0.5% (v/v). The characteristics of this novel ACOX enzyme are discussed in relation to other ACOXs and ACDHs.

Fatty acid β -oxidation is one of the characteristic functions of plant peroxisomes. Various forms of these organelles occur in plant cells, and the processes in which they are involved are largely dictated by their enzyme complement. Glyoxysomes are the site of β -oxidation and glyoxylate cycle enzymes that are responsible for fatty acid catabolism during post-germinative growth of oilseeds. Leaf peroxisomes are the site of photorespiratory and β -oxidation enzymes in photosynthetic organs. Unspecialized peroxisomes present in other organs have no clear function but still host β -oxidation enzymes.

Plant peroxisomes are capable of performing complete β -oxidation of fatty acids to acetyl-coenzyme A (CoA) (Gerhardt, 1992), while mammalian peroxisomes only partially oxidize long-chain fatty acids to octanoic acid (Van Veldhoven et al., 1992). The resulting short-chain fatty acids are then transferred to the mitochondria, where catabolism is completed. The first committed step in peroxisomal β -oxidation is carried out by the acyl-CoA oxidase (ACOX) enzyme, which is considered to be the rate-limiting step of the process in mammalian cells (Reubsæet et al., 1988). The first step of mitochondrial β -oxidation is carried out by the acyl-CoA dehydrogenase (ACDH) enzyme (Eaton et al., 1996).

The substrate specificity of plant ACOXs was analyzed using peroxisomal fractions from spinach and mung bean (Gerhardt, 1985) and from elm, rape, and

maize (Olsen and Huang, 1988), and employing both crude tissue extracts and peroxisomes isolated from normal, carbohydrate-starved, and carbohydrate-fed maize root tips (Hooks et al., 1995). A short-chain ACOX was identified and purified to apparent homogeneity from maize plantlets. It was reported that maize short-chain ACOX exhibits a native molecular mass of 60 kD and a subunit molecular mass of 15 kD (Hooks et al., 1996). This subunit molecular mass corresponds to approximately one-third to one-fifth of those of other ACOXs. In fact, the bacterial ACOX subunit is a dimeric enzyme of approximately 100 kD (Sztajer et al., 1993), that of the maize medium-chain ACOX a monomer of 62 kD (Hooks et al., 1996), and a subunit within the range of 69 to 79 kD characterizes cucumber and pumpkin (*Cucurbita pepo*) long-chain ACOX (Kirsh et al., 1986; De Bellis et al., 1999), mammalian ACOXs (Van Veldhoven et al., 1991; Hashimoto, 1996), and yeast ACOXs (Nicaud et al., 1998). Recently, we have characterized an Arabidopsis cDNA that encodes a 47-kD short-chain ACOX (Hayashi et al., 1999). Two additional full-length Arabidopsis cDNAs encoding distinct ACOXs were isolated by Hooks et al. (1999). The two corresponding enzymes are active on medium-chain and long-chain saturated fatty acyl-CoAs, respectively, and the deduced subunit molecular masses are approximately 73 and 77 kD, respectively.

With the aim of resolving the discrepancy among the reported molecular masses of ACOXs from different sources, and to further clarify the role of β -oxidation enzymes in plant tissues, we purified

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ACOX isoforms from pumpkin cotyledons. A cDNA coding for pumpkin long-chain ACOX was cloned and characterized previously (Hayashi et al., 1998a) and the native enzyme was also subsequently purified (De Bellis et al., 1999). We report the purification of a short-chain ACOX from pumpkin, and, based on the characteristics of this enzyme, describe how plant peroxisomes can perform the complete β -oxidation of fatty acids.

RESULTS

ACOX Isoforms in Pumpkin Cotyledons

To clarify which ACOX isoform was encoded by a pumpkin putative ACOX cDNA that showed high homology with mammalian ACOXs, we developed a hydrophobic interaction chromatography procedure to separate ACOX isoforms. An ACOX active on medium-long-chain substrates (C14–C16) was clearly separated from an isoform active on short-chain acyl-CoA esters (Hayashi et al., 1998a). The fractions collected after the hydrophobic interaction chromatography were subjected to western blotting and probed with two different antisera. The first was raised against a pumpkin long-chain ACOX (Hayashi et al., 1998a), the second against a His tag fusion protein expressed in *Escherichia coli* and employing as a template an Arabidopsis cDNA clone (GenBank accession no. T46525, which exactly matches the U72505 sequence submitted in the database by F. Grellet, P. Gaubier, H.-J. Wu, M. Laudie, C. Berger, and M. Delseny as a putative ACDH) that showed high homology with a glutaryl-CoA dehydrogenase. Therefore, we believed that this antiserum would reveal the presence of plant ACDHs.

Following staining of the membranes, we noted that the first antiserum revealed bands at a position corresponding to 72 kD, the subunit molecular mass of the mature long-chain ACOX (Hayashi et al., 1998a; De Bellis et al., 1999), but only in lanes representing fractions showing long-chain ACOX activity (palmitoyl-CoA as substrate); instead, the use of the second antiserum resulted in stained bands at a position of approximately 47 kD exclusively in lanes representing fractions characterized by short-chain ACOX activity (hexanoyl-CoA as substrate) (Fig. 1). No other fractions out of the range illustrated in Figure 1 (with the exception of fractions 56–58, representing the tail of the long-chain ACOX peak) showed ACOX activities or the presence of cross-reactive bands of the appropriate M_r . These results suggested that the putative ACDH cDNA from Arabidopsis encodes a short-chain ACOX, and we were subsequently able to demonstrate this finding expressing an active short-chain ACOX in insect cells (Hayashi et al., 1999). Moreover, the western-blot results were in contrast to those reported by Hooks et al. (1996) on a much lower subunit molecular mass (15 kD) for the short-chain ACOX of maize.

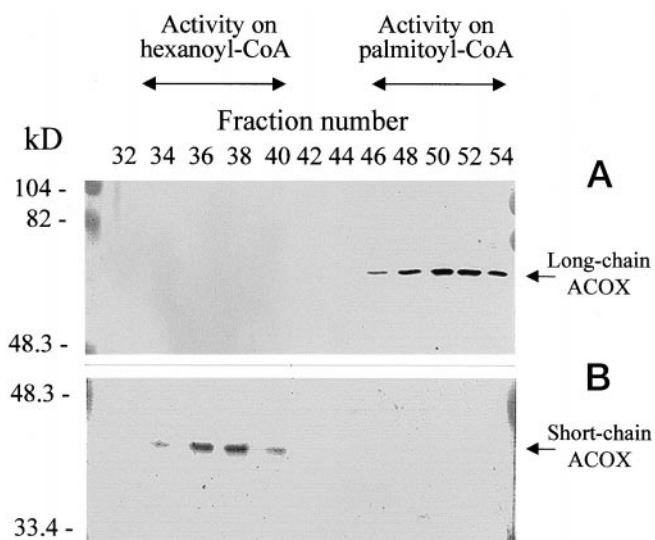


Figure 1. Western blot following hydrophobic interaction chromatography. A crude extract from 5-d-old dark-grown pumpkin cotyledons was loaded onto an Octyl Sepharose 4FF 1-mL column and protein eluted by decreasing the ammonium sulfate concentration and simultaneously increasing the ethylene glycol concentration. The presence of ACOX activity was tested employing short-chain (hexanoyl-CoA) and medium-/long-chain (decanoyl-CoA and palmitoyl-CoA) substrates. Approximately 20 μ L from each even fraction was loaded on each lane. Specific antibodies raised against pumpkin long-chain ACOX (A) and Arabidopsis short-chain ACOX (B) were employed. The positions of the prestained molecular mass standards are indicated on the left.

Purification of Short-Chain ACOX

The pumpkin short-chain ACOX was purified to apparent homogeneity by successive chromatographies on Phenyl-Supherose, hydroxyapatite, Hi-Trap Blue, and Mono S columns. The procedure, considerably different from the previously published method of Hooks et al. (1996), relied on the method employed for the purification of long-chain ACOX (De Bellis et al., 1999). The procedure resulted in an approximately 1,400-fold purification of short-chain ACOX to a specific activity of 71.7 μ mol min^{-1} mg^{-1} (Table I). Following the last step of purification (cation exchange chromatography), the fractions showing short-chain ACOX activity were subjected to SDS-PAGE, and proteins were detected by silver-staining and immunoblotting (Fig. 2). Silver-staining revealed that the purified enzyme is characterized by a subunit of approximately 47 kD and western blot indicated that the polypeptide was clearly recognized by the antiserum raised against the Arabidopsis ACOX fusion protein expressed in *Escherichia coli* (Hayashi et al., 1999). Mono S fractions not included in Figure 2 did not have ACOX activities and no cross-reactive bands were revealed.

Peak fractions from the Mono S chromatography were pooled and subjected to gel-filtration chromatography on a Superdex 200 16/60 column to determine the native molecular mass, which was esti-

Table I. Purification of short-chain acyl-CoA oxidase from pumpkin cotyledons

Purification Step	Total Protein	Total Activity	Recovery	Specific Activity	Purification
	mg	$\mu\text{mol min}^{-1}$	%	$\mu\text{mol min}^{-1} \text{mg}^{-1}$	-fold
Crude extract	1,500	75.7	100	0.05	–
Phenyl Supherose	25.2	32.2	42.5	1.28	25.6
Hydroxyapatite	1.2	16.4	21.6	9.5	190
Hi-Trap Blue	0.41	11.8	15.6	28.8	576
Mono S	0.12	8.6	11.4	71.7	1,434

ated to be approximately 180 kD (Fig. 3). Therefore, the pumpkin short-chain ACOX is suggested to be a tetrameric protein with identical subunits of 47 kD.

Biochemical Properties

The purified protein was employed to determine the substrate specificity of the enzyme. The pumpkin short-chain ACOX showed a maximum activity with C6 and C4 substrates, lower activity with C8 and C10 fatty acid esters, and practically no activity on C12 or longer straight chain substrates (Fig. 4). This clearly justified the name short-chain ACOX for the purified enzyme. No activity was detected that employed as substrates isobutyryl-CoA and isovaleryl-CoA (branched chain) or glutaryl-CoA (dicarboxylic). Moreover, the purified enzyme did not show any ACDH activity. These data are summarized in Table II.

The K_m value for substrate (hexanoyl-CoA) was estimated to be 1.7 μM and the pH optimum 8.3

(Table II), both values being similar to those determined for the pumpkin long-chain ACOX (De Bellis et al., 1999). The pI was not clearly determined (data not shown), but was estimated to be higher than 9.0 (Table II). The presence of albumin did not cause any effects on short-chain ACOX activity at increasing concentrations of hexanoyl-CoA, in contrast to the stimulation of the enzyme activity determined by albumin on the pumpkin long-chain ACOX (De Bellis et al., 1999); the purified enzyme displayed a substrate inhibition only at millimolar substrate concentrations (Fig. 5).

The effects of some chemical compounds on short-chain ACOX activity were tested: EDTA 10 mM, NaN_3 10 mM, and PMSF 1 mM did not influence the enzyme activity; N-ethylmaleimide (NEM) 1 mM and salicylhydroxamate (SHAM) 2 mM showed a slight inhibition (20%–25%) of the enzyme activity; Triton X-100 caused a 10% inhibition only at the concentration of 0.5% (w/v) (data not shown).

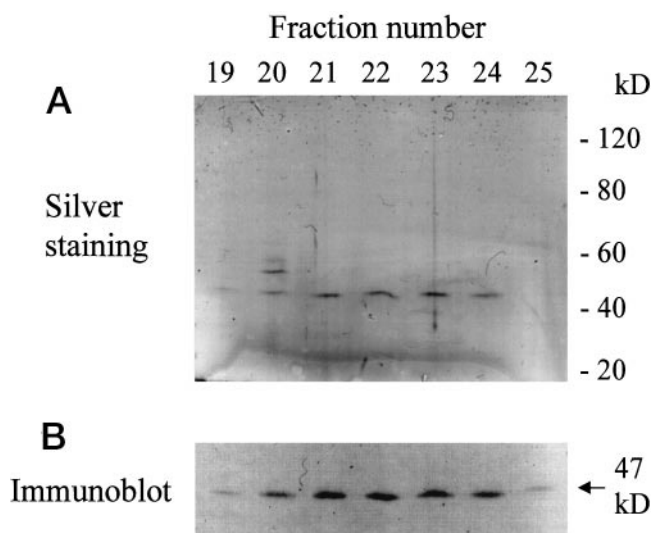


Figure 2. SDS-PAGE and western-blot analyses of short-chain ACOX on a PhastGel gradient of 10% to 15% following ion-exchange chromatography. Peak fractions (nos. 19–25) following a chromatography on a Mono-S column were subjected to SDS-PAGE and transferred onto a PVDF membrane. Approximately 1 μL from each fraction was loaded per lane. Molecular mass marker positions are indicated on the right. The gel was silver-stained (A) or short-chain ACOX was detected employing antibodies raised against Arabidopsis short-chain ACOX (B).

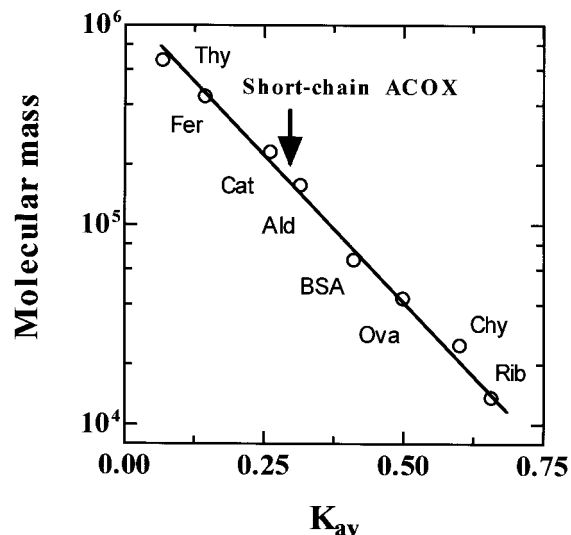


Figure 3. Determination of the molecular mass of the purified short-chain ACOX by size exclusion chromatography. A sample obtained after Mono-S chromatography was subjected to gel filtration on a Superdex 200 16/60 column. The column was calibrated with thyroglobulin, 669 kD (Thy); ferritin, 440 kD (Fer); catalase, 240 kD (Cat); aldolase, 158 kD (Ald); bovine serum albumin, 67 kD (BSA); ovalbumin, 43 kD (Ova); chymotrypsinogen, 25 kD (Chy); and ribonuclease A, 13.7 kD (Rib). The arrow indicates the elution position of pumpkin short-chain ACOX.

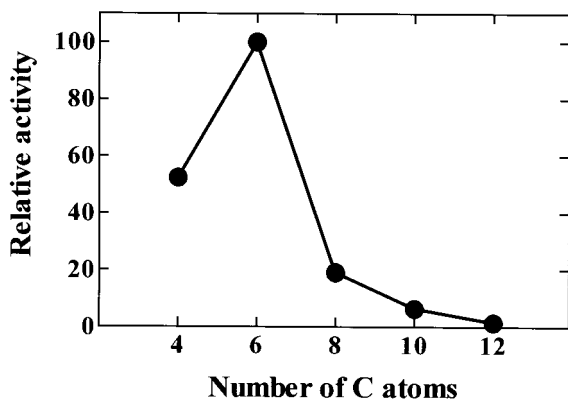


Figure 4. Substrate specificity profiles of purified pumpkin short-chain ACOX. Selectivity for the chain length of the fatty acids acyl-CoA as substrate. All activities were determined using 25 μM acyl-CoA as substrates. The highest activity was arbitrarily set at 100. The SE did not exceed 10%.

Subcellular Localization

The subcellular localization of short-chain ACOX was confirmed by Suc gradient centrifugation. Figure 6 shows short-chain ACOX activity (hexanoyl-CoA as substrate) and long-chain ACOX activity (palmitoyl-CoA as substrate) in the gradient fractions. Mitochondrial and glyoxysomal fractions were subjected to SDS-PAGE and western blot employing the antiserum raised against the Arabidopsis short-chain ACOX. Since the Arabidopsis short-chain ACOX shares high homology (up to 37%) at the amino acid level with mammalian ACDH, it would be interesting to detect some cross-reactivity with mitochondrial proteins in the range 40 to 50 kD, but no significant cross-reactivity was detected in lanes corresponding to mitochondrial fractions (Fig. 6) and in fractions characterized by lower density (data not shown).

Cross-Reactivity of Arabidopsis Antibodies with Enzymes from Other Plants

We tested whether the antibodies raised against the Arabidopsis short-chain ACOX cross-react with the equivalent enzyme from other plants. Figure 7 indicates that polypeptides of approximately 47 kD are

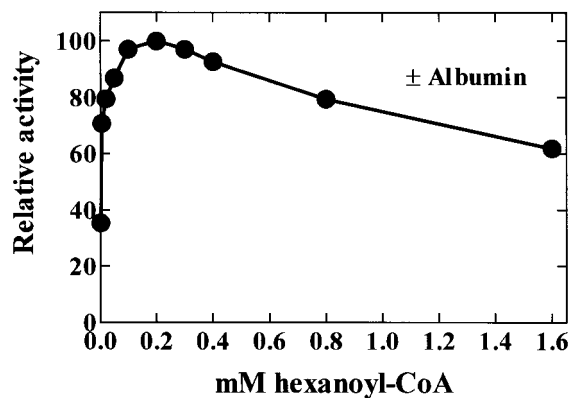


Figure 5. Activity of the purified short-chain ACOX as a function of substrate concentration in presence or absence of albumin (0.06%, w/v). Enzyme activity was measured with increasing concentration of hexanoyl-CoA with and without albumin. The highest activity was arbitrarily set at 100. The SE did not exceed 15%.

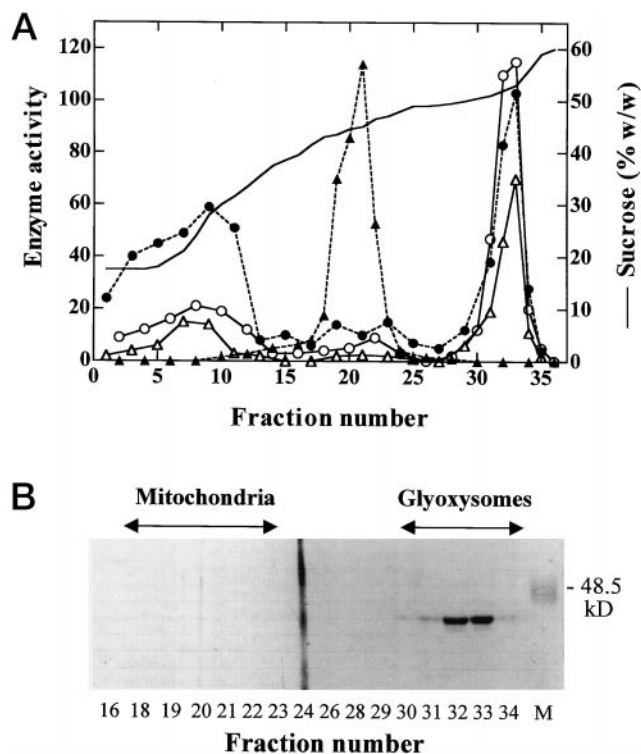


Figure 6. Subcellular localization of short-chain ACOX in pumpkin cotyledons. A, An extract from 5-d-old dark-grown pumpkin cotyledons was fractionated by Suc density gradient centrifugation and fractions were tested for enzyme activity: ●, catalase ($\mu\text{mol min}^{-1} \text{mL}^{-1} \times 30$); ▲, cytochrome *c* oxidase ($\text{nmol min}^{-1} \text{mL}^{-1} \times 4$); △, long-chain ACOX ($\mu\text{mol min}^{-1} \text{mL}^{-1}$); ○, short-chain ACOX ($\mu\text{mol min}^{-1} \text{mL}^{-1}$); solid line, Suc concentration. Long-chain ACOX and short-chain ACOX were tested employing palmitoyl-CoA and hexanoyl-CoA, respectively. B, Immunological detection of pumpkin short-chain ACOX by the use of antibodies raised against Arabidopsis short-chain ACOX. Fractions corresponding to mitochondria and peroxisomes are indicated. The position of the prestained molecular mass standard (48.5 kD) is indicated on the right.

Table II. Properties of short-chain acyl-CoA oxidase purified from pumpkin cotyledons

Native molecular mass	180 kD
Subunit molecular mass	47 kD
pI	>9.0
K_m (hexanoyl-CoA)	1.7
pH optimum (hexanoyl-CoA)	8.3
Activity on branched-chain acyl-CoAs (isobutyryl-CoA and isovaleryl-CoA)	ND ^a
Activity on glutaryl-CoA (dicarboxylic)	ND
Acyl-CoA dehydrogenase activity	ND
Subcellular localization	Peroxisomes

^a ND, Not detected.

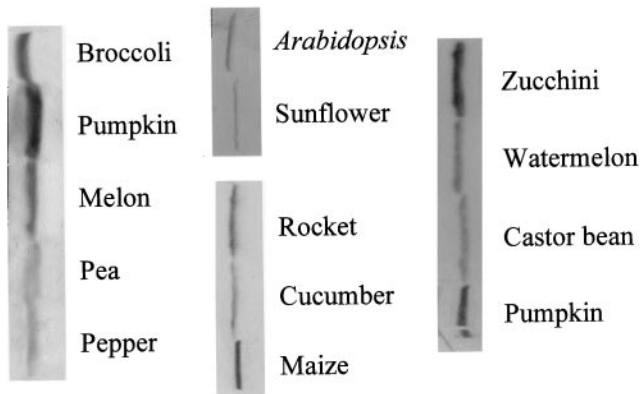


Figure 7. Immunoblots showing the cross-reaction of the antiserum raised against the Arabidopsis short-chain ACOX with the corresponding enzyme present in other plants. Crude extracts were obtained from etiolated zucchini cotyledons, etiolated watermelon cotyledons, etiolated castor bean endosperm, etiolated pumpkin cotyledons, Arabidopsis plantlets, etiolated sunflower cotyledons, rocket seedlings, etiolated cucumber cotyledons, maize embryos, broccoli seedlings, etiolated melon cotyledons, etiolated pea cotyledons, and pepper seedlings. Similar amounts of proteins (approximately 100 μg) were loaded on each lane.

detected in homogenates obtained from etiolated cotyledons of zucchini, watermelon, pumpkin, sunflower, cucumber, and melon; from castor bean endosperm; from rocket and broccoli seedlings; from maize embryos; and from 2-week-old Arabidopsis plants. Instead, only faint bands were detected in pea cotyledons and pepper seedlings.

DISCUSSION

Subunit and Native Molecular Masses

The short-chain ACOX was purified approximately 1,400-fold to yield a specific activity greater than 70 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. This value corresponds to that indicated by Sztajer et al. (1993) following the purification of an inducible short-chain ACOX from *Arthrobacter nicotianae*. Lower specific activities were reported by Kirsh et al. (1986) and De Bellis et al. (1999) for the long-chain ACOX purified from cucumber and pumpkin (27 and 13.5 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively), and by Hooks et al. (1996) for medium-chain and short-chain ACOX from maize seedlings (5.3 and 0.37 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively). The subunit molecular mass (Fig. 2) is identical to that reported by Hayashi et al. (1999) for the Arabidopsis short-chain ACOX, but clearly different from the pumpkin long-chain ACOX (Fig. 1). By size exclusion chromatography (Fig. 3), the pumpkin short-chain ACOX was identified as a tetrameric enzyme of 47 kD, which agrees with previous reports on the Arabidopsis short-chain ACOX (Hayashi et al., 1999), but not with results by Hooks et al. (1996) indicating a 60-kD tetramer in maize. Furthermore, the molecular mass and tetrameric organization of pumpkin short-

chain ACOX and mammalian short-, medium-, and long-chain ACDHs (Eaton et al., 1996) are similar.

Biochemical Properties

The major difference that was detected between the short-chain ACOX from pumpkin (Table II) and that of maize (Hooks et al., 1996) was in the pI values of >9.0 and 7.8, respectively. Both enzymes are active on C4 to C8 substrates, with the maize enzyme slightly more active on octanoyl-CoA. The K_m values determined with hexanoyl-CoA as substrate are relatively close: 1.7 μM for the pumpkin enzyme and 6 μM for the maize enzyme.

Pumpkin short-chain ACOX did not display a marked substrate inhibition at increasing concentrations of hexanoyl-CoA, and the presence of albumin in the assay medium also did not affect the activity. This represents a marked difference from the pumpkin long-chain ACOX, whose activity was stimulated by the presence of albumin and inhibited at palmitoyl-CoA concentrations higher than 100 μM (De Bellis et al., 1999). Sensitivity of the different oxidases to Triton X-100 also differs significantly. Concentrations of the detergent higher than 0.02% (v/v) inhibited long-chain ACOX (Gerhardt, 1987; De Bellis et al., 1999), while a concentration of 0.5% (v/v) is required to cause a 10% inhibition of pumpkin short-chain ACOX (data not shown). In contrast, NEM and SHAM inhibited at similar levels both short- and long-chain pumpkin ACOXs (De Bellis et al., 1999). These data indicate that NEM is an appropriate compound to trap free sulfhydryl groups in the assay of ACDH activity, as previously suggested by Furuta et al. (1981), and that SHAM should be carefully employed to discriminate between mitochondrial and peroxisomal β -oxidation in plants.

ACOX Isoforms in Plant Glyoxysomes

The data presented demonstrate that pumpkin glyoxysomes host a tetrameric short-chain ACOX characterized by 47-kD monomers, together with a dimeric long-chain ACOX with monomers of approximately 72 kD (Hayashi et al., 1998a; De Bellis et al., 1999). This observation can be extended to all higher plants because: (a) Kirsh et al. (1986) purified a dimeric (140-kD) cucumber long-chain ACOX; (b) the cloned Arabidopsis medium- and long-chain ACOX are both dimeric proteins with molecular masses of approximately 140 kD (Hooks et al., 1999); and (c) the Arabidopsis short-chain ACOX recently reported by Hayashi et al. (1999) is a tetrameric protein composed of 47-kD subunits. It remains to be determined whether a further monomeric (62-kD) medium-chain ACOX isoform and a tetrameric short-chain ACOX characterized by a native molecular mass of 60 kD are present in higher plant peroxisomes, as reported by Hooks et al. (1996). Therefore, plant peroxisomal

ACOXs appear more heterogeneous in their molecular size and structure than mammalian peroxisomal ACOXs (Hashimoto, 1996) and yeast ACOXs, which are characterized by subunits of approximately 79 kD (Nicaud et al., 1998). Such evidence suggests that higher plant peroxisomes evolved in a unique way. While mammalian peroxisomes never acquired the ability to perform a complete β -oxidation of fatty acids because of the absence of a short-chain ACOX (Hashimoto, 1996), higher plant peroxisomes acquired a short-chain ACOX activity that contributed to their ability to perform a complete fatty acid β -oxidation pathway in higher plant cells.

We propose that in plants a short-chain ACDH previously targeted to mitochondria evolved, through the acquisition of the appropriate targeting signal, to become a peroxisomal ACOX that contributed to the complete breakdown of long-chain fatty acids in a single organelle. Several lines of evidence support this hypothesis.

First, the phylogeny of algal peroxisomes indicates that both peroxisomes and mitochondria of ancient algae possess enzymes of the β -oxidation pathway. However, in other algae of the evolutionary line toward higher plants, such as *Mougeotia* (*Charophyceae*), the mitochondrial β -oxidation enzymes are absent, and ACOX activity is present in peroxisomes (Winkler et al., 1988; Stabenau, 1992). These results indicate that ACOXs appeared in plant peroxisomes relatively late in evolution (Stabenau, 1992). Second, the mammalian mitochondrial short-chain ACDH can function as an oxidase in the absence of suitable electron acceptors (Vanhove et al., 1993). Third, there is high homology between the Arabidopsis short-chain ACOX and the mammalian ACDHs and this is reflected by identical subunit molecular mass, tetrameric structure, and the presence of the two typical ACDH protein signatures (PS1 and PS2) in the Arabidopsis short-chain ACOX amino acid sequence (Hayashi et al., 1999). Fourth, the identification by Däschner et al. (1999) of a putative Arabidopsis isovaleryl-CoA (branched-chain) dehydrogenase localized in plant mitochondria, which shares high homology (>60%) at the amino acid level with mammalian isovaleryl-CoA dehydrogenases but considerably lower homology (28%) with the Arabidopsis short-chain ACOX. Furthermore, the putative Arabidopsis isovaleryl-CoA dehydrogenase presents a presequence characteristic of a mitochondrial signal peptide and not a peroxisomal PTS1 (Hayashi et al., 1997) or PTS2 (Kato et al., 1996, 1998) targeting signal.

If our hypothesis is correct, then a short-chain ACDH should be absent from higher plant mitochondria. Recently, Bode et al. (1999) reported the partial characterization and purification of ACDHs from maize root tips and sunflower stems. The authors presented data indicating that a dehydrogenase is the first enzyme of the plant mitochondrial β -oxidation

pathway, as is the case for mammalian mitochondria. They also reported two ACDHs with substrate specificities toward medium- and long-chain fatty acid esters, respectively. Previously, Anderson et al. (1998) showed that a branched-chain ACDH is involved in the mitochondrial Leu catabolism. We suggest that the plant mitochondrial β -oxidation enzymes reported so far represent the expression of higher plant genes somehow "forgotten" during evolution and switched on exclusively by particular developmental conditions. There have been various failed attempts over the years to measure ACDH activity in plant mitochondria, particularly in oilseed plants at the seedling stage, when lipid β -oxidation is active. This indicates that plant mitochondria are probably not involved in the massive degradation of fatty acids taking place during the post-germinative growth of oilseeds. In fact, Arabidopsis mutants showing defects in peroxisomal fatty acid β -oxidation do not show any post-germinative growth in the absence of an exogenous Suc supply (Hayashi et al., 1998b). Furthermore, in Figure 6 we reveal an absence of cross-reactivity of the polyclonal antibodies raised against Arabidopsis short-chain ACOX with pumpkin mitochondrial proteins in the range 35 to 60 kD in spite of the high homology with mammalian ACDHs. We therefore conclude that the short-chain ACOX is unique to plant peroxisomes and is essential for the complete degradation of fatty acids in this organelle.

MATERIALS AND METHODS

Plant Material and Chemicals

Pumpkin (*Cucurbita pepo* var. Amakuri Nankin) seeds were soaked overnight and germinated in the dark in a growth chamber for 5 d at 25°C. A similar procedure was employed for zucchini (summer squash, *Cucurbita pepo* L. Alberello di Sarzana), watermelon (*Citrullus vulgaris* Schrad., Crimson Sweet), castor bean (*Ricinus communis* L. CSCC 86), cucumber (*Cucumis sativus* L. Marketer), and melon (*Cucumis melo* L. Supermarket). The soaking step was not performed for sunflower (*Helianthus annuus* L. AC 2224), rocket (*Eruca sativa* Mill., Coltivata), maize (*Zea mays* L. Rival Hy), broccoli (*Brassica oleracea* L. Toscano), pea (*Pisum sativum* Asch. et Gr., Shuttle), or pepper (*Capsicum annuum* L. Quadrato d'Asti Giallo). All plants were grown for 5 d, except maize (3 d), broccoli (10 d), and rocket (10 d). Arabidopsis (ecotype Columbia) seeds were sterilized and imbibed in the dark at 4°C for 3 d before being sown onto 0.8% (v/v) agar plates containing one-half Murashige and Skoog salts (Murashige and Skoog, 1962). Plates were subsequently transferred in the light (21 W m⁻² irradiance) at 25°C for 2 weeks.

The CoA esters of various fatty acids were purchased from Sigma-Aldrich (St. Louis). Econo-Pac CHT-II Cartridge (5 mL) and Econo-Pac 10DG columns were purchased from Bio-Rad Laboratories (Hercules, CA); Octyl Sepharose 4 Fast Flow, Phenyl Sepharose High Performance, HiTrap Blue (1 mL), Mono S HR5/5, and other gels

for chromatography were obtained from Pharmacia (Uppsala).

Suc Density Gradient Centrifugation

Three grams of etiolated 5-d-old pumpkin cotyledons were homogenized in a Petri dish by chopping with razor blades in 10 mL of a medium containing 0.5 M Suc, 150 mM *N*-[2-hydroxy-1,1-Bis(hydroxymethyl)ethyl]glycine (Tricine), pH 7.5, and 1 mM EDTA. The homogenate was passed through four layers of cheesecloth, centrifuged at 600g for 10 min, and the supernatant was layered onto a discontinuous Suc gradient that contained 1 mM Tricine, pH 7.5, and 1 mM EDTA. The gradient was composed of 60% (2 mL), 56% (3 mL), 49% (10 mL), 43.5% (4 mL), 37.5% (6 mL), and 30% (6 mL) Suc (w/w). The gradient was centrifuged at 70,000g for 4 h in a rotor (model SW 28, Beckman Instruments, Palo Alto, CA), and gradient fractionated (model 185, ISCO, Lincoln, NE) into 1-mL fractions.

ACOX Purification

Five-day-old etiolated pumpkin cotyledons were homogenized in 3 mL of grinding medium per gram of fresh weight. The grinding medium was composed by 150 mM Tris, pH 7.6, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 μ M FAD, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% (v/v) glycerol. The homogenate was centrifuged for 20 min at 12,000g at 4°C. The supernatant, deprived of the top layer of lipids, was mixed with an equal amount of 3.4 M ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.2. It was then applied directly to a column (16 \times 150 mm) of Phenyl Sepharose High Performance (or in preliminary experiments on a Octyl Sepharose 4 Fast Flow column) equilibrated with buffer A (1.7 M ammonium sulfate, 20 mM sodium phosphate, 10 μ M FAD, and 10% [v/v] glycerol, pH 7.2). The column was washed with buffer A and eluted with a linear ethylene glycol gradient (0%–60%). Long- and short-chain ACOX were efficiently separated by this chromatographic step. Fractions with higher short-chain ACOX activity were pooled and desalted using Econo-Pac 10DG columns equilibrated with buffer B (10 mM Na-phosphate, 10 μ M FAD, and 10% [v/v] glycerol, pH 7.2). The sample was loaded on a Econo-Pac CHT-II Cartridge (5 mL, ceramic hydroxyapatite) equilibrated with buffer B, and the proteins were eluted by increasing the concentration of sodium phosphate to 0.5 M. Enzymatically active fractions were dialyzed against buffer B and loaded on a Hi-Trap Blue 1-mL column. Proteins were eluted with a linear NaCl gradient (0–3 M) in buffer B. Finally, enzymatically active fractions were dialyzed against buffer C (50 mM sodium phosphate, 10 μ M FAD, and 10% [v/v] glycerol, pH 6.7) and loaded on a MonoS HR 5/5 column. Proteins were eluted with a linear NaCl gradient (0–0.2 M) in buffer C. Gel filtration was performed with a Superdex 200 16/60 column (Pharmacia) equilibrated with a buffer composed of 50 mM Tris, pH 8.0, 150 mM NaCl, 10 μ M FAD, and 10% (v/v) glycerol.

Electrophoresis and Immunoblotting

SDS-PAGE analysis was performed at 15°C using a Phast System apparatus with PhastGel gradient 4 to 15 (Pharmacia) or in a traditional apparatus. PhastGels were silver-stained in the developer unit of the Phast System, according to the instructions of the manufacturer, or the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by diffusion blotting at 70°C. Fractions obtained by subcellular fractionation were subjected to electrophoresis on a 10% (v/v) SDS-polyacrylamide gel and protein transferred to a PVDF membrane in a semidry electroblotting system. Immunoreactive bands were visualized by alkaline-phosphatase reaction employing 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reagents.

Enzyme Assays

ACOX assays were performed by following the H₂O₂ formation coupled with a peroxidatic reaction (Gerhardt, 1987). The assay mixture consisted of 175 mM Tris, pH 8.5, 50 μ M FAD, 13 mM *p*-hydroxybenzoic acid, 1 mM 4-aminoantipyrine, 1 mM NaN₃, 5 units horseradish peroxidase, and 25 μ M acyl-CoA substrate. This assay was also used to determine *K_m* values and inhibition by different chemicals. Bis-Tris-propane (175 mM) in the range of 6.5 to 9.5, and Tris (175 mM) in the range of 7.5 to 8.8 were used to determine the pH optimum of the purified enzyme. ACDH assays were performed following two slightly different methods, as reported by Dommes and Kunau (1976) and Furuta et al. (1981), respectively.

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