Acyl-CoA Oxidase from *Candida tropicalis*†

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ABSTRACT: Acyl coenzyme A oxidase (acyl-CoA oxidase) has been isolated in good yield from *Candida tropicalis* pK 233 grown on *n*-alkanes. Gel filtration, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and measurement of flavin content suggest that the oxidase is an octamer of M_r 75 000 subunits each containing one flavin. The oxidase yields the red semiquinone form on dithionite or photochemical reduction, slowly forms an N-5 adduct with 0.16 M sulfite at pH 7.4, and is rapidly reduced by borohydride, forming the 3,4-dihydroflavin isomer. The red flavosemiquinone is only kinetically stabilized with respect to disproportionation in the free enzyme but is thermodynamically stabilized on binding enoyl-CoA derivatives. The enzyme is reduced by butyryl-, octanoyl-, and palmitoyl-CoA without formation of prominent long-wavelength bands. Acyl-CoA oxidase and the acyl-CoA dehydrogenases share many similarities in their interaction with CoA derivatives. For example, both enzymes stabilize the anionic radical on binding enoyl-CoA derivatives, both dehydrogenate 2-oxoheptadecyldethio-CoA but cannot utilize *S*-heptadecyl-CoA, both form long-wavelength bands with CoA persulfide species, and both enzymes are attacked by the suicide substrates 3,4-pentadienoyl-CoA and (methylenecyclopropyl)acetyl-CoA at the flavin prosthetic group.

The α–β unsaturation of acyl coenzyme A (acyl-CoA)[1] substrates of peroxisomal β-oxidation is catalyzed by the flavoprotein acyl-CoA oxidase (Stokes & Stumpf, 1976; Lazarow, 1978; Shimizu et al., 1979; Osumi et al., 1980; Inestrosa et al., 1980; Coudron & Frerman, 1982), whose mitochondrial counterparts are the acyl-CoA dehydrogenases (Beinert, 1963). These two classes of enzymes share the same reductive half-reaction: the removal of *pro*-2*R* and *pro*-3*R* hydrogens from their acyl thio ester substrates with reduction of bound flavin (Kawaguchi et al., 1980; Biellmann & Hirth, 1970a,b; Bucker et al., 1970):

$$E\cdot Fl_{ox} + RCH_2CH_2CO\text{–}SCoA \rightleftharpoons E\cdot Fl_{red} + RCH{=}CHCO\text{–}SCoA$$

but differ in their physiological electron acceptor. In mitochondria, the reduced dehydrogenase is reoxidized by a second flavoprotein, electron-transferring flavoprotein (ETF; Crane & Beinert, 1956; Hall & Kamin, 1975), which donates reducing equivalents to the electron-transport system at the level of a third component, ETF dehydrogenase (Ruzicka & Beinert, 1977). In contrast, the peroxisomal oxidase is reoxidized by molecular oxygen directly with the formation of hydrogen peroxide (Osumi & Hashimoto, 1978). Acyl-CoA oxidase activity has been demonstrated from a variety of sources, and the enzyme has been purified to homogeneity from alkane-utilizing yeast (Shimizu et al., 1979; Coudron & Frerman, 1982) and rat liver (Osumi et al., 1980; Inestrosa et al., 1980).

This paper reports an improved purification procedure for acyl-CoA oxidase from *Candida tropicalis* and a more detailed study of the properties of the flavin prosthetic group in this enzyme. Comparison of the properties of the oxidase with the corresponding acyl-CoA dehydrogenase may eventually lead to a clearer understanding of the molecular features which dictate the reactivity of bound dihydroflavins toward molecular oxygen. Second, in view of their oxidation of a common acyl-CoA substrate, it is of interest to examine to what extent the flavin environment of the oxidase resembles that of the acyl-CoA dehydrogenases. Lederer (1978) has suggested that lactate oxidase and flavocytochrome b_2 have similar environments in the C-4–N-5 region of the flavin prosthetic group because of marked similarities in behavior toward their common substrate, L-lactate, and toward the inhibitors D-lactate, oxalate, sulfite, and 2-hydroxy-3-butynoic acid.

Experimental Procedures

Materials

Candida tropicalis pK 233 was obtained from the American Type Culture Collection. CoA thio esters were obtained from P-L Biochemicals. Heptadecyl-SCoA and 2-oxoheptadecyldethio-CoA were gifts of Dr. Theodor Wieland and were synthesized as described previously (Ciardelli et al., 1981). Octenoyl-CoA was synthesized by the mixed anhydride method (Bernert & Sprecher, 1977) and was a gift of Robert Stern, University of Delaware. Horse radish peroxidase, *n*-decane, -dodecane, -tetradecane, and -hexadecane, glucose oxidase, catalase, apoferritin, β-galactosidase, Sepharose CL-4B, and sodium dodecyl sulfate molecular weight markers were obtained from Sigma Chemical Co. DE-52 was supplied by Whatman. Native molecular weight standards were from Bio-Rad. Pig kidney electron-transferring flavoprotein (Gorelick et al., 1982) was a gift from Robert Gorelick, University of Delaware.

Methods

General. Unless otherwise stated, all buffers contained 0.3 mM EDTA. Concentrations of acyl-CoA oxidase are expressed with respect to enzyme flavin by using an extinction coefficient of 14.2 mM^{-1} cm^{-1}, determined after release of the flavin with 4 M guanidine hydrochloride (Thorpe et al., 1979). Spectrophotometric experiments were conducted by using either Cary 219, Perkin-Elmer 552A, or Beckman DB instruments.

Enzyme Assays. The oxidase was assayed at 30 °C essentially as described previously (Allain et al., 1974; Kawamoto et al., 1978) in 0.8 mL of solution containing 50 mM phosphate, pH 7.4, 30 μM palmitoyl-CoA, 0.83 mM 4-amino-

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[1] Abbreviations: CoA, coenzyme A; ETF, electron-transferring flavoprotein; $NaDodSO_4$, sodium dodecyl sulfate; MCPA-CoA, (methylenecyclopropyl)acetyl-CoA; Fl, flavin; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide.

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antipyrine, 10.6 mM phenol, and 12 IU of peroxidase. Inclusion of FAD (Kawamoto et al., 1978) did not increase the rate of the assay.

Anaerobic Experiments. Anaerobic spectrophotometric experiments were conducted as described earlier (Mizzer & Thorpe, 1981). Enzyme solutions contained 80 nM glucose oxidase, 3.5 mM glucose, and 18 nM catalase to remove dissolved oxygen. In addition, a side arm contained a fluted piece of filter paper soaked with 0.2 mL of a 1 M glucose solution containing 40 units of glucose oxidase. Photoreductions were performed by using 12 μM oxidase in 0.75 mL of 50 mM phosphate buffer, pH 7.4, and 4 mM EDTA containing 3 μM 3,10-dimethyl-5-deazaisoalloxazine. The mixture was deoxygenated and illuminated at 25 °C, 5 cm from a 100-W tungsten bulb. Full reduction required 4 min, with maximal yield of red semiquinone after about 90 s of illumination.

The effect of 109 μM crotonyl-CoA or 39 μM heptadecyl-SCoA on the disproportionation equilibrium was evaluated as follows. The oxidase was partially photoreduced as described above such that approximately equimolar amounts of oxidized and fully reduced enzyme were obtained after disproportionation (see Results and Discussion). The CoA derivative was then added anaerobically to the mixture from a gas-tight syringe and the reappearance of red semiquinone followed spectrophotometrically.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) was performed by Robert Gorelick of this laboratory by applying 20 μg of oxidase or 10 μg of molecular weight markers to 5% gels. Proteins were stained with Coomassie Brilliant Blue R-250.

Gel Filtration. Estimation of the apparent molecular weight was performed by using a 1.5 × 60 cm Sepharose 6B column equilibrated with 100 mM phosphate buffer, pH 7.6, and run at 8 mL/h. Molecular weight standards were thyroglobulin, β-galactosidase, apoferritin, γ-globulin, bovine serum albumin, ovalbumin, myoglobin, and vitamin B_{12}.

Amino Acid Analysis. Amino acid analyses were run by Linda Parente, University of Delaware, using a Beckman 119C analyzer. Samples were treated at 110 °C with constant-boiling HCl (Moore & Stein, 1960).

Cultivation of Yeast. The growth medium was based on that described earlier (Kawamoto et al., 1978) and contained 5 g of $NH_4H_2PO_4$, 2.5 g of KH_2PO_4, 1.0 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $FeCl_3 \cdot 6H_2O$, 1 g of yeast extract, 0.5 g of Tween 85, and 10 g of a mixture of *n*-alkanes (10/40/30/20 decane/dodecane/tetradecane/hexadecane) per L, pH 5.4. A 4-L batch of medium was inoculated from a starter culture and grown in a 10-L vessel with vigorous aeration at 29 °C for 40 h. Typically, 15 g wet weight of cells is obtained per L, and the cells could be stored at −20 °C for several months without noticeable loss of enzyme activity. *Candida tropicalis* was maintained on nutrient agar slants.

Purification of Acyl-CoA Oxidase. Unless otherwise stated, all procedures were conducted at 4 °C. Ammonium sulfate saturation percentages refer to 25 °C. A suspension of cells (110 g wet weight in 370 mL of 10 mM phosphate buffer, pH 7.6, containing 1 mM EDTA, and 0.01% w/v phenylmethanesulfonyl fluoride) was sonicated by using a Bronwill Biosonik for 4 h, maintaining the solution lower than 10 °C. The cell debris was removed by centrifugation at 19000*g* for 30 min, and the supernatant was taken to 25% ammonium sulfate. After centrifugation, the oxidase was precipitated by the addition of 60% ammonium sulfate and redissolved in 90 mL of 10 mM phosphate buffer, pH 7.4. The enzyme was dialyzed overnight against 2 × 3 L of the same buffer and applied to a DE-52 column (4.5 × 24 cm) equilibrated with this buffer. The column was washed at 60 mL/h with 10 mM phosphate buffer, pH 7.4, containing 80 mM KCl until no further proteins emerged, and the oxidase was eluted by increasing the KCl concentration to 0.25 M. The active fractions were concentrated by using 50% ammonium sulfate, and the precipitate was redissolved in 20 mM phosphate buffer, pH 7.4, and dialyzed overnight against 4 L of the same buffer. The yellow solution (25 mL) was then applied to a Cibacron Blue–Sepharose column [1.8 × 13 cm; synthesized from free dye and Sepharose CL-4B by the procedure of Atkinson et al. (1981)] at a flow rate of 25 mL/h. The column was washed with 400 mL of 20 mM phosphate buffer until no further protein was eluted, and the oxidase eluted at 25 mL/h with a linear gradient formed by mixing 100 mL of 50 mM phosphate buffer, pH 7.4, with an equal volume of this buffer containing 1.5 M KCl. The oxidase was concentrated by ammonium sulfate precipitation, dialyzed vs. 100 mM potassium phosphate buffer, pH 7.4, and stored at −20 °C. The enzyme retains 80% and 100% of its activity on storage for 2 months in 100 mM potassium phosphate buffer, pH 7.4, at 4 and −20 °C, respectively.

Table I: Purification of Acyl-CoA Oxidase from *Candida tropicalis* pK 233

step	total protein (mg)	total units	sp act.	yield (%)
(1) cell-free extract from 110 g of yeast	3475	1936	0.56	100
(2) 25–60% ammonium sulfate ppt	827	1658	2	85
(3) DE-52 column	146	1507	10.3	78
(4) Cibacron Blue–Sepharose column	69	1321	19.1	68

Results and Discussion

Physical Properties of the Oxidase. Table I shows the purification outline for acyl-CoA oxidase from *Candida tropicalis*. The method differs most significantly from that described previously by the substitution of a Cibacron Blue–Sepharose chromatography step for the gel filtration column used by Shimizu et al. (1979). The specific activity of the enzyme obtained after this step (19.1 units/mg; Table I) is in excellent agreement with that reported by Shimizu et al. (1979) and corresponds to a turnover number of 1700 min^{-1} per flavin. Table I also indicates that the oxidase constitutes about 2.8% of the total soluble protein in *Candida tropicalis* grown on alkanes. The pure enzyme exhibits a single band on $NaDodSO_4$–polyacrylamide gel electrophoresis (M_r 75000; see Methods) in agreement with the results of Shimizu et al. (1979). Coudron & Frerman (1982) report that their preparations show several bands on $NaDodSO_4$–polyacrylamide gel electrophoresis with M_r values ranging from 76000 to 35000. The reason for this difference in behavior is not clear. The visible spectrum of the oxidase (not shown) shows an unresolved flavin chromophore very similar in general appearance to that of the general acyl-CoA dehydrogenase both in the position and extinction coefficient of the main absorbance peak at 446 nm and in the ratio of the two peak absorbances in the visible region (Table II). Freshly prepared oxidase exhibits a low trough absorbance at 310 nm although this increases on storage with the accumulation of small amounts of light-scattering material and the release of low

Table II: Summary of Properties of Acyl-CoA Oxidase and Pig Kidney General Acyl-CoA Dehydrogenase[a]

	Candida tropicalis acyl-CoA oxidase			pig kidney general acyl-CoA dehydrogenase
	Shimizu et al. (1979)	Coudron & Frerman (1982)	this work	Thorpe et al. (1979)
absorbance ratios	9.0/0.95/1.0 at 280/365/445	9.1/0.72/1.0 at 280/364/445	9.1/0.67/1.0 at 280/365/446	5.3/0.65/1.0 at 280/373/447
extinction coefficient (mM^{-1} cm^{-1})	11.3[b]	15.2	14.2	15.4
min M_r/FAD	70 000[b]	139 000	89 000	48 000
subunit M_r	74 000	76 000[c]	75 000	42 000
native M_r	600 000	550 000	600 000	160 000

[a] Taken from the indicated references. [b] Estimated by using an assumed extinction coefficient; see the text. [c] Smaller M_r fragments detected on $NaDodSO_4$-polyacrylamide gel electrophoresis.

Table III: Amino Acid Composition of Acyl-CoA Oxidase from *Candida tropicalis*[a]

amino acid	residues/ mol of FAD	residues/ M_r 75 000
aspartic acid	82	69
threonine	44	37
serine	45	38
glutamic acid	79	67
proline	35	30
glycine	57	48
alanine	63	53
half-cystine[b]	13	11
valine	53	45
methionine	18	15
isoleucine	45	38
leucine	68	57
tyrosine	30	25
phenylalanine	36	30
lysine	56	47
histidine	16	13
arginine	41	35
tryptophan[c]	12	10

[a] These values are the mean of duplicate analyses of samples after 24-, 48-, and 72-h hydrolyses (Moore & Stein, 1960). [b] Obtained after hydrolysis in the presence of dimethyl sulfoxide (Spencer & Wold, 1969). [c] Determined spectrophotometrically (Edelhoch, 1967) after exhaustive dialysis vs. 6 M guanidine hydrochloride.

levels of free FAD. The 280-nm/446-nm absorbance ratio of 9.1 for the oxidase and 5.3 for the dehydrogenase (Thorpe et al., 1979) reflects the increased content of tryptophan in the oxidase (12 vs. 4 per FAD, see later). In general, flavoprotein oxidases have an appreciably higher tryptophan content than flavoprotein dehydrogenases (Williams, 1976).

Gel filtration of the native oxidase on a calibrated gel filtration column (see Methods) suggests an apparent molecular weight of about 600 000, in agreement with data presented by Shimizu et al. (1979). Acyl-CoA oxidase is thus probably an octamer of about M_r 75 000 subunits.

The amino acid analysis of the yeast oxidase is shown in Table III. It is rather similar to that of the rat liver peroxisomal enzyme (Osumi et al., 1980) except that the mammalian enzyme is reported to have only 0.6 mol of half-cystine and five tryptophan residues per M_r 71 900 subunit. The composition in Table III corresponds to a minimum molecular weight per flavin of 89 000, somewhat higher than the subunit M_r of 75 000 determined by $NaDodSO_4$-polyacrylamide gel electrophoresis. This minimum M_r corresponds to an average of 0.84 FAD per subunit, or about 7 flavins per octamer. When allowance is made for the measured extinction coefficient for bound flavin (Table II), the data of Shimizu et al. (1979) indicate a very similar FAD content. Coudron & Frerman (1982) report a lower flavin content, corresponding to about four flavins per octamer.

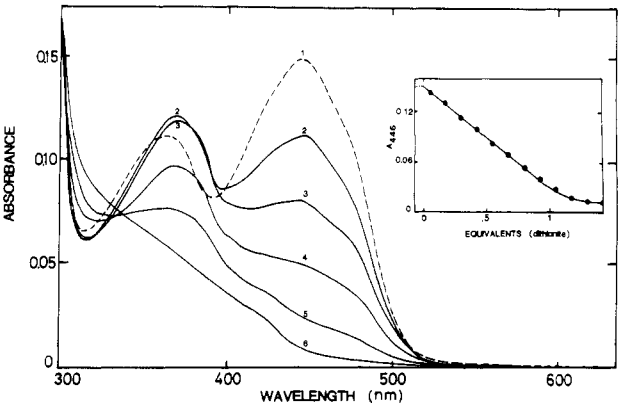

FIGURE 1: Dithionite titration of acyl-CoA oxidase. The enzyme (10.6 μM bound flavin in 0.85 mL of 100 mM phosphate buffer, pH 7.4, containing 80 nM glucose oxidase, 3.5 mM glucose, and 20 nM catalase) was deoxygenated and titrated with sodium dithionite at 25 °C (see Methods). Curves 1–6: 0, 2.7, 5.5, 8.2, 11.0, and 15.0 μM dithionite, respectively. Intermediate points have been omitted for clarity.

Reduction of Enzyme Flavin. A dithionite titration of acyl-CoA oxidase is shown in Figure 1. Full reduction requires the addition of 1.1 mol of dithionite per mol of FAD, suggesting that flavin is the only redox active group in this enzyme. During the first half of the titration, significant levels of the red anion radical were formed (see later), as evident by an immediate rise in absorbance at 370 nm after each addition of reductant (e.g., see curve 2). These absorbance changes were unstable, however, and declined while the spectrum was being scanned. This effect probably reflects disproportionation of the flavosemiquinone (see later) since the decrease at 370 nm is accompanied by a correspondingly smaller increase at 450 nm (data not shown). On completion of the titration (curve 6, Figure 1), the enzyme was partially reoxidized by the addition of a small volume of oxygen-saturated buffer, with the reappearance of 4.5 μM oxidized enzyme. This solution was maintained anaerobically for 2 days without the detectable formation of the characteristic red radical spectrum (Massey & Palmer, 1966; results not shown: see later). Thus, under these conditions, the red radical is not thermodynamically stable with respect to oxidized and fully reduced oxidase:

$$E\cdot Fl_{ox} + E\cdot Fl_{red} \rightleftharpoons 2E\cdot Fl_{sq}$$

Photoreduction using deazaflavin as catalyst (Massey & Hemmerich, 1978) similarly yields unstable mixtures of oxidized, semiquinone, and fully reduced forms at intermediate illumination times (data not shown); full reduction requires about 4 min under the conditions employed (see Methods). At approximately half reduction, disproportionation of the semiquinone component generated in these mixtures requires

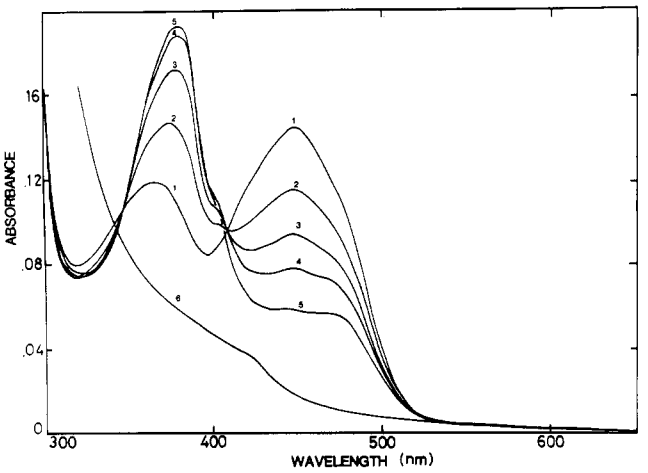

FIGURE 2: Dithionite titration of acyl-CoA oxidase in the presence of heptadecyl-SCoA. The oxidase (10 μM in 0.87 mL of 100 mM phosphate, pH 7.4, containing 48 μM heptadecyl-SCoA, 79 nM glucose oxidase, 3.4 mM glucose, and 20 nM catalase) was deoxygenated and titrated with dithionite at 25 °C. Curves 1–5: 0, 2.7, 4.0, 5.4, and 8.1 μM dithionite, respectively. Curve 6 was recorded after the addition of excess reductant.

about 90 min for completion. These results suggest that assignment of a pK for the radical based on the yield on photoreduction (Coudron & Frerman, 1982) may not be valid, since the system is not at equilibrium. The photoreduced enzyme is completely reoxidized by 2 equiv of ferricyanide, although this reaction is very sluggish, requiring several hours between additions toward the end of the back-titration. Spectra recorded during this reoxidation do not reveal the significant accumulation of radical.

In contrast to the relatively low yields of radical obtained on dithionite or photochemical reduction, the anionic radical can be trapped on reduction of the oxidase in the presence of heptadecyl-SCoA (Figure 2). This long-chain thio ether analogue is a potent competitive inhibitor of pig kidney general acyl-CoA dehydrogenase, inducing a marked resolution of the flavin chromophore without reduction of the enzyme (Thorpe et al., 1981). The spectral effects of this ligand on the oxidase are much less pronounced, and as expected from experiments with the dehydrogenase (Thorpe et al., 1981), no reduction of the enzyme occurs under anaerobic conditions (Figure 2, curve 1). Dithionite titration of the enzyme–thio ether complex generates the red semiquinone almost stoichiometrically (apparent ϵ_{380} = 19 mM^{-1} cm^{-1}) as judged by the preservation of the isosbestic point at 408 nm during the first phase of the titration. This result largely reflects a kinetic stabilization of the radical against further reduction by dithionite (see later). The rather slow disproportionation reaction encountered with the oxidase in all these reductive titrations indicates that there is probably no effective redox communication between the flavins in this oligomeric enzyme.

Treatment of acyl-CoA oxidase with sodium borohydride effects rapid reduction of the enzyme to the 3,4-dihydroflavin form (Müller et al., 1969) with maxima at 374, 391, and 415 nm (Figure 3). Similar spectral changes have been observed with L- and D-amino acid oxidases (Massey et al., 1968) and *Megasphaera elsdenii* butyryl-CoA dehydrogenase (Engel & Massey, 1971a) but not with general acyl-CoA dehydrogenase (Thorpe et al., 1979) and a variety of other flavoenzymes (Massey et al., 1968). Further additions of borohydride lead to the gradual accumulation of the tetrahydroflavin species (Müller et al., 1969), as indicated by bleaching of the absorbance at 390 nm and by the loss of the isosbestic point at 411 nm (Figure 3). In agreement with results obtained by

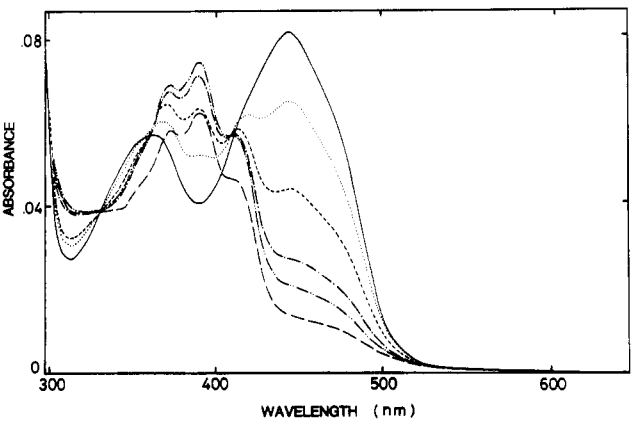

FIGURE 3: Reduction of acyl-CoA oxidase from *Candida tropicalis* by using sodium borohydride. The enzyme (5.6 μM in 0.8 mL of 100 mM phosphate buffer, pH 7.4) was titrated anaerobically with a solution containing 7.5 mg of sodium borohydride/mL at 25 °C. Spectra represent the addition of 0 (—), 1 (···), 2 (- - -), 4 (-·-), 5 (-··-), and 6 (– –) μL of reductant. Spectra were recorded after bubbling had ceased.

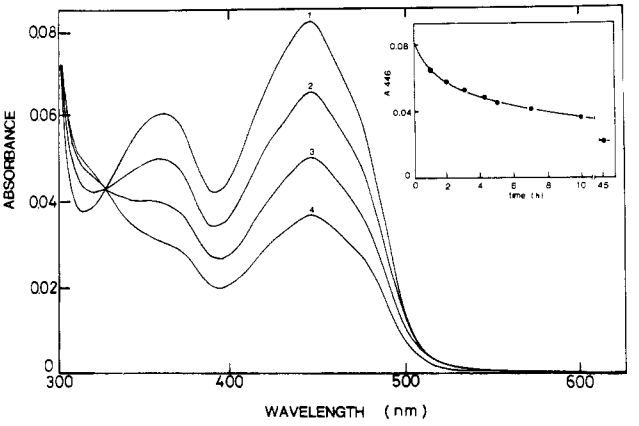

FIGURE 4: Effect of sulfite on acyl-CoA oxidase. Spectra of the enzyme (5.7 μM in 0.87 mL of 100 mM phosphate buffer containing 160 mM sodium sulfite adjusted to pH 7.4) were recorded at 25 °C after 0, 1, 3, and 10 h (curves 1–4, respectively). Intermediate points have been omitted for clarity (see inset).

using other flavoenzymes, the 3,4-dihydroflavin species is stable in air, and no significant reoxidation occurs over 12 h at 25 °C. Dilution of the 3,4-dihydroflavin species into a standard assay (see Methods) gives an acceleration in rate from about 15% initially to 49% native activity after 2 min. In contrast, enzyme which is overreduced to the extent shown in Figure 3 shows a more pronounced lag phase, exhibiting very low activity initially with a maximal activity of 40% attained after about 7 min. The aerobic addition of 66 μM palmitoyl-CoA to a sample of the 3,4-dihydroflavin oxidase leads to the partial reappearance (about 50%) of the native oxidized enzyme spectrum. Further additions of substrate do not effect additional reoxidation of the chromophore. Substrate-induced reoxidation of the 3,4-dihydroflavin form of L-amino acid oxidase has been described previously (Massey et al., 1968), and a detailed study of these effects with acyl-CoA oxidase has not been undertaken.

In common with many flavoprotein oxidases (Massey et al., 1969) and in contrast to the acyl-CoA dehydrogenases (Massey et al., 1969; Thorpe et al., 1979), acyl-CoA oxidase forms an N-5 adduct on incubation with sulfite with extensive bleaching of the 450-nm absorbance (Figure 4). This reaction is unusually slow: the changes shown are half-complete after about 5 h at 25 °C with 0.16 M sulfite and remain incomplete after a 40-h incubation (Figure 4, see inset). This sluggishness

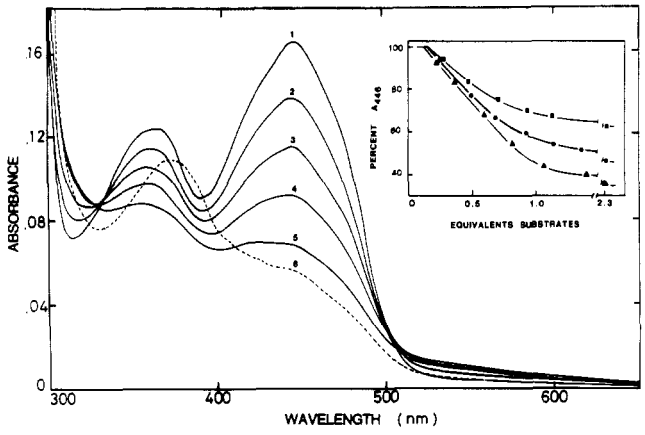

FIGURE 5: Titration of acyl-CoA oxidase with palmitoyl-, octanoyl-, and butyryl-CoA. The oxidase (11.6 μM in 0.85 mL of 100 mM phosphate buffer, pH 7.4, containing 80 nM glucose oxidase, 3.5 mM glucose, and 20 nM catalase) was titrated anaerobically with palmitoyl-CoA at 25 °C. Curves 1–5: 0, 4.2, 7.1, 9.9 and 12.7 μM palmitoyl-CoA, respectively. After the addition of 2.3 equiv of palmitoyl-CoA (▲; see inset), the solution was maintained at 25 °C overnight, yielding curve 6.

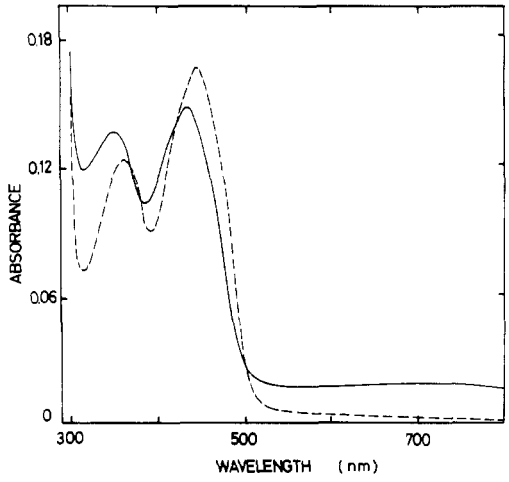

FIGURE 6: Effect of coenzyme A persulfide species on acyl-CoA oxidase. The oxidase (11.6 μM in 0.8 mL of 100 mM phosphate buffer, pH 7.4, 25 °C) before (- - -) and after (—) the addition of 0.1 mL of a solution containing 8.3 mM CoASH and 12.5 mM sodium sulfide preincubated for 1 h on ice.

precluded determination of the dissociation constant for sulfite binding and might also explain the failure of Coudron & Frerman (1982) to observe a sulfite adduct with this enzyme. The spectral changes shown in Figure 4 are not significantly reversed on dialysis for 20 h against 100 mM phosphate, pH 7.4, at 4 °C. However, a slight recovery of the oxidized flavin spectrum (about 5%) is observed after storage of the dialyzed enzyme for 1 week at 4 °C under aerobic conditions. Prior to storage, the dialyzed enzyme exhibited 33% of native activity, and, as expected from the above results, no significant reactivation occurred during the assay measurement.

Interaction of Acyl-CoA Oxidase with Thio Ester Substrates. Figure 5 shows an anaerobic titration of acyl-CoA oxidase with palmitoyl-CoA. Bleaching of the 450-nm absorbance is accompanied by the formation of a long-wavelength band of low intensity extending to about 650 nm. Similar spectral changes are observed with the rat liver peroxisomal enzyme, although the long-wavelength absorbance is more intense with the mammalian enzyme (Osumi et al., 1980). The spectral changes are substantially completed on the addition of 1 equiv of substrate (see inset). Reduction of the oxidase with butyryl- and octanoyl-CoA (see inset, Figure 5) yields similar bleaching of the flavin chromophore, but without the appearance of significant long-wavelength absorbance (spectra not shown). Reduction with all three substrates leads to the slow accumulation of significant levels of the red anion radical after prolonged anaerobic incubation. For example, curve 6 in Figure 5 is after 12 h in the presence of 27 μM palmitoyl-CoA. Considerably greater yields of semiquinone (apparent ϵ_{378} = 13.9 mM^{-1} cm^{-1} after 15 h) can be obtained if the oxidase is first reduced by 220 μM palmitoyl-CoA and then half is reoxidized by the addition of oxygen-saturated buffer to provide a greater concentration of oxidized flavin component for the comproportionation reaction:

$$E{\cdot}Fl_{ox} + E{\cdot}Fl_{red} \rightleftharpoons 2E{\cdot}Fl_{sq}$$

Thus, although this equilibrium lies to the left with the free enzyme, such that the semiquinone form is not evident in equilibrium mixtures of partially reduced enzyme as described above, the radical is thermodynamically stabilized on the addition of an appropriate ligand. A direct demonstration of the preferential binding of enoyl-CoA to the red semiquinone form of the oxidase was obtained by adding 109 μM crotonyl-CoA to an approximately equimolar solution of oxidized and dihydroflavin forms (see Methods). Spectra recorded over 2 days at 25 °C, before increasing turbidity interfered with measurements, demonstrate a slow comproportionation reaction leading to the appearance of sizable levels of red radical (apparent ϵ_{378} = 11.4 mM^{-1} cm^{-1}). In contrast to the marked stabilization afforded by enoyl-CoA derivatives, the long-chain thio ether analogue heptadecyl-SCoA was much less effective, yielding a maximal apparent ϵ_{378} of 8.8 mM^{-1} cm^{-1} (see Methods). However, as noted earlier, this analogue is able to effect a striking kinetic stabilization of the radical during dithionite titrations (Figure 2). As expected from its physiological role, acyl-CoA oxidase reduced with palmitoyl-CoA is unable to transfer reducing equivalents to mammalian ETF under anaerobic conditions (using 7 nM oxidase, 44 μM palmitoyl-CoA, and 13 μM ETF at pH 7.4). In addition, the oxidase shows no detectable activity in a standard assay system for the acyl-CoA dehydrogenase (Thorpe, 1981) in which phenazine methosulfate mediates the transfer of reducing equivalents to 2,6-dichlorophenolindophenol under aerobic conditions. Acyl-CoA oxidase from *Candida utilis* is similarly unable to utilize these dyes as acceptors (Stokes & Stumpf, 1976).

Effect of Other CoA Derivatives. Recently, the ligand responsible for the green color of butyryl-CoA dehydrogenase has been identified as a coenzyme A persulfide (Williamson et al., 1982a,b). The addition of mixtures of CoA- and S^0-containing species to pig kidney general acyl-CoA dehydrogenase also led to the appearance of a long-wavelength band centered at 710 nm, and experiments using flavin analogues (Williamson et al., 1982a) and resonance Raman spectroscopy (Williamson et al., 1982b) suggested that this new band be ascribed to a charge-transfer complex between oxidized flavin as the acceptor and bound persulfide as the donor. Surprisingly, acyl-CoA oxidase also reacts with $CoA/S^{2-}/S^0$ mixtures, generating a 710-nm band (Figure 6). Prolonged incubation of the oxidase with this mixture leads to the subsequent reduction of the enzyme flavin, probably due to the relatively high concentration of sulfide species in solution. General acyl-CoA dehydrogenase is also reduced by sodium sulfide (Thorpe & Massey, 1983). In contrast to the green form of butyryl-CoA dehydrogenase, in which the persulfide ligand is bound tenaciously (Steyn-Parvé & Beinert, 1958; Engel & Massey, 1971a,b), the equivalent complex with

Table IV: Comparison of Properties of Acyl-CoA Oxidase with Mammalian General Acyl-CoA Dehydrogenase

property	oxidase	dehydrogenase
flavin prosthetic group	FAD	FAD
subunit M_r	75 000	42 000
native M_r	600 000	160 000
visible spectrum	unresolved	unresolved
fluorescence	not significant	not significant
semiquinone type	red (kinetically stabilized); enoyl-CoA thermodynamically stabilizes this form	blue (thermodynamically stabilized); red radical stabilized by substrates or products
borohydride reduction	rapid 3,4-dihydroflavin formation	very slow reduction to 1,5-dihydroflavin species
reactivity with sulfite	N-5 adduct formed slowly	no significant reaction
reduction by substrates of optimal chain length	weak long-wavelength bands	more extensive reduction, pronounced charge-transfer bands
stereochemistry of substrate oxidation	removal of *pro*-2,3*R* hydrogens	removal of *pro*-2,3*R* hydrogens
inhibition by suicide substrates:		
3-octynoyl-CoA	yes	yes
3,4-pentadienoyl-CoA	rapid, irreversible	rapid, reversible
MCPA-CoA	slow	rapid
complex with CoA persulfide	yes, λ_{max} = 710 nm	yes, λ_{max} = 710 nm
reactivity of substrate-reduced enzyme with O_2	rapid	extremely slow

acyl-CoA oxidase (Figure 6) is completely dissociated on gel filtration on Sephadex G-25 at 4 °C. This difference is in keeping with the differing chain length specificities of the two enzymes.

Osumi et al. (1980) have reported that the mammalian acyl-CoA oxidase forms complexes with 3-keto-hexadecanoyl-CoA, with the appearance of a long-wavelength band (λ_{max} about 600 nm). The spectral changes resemble those observed with the acyl-CoA dehydrogenases on the addition of acetoacetyl-CoA (λ_{max} = 550–580 nm, depending on the source; Engel & Massey, 1971a,b; McKean et al., 1979; Thorpe & Massey, 1983) and probably reflect the formation of a charge-transfer complex between oxidized flavin as the acceptor and the enolate form of the 3-keto derivative as the donor. The yeast oxidase similarly yields a charge-transfer band centered at about 560 nm (apparent ϵ_{560} = 1.3 mM^{-1} cm^{-1}) on the addition of 120 μM acetoacetyl-CoA to 5.6 μM oxidase in 50 mM phosphate, pH 7.4.

The long-chain analogue 2-oxoheptadecyldethioCoA, in which the sulfur atom of CoA is replaced by a methylene group, has been recently found to be oxidized by pig kidney general acyl-CoA dehydrogenase at about 60% of the rate exhibited by palmitoyl-CoA in the standard dehydrogenase assay (Thorpe et al., 1981). This ketone analogue is also a substrate for the oxidase, showing 67% of the rate in the standard assay (see Methods).

Comparison of Acyl-CoA Oxidase with Acyl-CoA Dehydrogenase. Table IV presents a summary of some of the salient properties of the yeast oxidase and the mammalian general acyl-CoA dehydrogenase, the best understood representative of the acyl-CoA dehydrogenases. Many aspects of the interaction of these two enzymes with CoA derivatives are similar. For example, both catalyze the dehydrogenation of thio ester substrates with the same stereochemistry, both thermodynamically stabilize the red radical in the presence of enoyl-CoA, and both enzymes can be greened on the addition of CoA persulfide species. The thio ether analogue heptadecyl-SCoA is not dehydrogenated by either enzyme, whereas the long-chain dethio keto analogue is a good substrate for both the oxidase and the dehydrogenase. A further important similarity is that all of the suicide substrates recently found to inhibit the dehydrogenase are also effective against the oxidase. Thus, 3-alkynoyl thio ester derivatives irreversibly inhibit the dehydrogenase by covalent modification of the protein (Frerman et al., 1980; Gomes et al., 1981; Fendrich & Abeles, 1982), and 3-octynoyl-CoA has been recently reported to irreversibly inhibit the yeast oxidase (Coudron & Frerman, 1982). 3,4-Pentadienoyl-CoA rapidly inhibits general acyl-CoA dehydrogenase, forming an unstable adduct with the flavin prosthetic group (Wenz et al., 1982). The oxidase is also rapidly inactivated by this allenic thio ester, but in this case, dissociation of the resulting adduct has only been accomplished on denaturation of the enzyme (Z. Jiang, C. Thorpe, A. Wenz, and S. Ghisla, unpublished results). Lastly, (methylenecyclopropyl)acetyl-CoA, a metabolite of hypoglycin isolated from the Jamaican ackee fruit, is a suicide substrate for the acyl-CoA dehydrogenases (Wenz et al., 1981) and also irreversibly inactivates the yeast oxidase with similar bleaching of the flavin chromophore (Z. Jiang, C. Thorpe, A. Wenz, and S. Ghisla, unpublished results). These combined observations suggest that the oxidase, like the acyl-CoA dehydrogenases, probably initiates substrate dehydrogenation by abstraction of an α-proton by an active-site base residue. In view of these similarities, it is interesting to note that the oxidase, as expected from its classification (Massey & Hemmerich, 1980), forms the red semiquinone on one-electron reduction and yields N-5 adducts on sulfite treatment in clear contrast to the acyl-CoA dehydrogenase (Table IV). A recent resonance Raman spectroscopic study suggests significant differences in the H-bonding interactions between flavin and apoprotein in the yeast oxidase and liver general acyl-CoA dehydrogenase (Schmidt et al., 1983).

Once formed, the reduced enzyme–enoyl-CoA complexes of the oxidase do not exhibit the prominent long-wavelength charge-transfer bands characteristic of the acyl-CoA dehydrogenases (Beinert, 1963; Thorpe et al., 1979). In the case of the mammalian dehydrogenases, reduction with substrates, particularly those of optimal chain length, confers considerable protection against reoxidation of bound dihydroflavin by molecular oxygen (Beinert, 1963). Two observations illustrate that this protection is not simply a consequence of charge-transfer complex formation between reduced flavin and enoyl-CoA derivatives. First, butyryl-CoA-reduced general acyl-CoA dehydrogenase is only slowly reoxidized by oxygen, although this species does not exhibit significant long-wavelength absorbance (Thorpe et al., 1979). Second, the reduced enzyme–crotonyl-CoA complexes of both *Megasphaera elsdenii* and pig liver butyryl-CoA dehydrogenases exhibit charge-transfer bands, but whereas the mammalian complex is of exceptional stability (Steyn-Parvé & Beinert, 1958), the

bacterial complex is rapidly reoxidized by oxygen (Engel & Massey, 1971a). Since *Megasphaera elsdenii* is an obligate anaerobe, protection of the reducing equivalents from attack by molecular oxygen would not be useful under normal growth conditions (Engel, 1978, 1980).

This paper has presented an initial comparison of the yeast acyl-CoA oxidase with a mammalian general acyl-CoA dehydrogenase. The recent availability of preparations of butyryl-CoA dehydrogenase free of bound CoA derivatives (Williamson & Engel, 1982) should provide further insight into the reactivity of these enzymes with molecular oxygen.

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Registry No. MCPA-CoA, 56898-43-2; 3,4-pentadienoyl-CoA, 84061-72-3; 3-octynoyl-CoA, 75696-31-0; 2-oxoheptadecyldethio-CoA, 78939-48-7; butyryl-CoA, 2140-48-9; octanoyl-CoA, 1264-52-4; palmitoyl-CoA, 1763-10-6; sulfite, 14265-45-3; sodium borohydride, 16940-66-2; dithionite, 14844-07-6; acyl-CoA oxidase, 61116-22-1; CoA persulfide, 81918-99-2.

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