Higher-plant medium- and short-chain acyl-CoA oxidases: identification, purification and characterization of two novel enzymes of eukaryotic peroxisomal β-oxidation

Mark A. HOOKS*, Kornelia BODE and Ivan COUÉE†

Station de Physiologie Végétale, Institut National de la Recherche Agronomique, Centre de Recherches de Bordeaux, BP 81, 33883 Villenave d'Ornon Cedex, France

Medium- and short-chain acyl-CoA oxidases were identified in and subsequently purified from dark-grown maize plantlets. The oxidase showing preference for medium-chain fatty acyl-CoAs (C_{10}–C_{14}) was purified to homogeneity. The oxidase showing preference for short-chain fatty acyl-CoAs (C_4–C_8) was purified over 150-fold. Various catalytic properties confirmed these enzymes to be true acyl-CoA oxidases. They produced *trans*-2-enoyl-CoA and H_2O_2 from the saturated acyl-CoA, as verified by various independent assay techniques. They also exhibited FAD-dependent activity; i.e. removal of loosely bound FAD by gel filtration markedly reduced activity, which could be restored upon re-addition of FAD. They showed apparent K_m values between 2 and 10 μM for the acyl-CoA substrate giving maximal activity, no activity with the corresponding free fatty acid, high pH optima (8.3–8.6) and a peroxisomal subcellular location. The medium-chain acyl-CoA oxidase was determined to be a monomeric protein with a molecular mass of 62 kDa. The short-chain acyl-CoA oxidase was shown to have a native molecular mass of 60 kDa, but exhibited a labile multimeric structure, as indicated by the elution of multiple peaks of activity during several chromatographic steps, and ultimately by the purification of a subunit of molecular mass 15 kDa. The medium- and short-chain acyl-CoA oxidases were demonstrated to be distinct from the maize equivalent of the cucumber glyoxysomal long-chain acyl-CoA oxidase previously purified and characterized [Kirsch, Loffler and Kindl (1986) J. Biol. Chem. **261**, 8570–8575]. The maize long-chain acyl-CoA oxidase was partially purified to permit determination of its substrate specificity; it showed activity with a broad range of acyl-CoAs of chain length greater than C_8, and maximal activity with C_{16}. The implications of the existence of multiple acyl-CoA oxidases in the regulation of plant peroxisomal β-oxidation are discussed.

INTRODUCTION

Acyl-CoA oxidases (ACOXs; EC 1.3.3.6) convert fatty acyl-CoA into *trans*-2-enoyl-CoA as the first step of peroxisomal β-oxidation. ACOXs contain FAD as a cofactor, which is reduced upon oxidation of the fatty acyl-CoA and subsequently reoxidized by O_2, forming H_2O_2. Families of ACOXs exist in nearly all eukaryotic organisms, which have been shown to possess either enzymes with different substrate specificities or multiple ACOX genes. Mammals have two or three ACOXs, depending on the species and tissue type, which are active in the process of shortening long-chain acyl-CoAs and in other specialized oxidation reactions [1,2]. No ACOXs specific for medium- or short-chain acyl-CoAs have been described in mammalian tissues. By the cloning and sequencing of genes induced during growth on long-chain alkanes, the yeasts *Candida maltosa* {*C. maltosa* ACOX A [2a] and *C. maltosa* ACOX B (Y. Matsuda and M. Takagi, unpublished work; Genbank accession no. D21228)} and *Candida tropicalis* [3–5] were shown to possess ACOX gene families. The only plant ACOX to be purified and characterized is of the long-chain type (LCOX), showing activity primarily with acyl-CoAs longer than C_{12} and virtually no activity with acyl-CoAs shorter than C_8 [6]. The substrate specificity of this enzyme is different from that of the general ACOX activity of peroxisomes isolated from a number of plant species [7,8], and thus the current view is that the specificity of ACOXs is species- and, possibly, tissue type-dependent [9].

Several lines of evidence suggest that this difference is due to the occurrence of a family of ACOXs whose members differ in their specificity for acyl-CoAs of different chain lengths, and are differentially expressed. (i) Peroxisomes isolated from various species possess ACOX activity with different acyl-CoA specificities [7,8], including that for branched-chain acyl-CoAs [10]. (ii) Excised tissues and isolated peroxisomes were capable of the complete and efficient oxidation of long-, medium- and short-chain acyl-CoAs [11,12]. (iii) Glyoxysomes purified from cucumber cotyledons showed no significant accumulation of medium- or short-chain acyl-CoAs when supplied with labelled long-chain fatty acids [13]. (iv) The substrate specificity of ACOX activity in protein extracts, or isolated peroxisomes, of maize roots changed with the developmental or carbohydrate status of the tissue [14]. (v) The recent identification in *Arabidopsis thaliana* of two different ACOX genes provided direct evidence for a multigene family in higher plants, but the substrate specificity of the enzyme encoded by each gene is unknown [15].

We have identified in maize two novel ACOXs, MCOX and SCOX, that are specific for medium- and short-chain fatty acyl-CoAs respectively. MCOX was purified to homogeneity, whereas SCOX in its native form was partially purified and a subunit obtained in homogeneous form. The physical and catalytic

Abbreviations used: ACOX, acyl-CoA oxidase; LCOX, long-chain acyl-CoA oxidase; MCOX, medium-chain acyl-CoA oxidase; SCOX, short-chain acyl-CoA oxidase.

* To whom correspondence should be sent, at present address: Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

† Present address: Institute National de la Recherche Agronomique, Centre de Recherches d'Orléans, 45160 Ardon, France.

properties of each enzyme were determined from both pure and partially pure fractions. The identification of these enzymes in plants answers many questions raised by the efficiency of peroxisomal β-oxidation in relation to the enzymology currently understood, but complicates our understanding of the control of β-oxidation fluxes. It is evident that the balance in catabolism of short-, medium- and long-chain fatty acids must be strictly regulated, given the wide variety of known or proposed effects of the reactants and products of β-oxidation on plant developmental and metabolic processes. The implications of the existence of multiple ACOX enzymes that differ in substrate specificity are discussed in relation to the regulation of peroxisomal β-oxidation.

EXPERIMENTAL

Materials

Chemicals, enzymes and buffers were purchased from Sigma (St. Louis, MO, U.S.A.) or Serva (Paris, France). Fatty acyl-CoA esters were from Sigma. Phenyl-Sepharose and DEAE-Sepharose CL-6B chromatography gels, Mono-P FPLC chromatofocusing column, Polybuffer 96 and Percoll were from Pharmacia (Uppsala, Sweden). Hydroxyapatite-HT and Ultrogel AcA-34 were from Bio-Rad (Richmond, CA, U.S.A.) and Biosepra (IBF, Heidelberg, Germany) respectively.

Plant material

Maize (*Zea mays* L., cv. DEA; Pioneer France Maïs) seeds were soaked in flowing tap water for 3 h and germinated in the dark at 25 °C between sheets of moist Whatman (Maidstone, Kent, U.K.) 3MM chromatography paper [16]. After 5–6 days, the entire plantlet was separated from the seed and used for extraction of the ACOXs.

Buffers

Buffer A comprised 150 mM Tris/HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10 μM FAD, 10 % (v/v) glycerol and 0.1 mM PMSF. Buffer B consisted of 20 mM KH_2PO_4, pH 7.5, 10 % (v/v) glycerol, 10 μM FAD and 0.4 M $(NH_4)_2SO_4$. Buffer C was 20 mM KH_2PO_4, pH 7.5, and 10 % (v/v) glycerol. Buffer D was composed of 20 mM KH_2PO_4, pH 7.5, 10 μM FAD and 60 % ethylene glycol. Buffer E was 200 mM KH_2PO_4, pH 7.5, and 10 % (v/v) glycerol. Buffer F was 10 mM Tris/HCl, pH 8.6, and 10 % (v/v) glycerol. Buffer G was 10 mM Tris/HCl, pH 7.5, 10 % (v/v) glycerol and 0.1 M KCl.

Partial purification of enzymes

All purification steps were performed at 4 °C. Maize plantlets (400–500 g) were homogenized in a Waring blender with 600 ml of buffer A and 1 % (w/w of tissue) insoluble polyvinyl-polypyrrolidone. The resulting paste was squeezed through cheesecloth and the filtrate centrifuged at 16000 ***g*** for 30 min. The supernatant was adjusted to 30 % saturation at 2 °C (on ice) with solid ammonium sulphate added over 30 min, and stirred for another 30 min. The solution was cleared by centrifugation at 16000 ***g*** for 10 min. The supernatant was brought to 50 % saturation with solid ammonium sulphate as described for the 30 % saturation. The pH was kept constant at pH 7.5 by the addition of solid Tris base. The solution was centrifuged at 16000 ***g*** for 10 min, and the precipitate was resuspended in 50 ml of buffer B. The insoluble material was removed by centrifugation at 27000 ***g*** for 10 min and filtration through one layer of Miracloth (Calbiochem, San Diego, CA, U.S.A.). An independent tissue sample of 400–500 g of dark-grown maize plantlets was used for the purification of each enzyme.

Chromatographic separations

All chromatographic separations were performed on either a Pharmacia LCC-500 or a Bio-Rad Econo System liquid chromatography system, using Pharmacia K9 or XK, or Biosepra, glass columns.

Separation of SCOX and MCOX from LCOX

The filtered 30–50 % ammonium sulphate fraction was loaded on to a phenyl-Sepharose column (1.5 cm × 35 cm) equilibrated with buffer B. The column was washed with buffer B until the absorbance at 280 nm returned to baseline. Buffer B was replaced with buffer C containing 10 μM FAD, and a stepwise gradient between 0 and 100 % buffer D was used to elute proteins at 15 %, 35 % and 60 % ethylene glycol. Bound proteins were eluted at a flow rate of 1 ml/min and collected in 10 ml fractions. The fractions from the 35 % ethylene glycol eluate were pooled and used for the further purification of SCOX and MCOX.

Separation of SCOX and MCOX

The 35 % ethylene glycol fraction was diluted 2-fold in buffer C and loaded at a flow rate of 0.5 ml/min on to a hydroxyapatite-HT column (2.5 cm × 2.5 cm) equilibrated in the same buffer. Non-bound proteins were collected in 10 ml fractions and pooled for the further purification of MCOX. Bound proteins, including SCOX, were eluted at a constant flow rate of 0.4 ml/min over 80 min with a linear gradient of 0–100 % buffer E, which was then kept constant at 100 % for another 80 min. The eluate was collected in 3 ml fractions and those containing SCOX activity were pooled and diluted 5-fold with buffer F.

Further purification of MCOX

The fractions of non-bound protein from the hydroxyapatite column were pooled, adjusted to pH 8.6 with Tris base and loaded at a flow rate of 1 ml/min on to a DEAE-Sepharose CL-6B column (0.5 cm × 15 cm) equilibrated in buffer F. MCOX was eluted using a simultaneous linear gradient of decreasing pH and increasing ionic strength (0–100 % buffer G) for 150 min at a flow rate of 0.4 ml/min [17]. The fractions containing MCOX activity were pooled and the proteins precipitated with solid ammonium sulphate at 80 % saturation. The precipitate was collected by centrifugation and resuspended in 2 ml of buffer C containing 10 μM FAD. The sample was gel-filtered at a flow rate of 0.5 ml/min on an Ultrogel AcA-34 column (2.5 cm × 75 cm) equilibrated with buffer C, and the eluate was collected in 5 ml fractions.

Further purification of SCOX

The diluted SCOX sample from the hydroxyapatite column was adjusted to pH 8.6 with solid Tris base and loaded at a flow rate of 1 ml/min on to a DEAE-Sepharose CL-6B column (0.5 cm × 15 cm) equilibrated with buffer F. Non-bound proteins were collected in 5 ml fractions. Since the amount of SCOX that bound to the column was highly dependent on the column bed volume, the proportion of SCOX activity remaining bound was determined by eluting the bound proteins with simultaneous linear gradients of decreasing pH and increasing ionic strength as described above. Fractions were collected in 3 ml volumes. The fractions of non-bound protein containing SCOX activity were pooled and the protein was concentrated by precipitation with

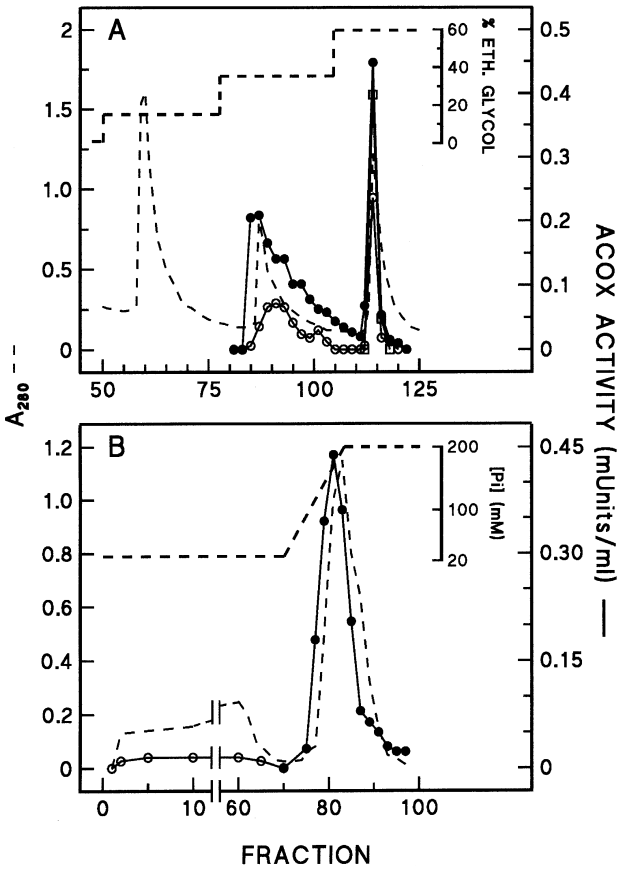

Figure 1 Representative separation of MCOX and SCOX from LCOX by column chromatography on phenyl-Sepharose (A) and hydroxyapatite-HT (B)

Symbols: ●, SCOX; ○, MCOX; □, LCOX. Symbols have been omitted for fractions not exhibiting ACOX activity. (**A**) The resuspended precipitate from the 30–50% ammonium sulphate fractionation was loaded on to a phenyl-Sepharose column, and the bound proteins were eluted stepwise with increasing concentrations of ethylene glycol. MCOX and SCOX were further purified from the peak fractions eluting at 35% ethylene glycol. (**B**) The pooled fractions from the 35% ethylene glycol eluate were diluted 2-fold with buffer B and loaded on to the column. MCOX was eluted in the fractions corresponding to non-adsorbed protein. SCOX, which remained bound, was eluted with a linear phosphate gradient between 20 and 200 mM.

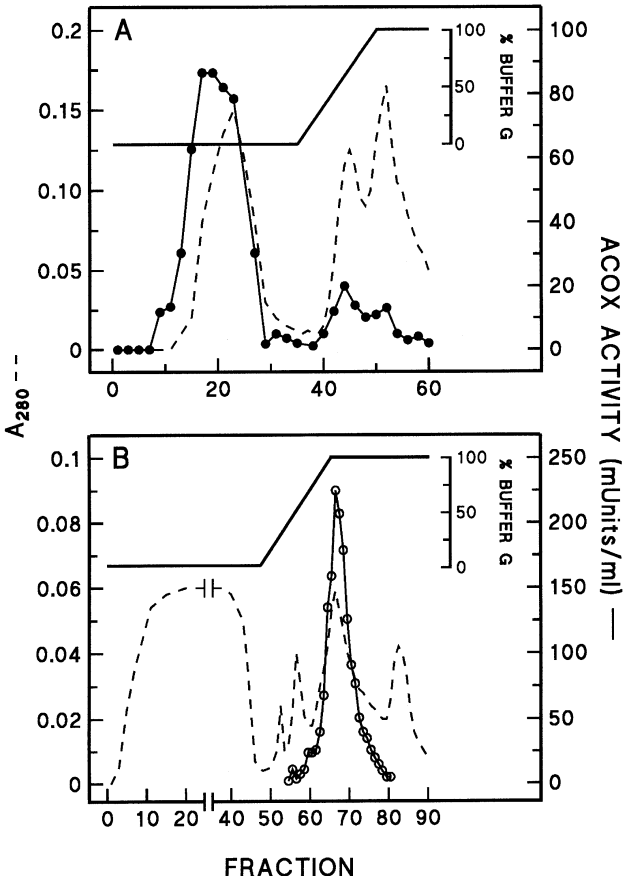

Figure 2 Further purification of MCOX and SCOX on DEAE-Sepharose

Symbols: ●, SCOX; ○, MCOX. Symbols were omitted for fractions not exhibiting ACOX activity. (**A**) Fractions containing SCOX activity from the hydroxyapatite column were combined and diluted 2-fold with buffer C. After adjusting the pH to 8.6 with KOH, the solution was loaded on to the DEAE-Sepharose column. SCOX was eluted with the non-adsorbed protein fractions. (**B**) The fractions containing MCOX activity from the hydroxyapatite column were combined, adjusted to pH 8.6 with KOH and loaded on to the DEAE-Sepharose column. MCOX activity was eluted with simultaneous linear gradients of decreasing pH and increasing ionic strength, as described in the Experimental section.

ammonium sulphate at 60% saturation. The precipitate was collected by centrifugation, resuspended in 2 ml of buffer C containing 10 μM FAD and gel-filtered on Ultrogel AcA-34 as described for MCOX.

Enzyme assays

ACOX (EC 1.3.3.6) activities in column fractions were measured by a peroxidase-coupled reaction using *p*-hydroxybenzoic acid as the chromogenic peroxidase substrate [18], and the substrates C_6-CoA for SCOX, C_{10}- or C_{12}-CoA for MCOX and C_{16}-CoA for LCOX. This assay with the corresponding substrates was also used to determine SCOX and MCOX specific activities after each purification step, and their isoelectric points, K_m values and FAD binding properties. SCOX and MCOX activities were also determined by monitoring the increase in absorbance at 260 nm ($\epsilon = 6700\ M^{-1}\cdot cm^{-1}$ [19]) due to the desaturation of the acyl-CoA, and by the enoyl-CoA hydratase (EC 4.2.1.74)/3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) coupled reaction [20]. The former assay, employing an Aces/Tris/ethanolamine constant-ionic-strength buffer system for pH 7–9 [21], was used to determine the pH-dependence of SCOX and MCOX activity. For each of the above assays, an acyl-CoA concentration of 50 μM was routinely used. This concentration was saturating for the enzymes, while not underestimating LCOX activity due to micelle formation with C_{16}-CoAs [22]. In addition, use of this concentration permitted direct comparison with previously published ACOX activity determinations [6,23]. Acyl-CoA dehydrogenase (EC 1.3.99.3), fumarase (EC 4.2.1.2), catalase (EC 1.11.1.6) and glucose-6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase (EC 1.1.1.49/EC 1.1.1.44) were assayed as previously described [24]. For all assays, 1 unit of enzyme activity was defined as 1 μmol of product formed during 1 min of reaction at 25 °C. Protein was determined according to the method of Bradford [25].

Characterization of physical properties

Native molecular masses of the ACOXs were determined by gel-filtration chromatography on Ultrogel AcA-34. Subunit molecular masses were determined by SDS/PAGE [26] using Sigma molecular mass markers. Isoelectric points were determined by

chromatofocusing according to the Pharmacia Chromatofocusing handbook, using a Mono-P column and Polybuffer 96.

Analysis of *trans*-2-enoyl-CoAs by HPLC

Saturated and mono-unsaturated acyl-CoA esters were separated by HPLC as described by Dieuaide et al. [27], except that aliquots of the reaction mixture were injected without prior purification. The peaks were collected and assayed for *trans*-2-enoyl-CoA using the enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase coupled assay. The *trans*-2-enoyl-CoA esters were always found to elute slightly before the corresponding saturated acyl-CoA ester.

Isolation of organelles

Maize root tips (3 mm) were excised from 3-day-old maize seedlings and incubated for 48 h in nutrient solution without exogenous carbohydrate, bubbled continuously with O_2/N_2 (1:1) [16]. Carbohydrate starvation served to minimize the heterogeneity of mitochondrial populations [24]. Organelles were separated by isopycnic centrifugation on self-generating Percoll gradients [24], except that the initial centrifugation step after filtering was decreased from 1000 ***g*** to 600 ***g*** and the resulting supernatant was layered directly on to the Percoll. The different fractions were analysed for SCOX, MCOX, LCOX, catalase, fumarase and glucose-6-phosphate dehydrogenase/6-phosphogluconate dehydrogenase activities.

RESULTS

Separation of SCOX, MCOX and LCOX

MCOX and SCOX were purified using a five-step purification protocol consisting of precipitation with ammonium sulphate, and subsequent column chromatography on phenyl-Sepharose, hydroxyapatite-HT, DEAE-Sepharose and Ultrogel AcA-34. Elimination of the heating step and addition of the anion-exchange chromatography on DEAE-Sepharose were the main differences between our procedure and that of Kirsch et al. [6]. The heating step, commonly used in the purification of ACOXs

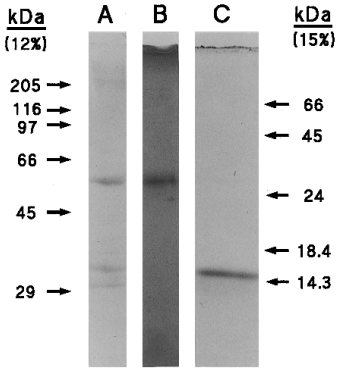

Figure 3 SDS/PAGE analysis of SCOX and MCOX after purification

Lane A, Coomassie Blue-stained SDS/PAGE gel (12%) containing 5 μg of protein from the pooled DEAE-Sepharose fractions showing MCOX activity. Lane B, silver-stained SDS/PAGE gel (12%) containing approx. 0.5 μg of protein from the pooled and concentrated gel-filtration fractions (38–50) showing MCOX activity. Lane C, SDS/PAGE gel (15%) containing 3.5 μg of protein from the pooled and concentrated gel-filtration fractions (58–66) containing SCOX activity. The arrows at the left and right denote the migration positions of the molecular mass markers for the 12% and 15% gels respectively.

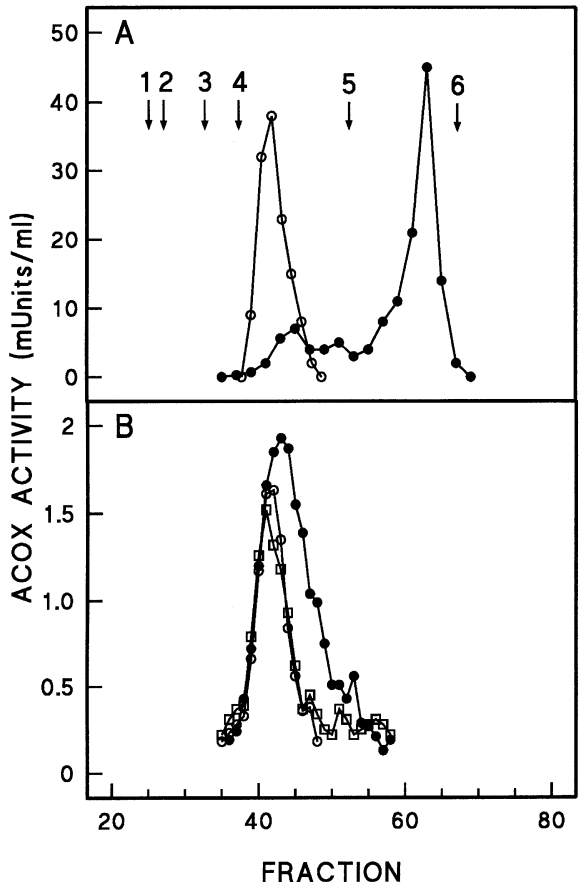

Figure 4 Gel-filtration chromatography of ACOX preparations on Ultrogel AcA-34

Symbols: ●, SCOX; ○, MCOX; □, LCOX. Symbols were omitted for fractions not exhibiting ACOX activity. Arrows represent the elution positions of the following molecular mass markers: 1, apoferritin (443 kDa); 2, β-amylase (220 kDa); 3, yeast alcohol dehydrogenase (150 kDa); 4, BSA (66 kDa); 5, carbonic anhydrase (29 kDa); 6, cytochrome *c* (12.5 kDa). (**A**) Final purification of MCOX and SCOX. Separate columns were run for each enzyme. For MCOX, fractions 60–80 from the DEAE-Sepharose column were combined and the protein was precipitated with 80% ammonium sulphate. After resuspension in 2 ml of buffer C containing 10 μM FAD, the sample was loaded on to the gel-filtration column. For SCOX, the fractions corresponding to non-absorbed protein from the DEAE-Sepharose column were pooled and the protein was precipitated with 60% ammonium sulphate. A 2 ml sample containing approx. 3.5 units of SCOX activity was loaded on to the column. (**B**) A homogenate of 2 g of frozen dark-grown maize plantlets was fractionated with ammonium sulphate between 30 and 50% saturation, and 2 ml of the resuspended pellet (approx. 125 m-units of ACOX activity determined with C_6-CoA) was loaded on to the column. ACOX activities were determined at 50 μM acyl-CoA. Baseline activity for the homogenate without substrate was 0.2 m-unit/ml.

[6,17,28], was found to result in aggregation of SCOX, as shown by gel-filtration chromatography, and loss of activity. The absence of β-mercaptoethanol and polyvinylpolypyrrolidone during homogenization also resulted in increased SCOX aggregation and loss of activity. Anion-exchange chromatography, routinely employed in the purification of the ACOXs from mammals [17] and yeast [23], was necessary in order to separate completely SCOX or MCOX from contaminating amounts of the other ACOXs.

Stepwise elution of protein from the phenyl-Sepharose column with increasing concentrations of ethylene glycol produced MCOX and SCOX essentially free from LCOX (Figure 1A). The 35% ethylene glycol eluate was used for the subsequent purification of both MCOX and SCOX; these were separated using

Table 1 Purification of MCOX and SCOX

MCOX and SCOX were independently purified from two different samples of dark-grown maize seedlings.

	Activity (nmol/min)		Protein (mg)		Specific activity (nmol/min per mg of protein)		Purification (fold)		Yield (%)	
Step	MCOX	SCOX	MCOX	SCOX	MCOX	SCOX	MCOX	SCOX	MCOX	SCOX
16000 ***g*** supernatant	22200	33200	4800	3500	4.6	9.5	–	–	100	100
$(NH_4)_2SO_4$ fractionation	24800	36900	3200	1500	7.8	25	1.7	2.6	111	112
Phenyl-Sepharose	10900	22400	158	167	69	135	15	14	49	67
Hydroxyapatite	10900	13600	76	39	143	350	31	37	49	41
DEAE-Sepharose	2500	4200	1.5	2.6	1700	1600	370	168	11	8
$(NH_4)_2SO_4$ precipitation and Ultrogel AcA-34	2100	600	0.4	1.6	5300	375	1200	39	9	2

hydroxyapatite-HT, to which SCOX remained bound but MCOX did not (Figure 1B). Both MCOX and SCOX were further purified by ion-exchange chromatography on DEAE-Sepharose (Figure 2). The retention of mammalian ACOXs is dependent on the presence of FAD [17]. In contrast, whether or not maize MCOX or SCOX were retained by the column was independent of FAD, but dependent on the bed volume. The optimal purification was obtained using approx. 3 ml bed volume per 100 mg of total protein, which resulted in the elution of SCOX (Figure 2A) and the retention of MCOX (Figure 2B). After DEAE-Sepharose chromatography, the MCOX preparation contained one predominant protein (Figure 3, lane A), while the SCOX preparation contained seven bands of approximately equal intensity, including one of 15 kDa (results not shown). MCOX was subsequently purified to homogeneity by gel filtration on Ultrogel AcA-34 (Figure 4A), as determined by SDS/PAGE (Figure 3, lane B). Gel filtration of the SCOX preparation from the DEAE-Sepharose column consistently resulted in dissociation, giving only a homogeneous subunit of 15 kDa (compare Figures 4A, 4B and Figure 3, lane C). This also resulted in corresponding drastic decreases in specific activity and greater than 80 % loss of the activity applied to the column. In contrast, MCOX total activity decreased by no more than 20 % when gel-filtered in the presence of FAD.

The best results obtained with this purification procedure yielded 2.1 units of MCOX, with a specific activity of 5.3 units/mg of protein (total recovery = 9 %; Table 1). This value was approx. 5- and 4-fold lower than those of the cucumber glyoxysomal LCOX [6] and *Candida tropicalis* ACOX [23] respectively, determined with their substrates exhibiting maximal activity. We cannot exclude the possibility that partial inactivation of MCOX during purification contributed to its comparatively lower specific activity. The best results in purifying SCOX yielded 4.2 units of enzyme after the DEAE-Sepharose column (total recovery = 8 %; Table 1). Its specific activity of 1.6 units/mg of protein was an underestimate, due to the presence of other proteins in the preparation.

MCOX and SCOX were confirmed to be true ACOXs by independent analyses of hydrogen peroxide and *trans*-2-enoyl-CoA formation. The acyl-CoA-dependent formation of hydrogen peroxide was evident from our routine use of the peroxidase-coupled reaction during the purification and characterization of the enzymes. *trans*-2-Enoyl-CoA production was monitored by the increase in absorbance at 260 nm due to the introduction of the double bond in the 2-position of the fatty acid moiety, by the coupled reaction with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, and directly by HPLC analysis of the reaction products. Neither enzyme exhibited acyl-CoA dehydrogenase activity.

Table 2 Biochemical properties of MCOX and SCOX

MCOX and SCOX K_m values were determined with C_{12}-CoA and C_6-CoA respectively as substrate.

Parameter	MCOX	SCOX
Native molecular mass (kDa)	62	60
Subunit molecular mass (kDa)	60	15
Isoelectric point	6.5	7.3*
K_m (μM)	2	6
pH optimum	8.6	8.3–8.6
FAD-dependent activity	Yes	Yes
Active with free fatty acids	No	No
Subcellular location	Peroxisomes	Peroxisomes

* Apparent subunit pI.

Characterization of MCOX

Native MCOX was shown to be a 62 kDa monomeric protein, since gel filtration (Figure 4A) and SDS/PAGE (Figure 3, lane B) produced a nearly identical molecular mass. Gel-filtration chromatography of an ammonium sulphate-fractionated homogenate confirmed the native molecular mass to be approx. 62 kDa (Figure 4B). Chromatofocusing of a sample of MCOX from the DEAE-Sepharose column yielded one peak of activity with an apparent pI of 6.5. The elimination of FAD by double gel filtration of pure MCOX resulted in elimination of nearly 90 % of its activity. The re-addition of FAD restored activity, the level of which depended on the concentration of FAD added. The optimum pH for MCOX activity was approx. 8.6, and there was a 2-fold decrease in activity between the optimum and pH 9.3. Activity on the acidic side of the pH optimum showed a 1.5-fold difference between pH 7.2 and 8.6. Density-gradient centrifugation of organellar extracts indicated MCOX to be localized to peroxisomes, due to its co-migration with catalase, the peroxisomal marker enzyme. The above results are summarized in Table 2.

Characterization of SCOX

The homogeneous preparation of SCOX was found to possess a native and subunit molecular mass of 15 kDa, as shown by both

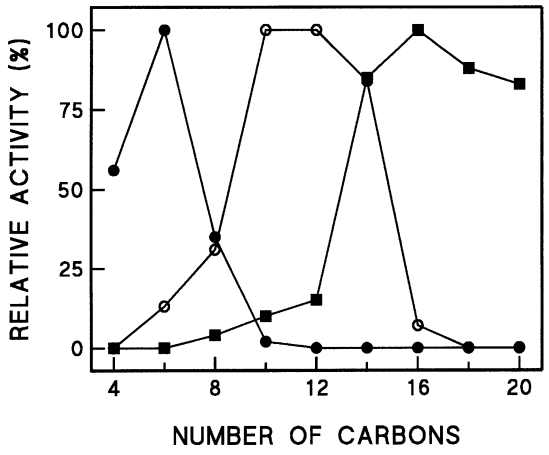

Figure 5 Substrate specificity profiles of SCOX, MCOX and LCOX

Symbols: ●, SCOX; ○, MCOX; ■, LCOX. LCOX fractions free of MCOX activity were obtained by column chromatography of the 60% ethylene glycol eluate from the phenyl-Sepharose column on DEAE-Sepharose. All activities were determined using 50 μM acyl-CoA. The highest activity for each enzyme was arbitrarily set at 100.

gel filtration (Figure 4A) and SDS/PAGE (Figure 3, lane C). In contrast, gel filtration of an ammonium sulphate-fractionated homogenate, under conditions most likely to keep quaternary structures intact, resulted in elution of SCOX with an apparent molecular mass of 60 kDa (Figure 4B), thus suggesting that the native enzyme is homotetrameric. The isoelectric point of SCOX was determined by chromatofocusing of an aliquot of the enzyme from the DEAE-Sepharose column. SCOX activity was eluted in two peaks, the first comprising 20% of SCOX activity with a pI above 8, while the other 80% exhibited a pI of 7.2. The multiple peaks of activity represented different aggregate forms of SCOX, since the preparation used was free from both MCOX and LCOX activities. SCOX exhibited FAD-dependent activity, in that elimination of loosely bound FAD by double gel filtration on Sephadex G-15 resulted in an approx. 70% decrease in activity that could be restored upon re-addition of FAD; the restoration of activity was concentration-dependent. An analysis of the pH-dependence of SCOX activity showed its optimum to be between pH 8.3 and 8.6, with a 3-fold increase in activity moving from pH 7.2 to 8.3 and a 2-fold decrease in activity moving from pH 8.3 and 9.3. SCOX migrated with MCOX and catalase on density gradients, thereby indicating a peroxisomal location. The above results are summarized in Table 2.

Substrate specificities of SCOX, MCOX and LCOX

MCOX and SCOX were distinguished from LCOX by their preferences for medium- and short-chain fatty acyl-CoAs respectively (Figure 5). MCOX showed nearly equal activity with C_{10}-, C_{12}- and C_{14}-CoAs, and sharp decreases in activity with longer or shorter chain lengths. SCOX was active only with acyl-CoAs of length C_8 and shorter, and maximally active with C_6. The apparent K_m values of MCOX for C_{12}-CoA and SCOX for C_6-CoA were 2 μM and 6 μM respectively. Neither enzyme was active with the free fatty acid. LCOX exhibited a substrate specificity (Figure 5) similar to that of the LCOX of cucumber cotyledons (compare with Figure 7 in [6]), showing reactivity primarily with fatty acyl-CoAs longer than C_{14}. Comparable activity, which similarly decreased with increasing degree of unsaturation, was observed with long-chain unsaturated fatty acyl-CoAs.

DISCUSSION

These results demonstrate the existence in higher plants of two previously unknown ACOXs, one specific for medium-chain fatty acyl-CoAs and the other specific for short-chain fatty acyl-CoAs. Their purification and the subsequent characterization of their catalytic properties confirmed them to be true acyl-CoA oxidases distinct from the long-chain fatty acyl-CoA-specific enzyme. It is generally accepted that β-oxidation is ubiquitous in plant peroxisomes [29]. The subsequent proposal of the ubiquitous presence of an LCOX [6] was reasonable in that this enzyme represents a starting point for the degradation of long-chain acyl-CoAs. Correspondingly, maize contains an LCOX, which was identified by partial purification and determination of its substrate specificity. The available evidence on peroxisomal β-oxidation and ACOX activities in various tissues from different plant species suggests that MCOX and SCOX should also be ubiquitous in all higher-plant peroxisomes. The substrate specificity profile of ACOX activity in extracts or peroxisomes isolated from various tissues of dark-grown maize plantlets [14] is generally similar to that determined in peroxisomes from mung bean hypocotyls and spinach leaves, although those from spinach leaves show slightly less SCOX activity [7]. The lack of accumulation of medium- or short-chain fatty acids or acyl-CoAs in glyoxysomes from cucumber cotyledons fed with long-chain fatty acids [13] indicates that the magnitude of the activities of both SCOX and MCOX may be comparable with that of LCOX, even in this fatty tissue.

Comparative functional and structural characteristics of ACOXs in maize and other organisms

The substrate specificities of both MCOX and LCOX are similar to those of ACOXs previousy characterized. The substrate specificity and apparent K_m values of MCOX for C_{10}-, C_{12}- and C_{14}-CoAs are equivalent to those of ACOXs described for yeasts [23,30], but the maize enzyme shows sharper decreases in activity between C_{10}- and C_8-CoA and C_{14}- and C_{16}-CoA. The maize LCOX possesses a substrate specificity profile similar to that of the cucumber glyoxysomal enzyme [6], but shows greater activity with C_{18}-CoA and C_{20}-CoA relative to C_{16}-CoA. The combined specificities of MCOX and LCOX are roughly equivalent to the broad substrate specificity of the mammalian palmitoyl-CoA oxidase [22,31–33]. SCOX possesses a substrate specificity that is unique among eukaryotic ACOXs, but which is similar to those of the prokaryotic SCOX [34] and the mammalian mitochondrial short-chain acyl-CoA dehydrogenase [35].

The loose binding of FAD by maize MCOX and SCOX was similar to that observed by Inestrosa et al. [31] for rat palmitoyl-CoA oxidase, but contrasted with the result of Osumi et al. [28], who showed that FAD could not be removed from the rat liver enzyme by even long-term dialysis. The inability of added FAD to stimulate gel-filtered *Candida utilis* ACOX led Stokes and Stumpf [36] to conclude that this enzyme binds FAD tightly. Rat pristanoyl-CoA oxidase also binds FAD tightly [37]. The partial removal of FAD from maize SCOX might have reflected the labile quaternary structure of the enzyme (see below), where the FAD affinity may depend on the complexation state of the enzyme.

The major differences between the maize ACOXs and other known ACOXs involve their quaternary structures, with both MCOX and LCOX being monomeric and SCOX multimeric. However, diverse quaternary structures seem to be a general

property of ACOXs. LCOX from cucumber cotyledons is a homodimer of molecular mass 150 kDa [6]. The ACOXs from *Candida maltosa* and *Candida tropicalis* [5,23,38,39] and rat pristanoyl-CoA oxidase [37] are octameric species of molecular masses 513–640 kDa. The latter is similar to maize SCOX in that it also has a labile quaternary structure [37]. Mammalian palmitoyl-CoA oxidase is a heterotrimer of molecular mass 140 kDa [28], whereas the human liver branched-chain acyl-CoA oxidase is a 70 kDa monomeric enzyme [2]. Kirsch et al. [6] obtained evidence for a monomeric ACOX form in the cytosol of cucumber cotyledons by immunoprecipitation, but no activity was detected. They ascribed this monomeric form to a cytosolic precursor of the glyoxysomal LCOX. Maize LCOX is probably monomeric, since its native molecular mass, between 62 and 65 kDa (Figure 4B), is approximately equal to that of the individual subunits of other ACOXs, virtually all of which have subunit molecular masses of at least 70 kDa. Besides the 51 kDa and 23 kDa subunits of mammalian palmitoyl-CoA oxidase (cleavage products of an approx. 70 kDa precursor) [1], the major exception is maize SCOX, which has a subunit with a molecular mass of approx. 15 kDa.

The 60 kDa form of SCOX appears to be the active form *in vivo*, as either further aggregation or dissociation reduces activity. Its labile quaternary structure is indicated by its heterogeneous hydrophobic nature and by its dissociation during chromatofocusing or gel filtration. The question may still remain as to whether the 15 kDa subunit could function as an acyl-CoA oxidase; however, several lines of evidence indicate the purified subunit to be the active subunit. (i) The activity profile shown in Figure 4(A) shows that the 15 kDa subunit is required for SCOX activity. Furthermore, the pooled fractions from the Ultrogel AcA-34 gel-filtration column have SCOX activity showing substrate specificity identical with that of SCOX in fractions from the previous two purification steps. (ii) No protein impurities are visible in the pooled fraction containing both the SCOX activity and the 15 kDa protein. (iii) It is not likely that the SCOX subunit is a proteolytic artefact resulting from purification, since this proteolysis would have had to occur during the last steps of purification. Gel-filtration chromatography analysis of the crude homogenate, resuspended ammonium sulphate precipitate or phenol-Sepharose eluate did not show any SCOX activity eluting at 15 kDa. Furthermore, SCOX-containing fractions from the DEAE-Sepharose did not show significant decreases in activity upon storage for several days at 4 °C. (iv) Examples exist where small proteins possess properties similar to those that would be required for acyl-CoA oxidase activity. NADH oxidase, which can bind two dinucleotide cofactors concurrently, has a molecular mass of 25 kDa [40], and acyl-CoA binding proteins, which can bind even acyl-CoAs of chain length greater than 16 carbons, have molecular masses of approx. 10 kDa [41]. Therefore the evidence indicates that the 15 kDa subunit is the basic subunit and shows greatly enhanced activity when in the tetrameric state.

The unique substrate specificity of SCOX raises interesting questions as to its evolutionary origins in plants in relation to its physiological role. An enzyme with such a specificity may be essential for the efficient and complete β-oxidation of fatty acids as a source of metabolic fuel [42] or to remove potentially toxic short-chain fatty acids [43]. The hypothesis stems from the existence of a short-chain acyl-CoA dehydrogenase in mammalian mitochondrial β-oxidation, which is known to be instrumental in completely catabolizing fatty acids [42], and whose expression, along with that of medium-chain acyl-CoA dehydrogenase and long-chain acyl-CoA dehydrogenase, is controlled by a number of developmental, nutritional and hormonal factors [44]. A direct response to nutritional factors has also been shown for general acyl-CoA dehydrogenase activity in maize [27]. Such a hypothesis for SCOX in plants is supported by our evidence demonstrating differential expression of SCOX, relative to the other ACOXs, that depends on tissue metabolic activity [14].

ACOXs in the regulation of peroxisomal β-oxidation flux

It is likely that plants require varied levels of flux through β-oxidation at different stages of development to regulate the levels of either its substrates or products, given their possible effects on other aspects of plant metabolism. Different tissues require different levels of energy and carbon output for metabolism, supplied by a number of catabolic pathways, thus varying the demand for fatty acid-derived acetyl-CoA as a source of energy and carbon. Acetyl-CoA is also a precursor of acetate, which has been shown to repress the expression of certain photosynthetic genes, and may function in part as a switch in the heterotrophic/autotrophic transition to ensure the complete use of stored fatty acids before a plant becomes photosynthetic [45]. Short-chain fatty acids have been shown to have a wide variety of physiological effects (see [46] and references therein). Fatty acylation of proteins [47] and enzymes [48] has dynamic effects on their function, where the relative abundance of the different acyl-CoAs may dictate the type of fatty acid attached [49]. ACOXs can be directly implicated in a plant's response to pathogen attack by producing H_2O_2, in relation either to systemic acquired resistance [50] or lignin synthesis [51]. β-Oxidation has been directly implicated in the formation of the plant hormone jasmonic acid [52], which has been shown to have numerous physiological effects (for a review, see [53]). Therefore, knowledge of the mechanisms involved in regulating fluxes through peroxisomal β-oxidation is essential for a more comprehensive understanding of the involvement of β-oxidation in other fundamental plant processes.

Previous studies with animal and plant systems have implicated ACOX as the primary step controlling the flux through this pathway by showing either that the substrate specificity of β-oxidation of isolated rat liver peroxisomes mimics the substrate specificity of ACOX activity [18,54], or that purified yeast ACOX alone, when added to extracts of rat [22] or plant tissues [55], results in increased production of acetyl-CoA from long-chain acyl-CoAs. Recent studies using recombinant hepatoma cell lines that differentially express very-long-chain ACOX have shown this enzyme to be essential in controlling the rate of β-oxidation of the corresponding class of fatty acids [56]. If the above results are applicable to all the ACOX enzymes in plants, two levels of flux control are possible. One represents a global control, whereby the co-ordinate induction or repression of the three forms would increase or decrease the total flux, while maintaining the relationships between the concentrations of the ACOXs and acyl-CoAs. The other represents a selective control, whereby the differential expression of one or more ACOX form(s) will dictate the preferential use or formation of a particular class of fatty acyl-CoA. Subsequently, the flux of ACOX products into the next steps of β-oxidation will depend on the relative abundance of ACOX forms and available acyl-CoAs. A number of other factors could then be involved in the regulation of flux at the level of ACOX activity, such as substrate competition or end-product inhibition.

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