Structure–Function Correlation of Fatty Acyl-CoA Dehydrogenase and Fatty Acyl-CoA Oxidase†

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ABSTRACT: We have employed a new pseudosubstrate, β-(2-furyl)propionyl coenzyme A (FPCoA), to study the functional properties of two enzymes, fatty acyl-CoA dehydrogenase from porcine liver and fatty acyl-CoA oxidase from *Candida tropicalis*, involved in the oxidation of fatty acids. Previous studies from our laboratory have shown that the dehydrogenase exhibits oxidase activity at the rate of dissociation of the product charge-transfer complex. This raises the question of the difference in functionality between these two flavoproteins. To investigate these differences, we have compared the pH dependence of product formation, the isotope effects using tetradeuterio-FPCoA, and the spectral properties and chemical reactivity of the product charge-transfer complexes formed with the two enzymes. The pH dependencies of the reaction of FPCoA with electron-transfer flavoprotein (ETF) for the dehydrogenase and of the reaction of FPCoA with O_2 for the oxidase are quite similar. Both reactions proceed more rapidly at basic pH values while substrate binds more tightly at acidic pH values. These data for both enzymes are consistent with a mechanism in which enzyme is involved in protonation of the carbonyl group of substrate followed by base-catalyzed removal of the C-2 proton from substrate. The C-2 anion of substrate may then serve as the active species in reduction of enzyme-bound flavin. The deuterium isotope effects for both enzyme systems are primary across the entire pH range, assuring that the chemically important step of substrate oxidation is rate limiting in these steady-state kinetic experiments. The two enzymes differ in the chemical reactivity of their product charge-transfer complexes. The complex formed during reaction of the dehydrogenase and FPCoA is unstable with a dissociation rate constant of 0.01 s^{-1} and reacts with ETF but not with O_2. The complex formed from the reaction of oxidase with FPCoA is stable, showing no dissociation; it reacts rapidly with O_2 but does not react with ETF. The functional difference of these two flavin adenine dinucleotide containing flavoproteins resides in the difference in chemical reactivity of the product charge-transfer complexes.

We have been investigating the mechanism of the reactions of a new pseudosubstrate, β-(2-furyl)propionyl coenzyme A (FPCoA), with fatty acyl-CoA dehydrogenase from porcine liver (McFarland et al., 1982a). This substrate produces a chromophoric product, *trans*-β-(2-furyl)acryloyl-CoA (FACoA), which absorbs at 340 nm, a wavelength at which flavin species are isosbestic. Fatty acyl-CoA oxidase from *Candida tropicalis* also reacts with FPCoA to produce the same oxidized product, FACoA. Since both enzymes react with the same substrate but by different mechanisms, this system permits the functional differences between these two related enzymes to be characterized in a unique way.

Fatty acyl-CoA dehydrogenase was first characterized by Green and Beinert (Crane et al., 1956); three related enzymes with different specificity were shown to react with the protein substrate electron-transfer flavoprotein (ETF) (Crane & Beinert, 1956). The reaction has been shown to generate the red anionic semiquinone of ETF as the kinetically important product (Auer & Frerman, 1980; Hall et al., 1979; Reinsch et al., 1980b). The steady-state kinetic experiments designed to establish the order of substrate binding to protein are consistent with an ordered binding mechanism in which ETF binds only to the binary complex between enzyme and fatty acyl-CoA ester and in which reaction takes place within this ternary complex (McKean et al., 1979). Furthermore, studies of isotope effects and pH dependencies of the reactions of butyryl-CoA are consistent with a mechanism in which fatty acyl-CoA substrate is protonated by an acid catalyst on the enzyme, facilitating the base-catalyzed removal of the C-2 proton by a base catalyst on the enzyme (Schmidt et al., 1981; Reinsch et al., 1983). Finally, studies on the reaction of FPCoA with the dehydrogenase have established that the enzyme catalyzes two unexpected reactions of substrate at the rate of dissociation of the product charge-transfer complex, namely, the oxidase and transhydrogenase reactions (McFarland et al., 1982b). The demonstration of oxidase activity in the dehydrogenase raises the interesting question of the fundamental difference between these two enzymes. Since the oxidase is a clearing enzyme for high levels of saturated fatty acid while the dehydrogenase is part of a system to store energy derived from fatty acid oxidation, a comparison of the two enzymes is of metabolic interest as well. Accordingly, we report the pH dependence, isotope effects, and properties of the charge-transfer product complexes for the reaction of FPCoA with fatty acyl-CoA dehydrogenase and fatty acyl-CoA oxidase. In addition, we report the correlation of these functional properties with earlier structural work (resonance Raman spectroscopy) on these two proteins (Schmidt et al., 1983).

Experimental Procedures

Enzymes and Reagents. Fatty acyl-CoA oxidase, fatty acyl-CoA dehydrogenase, and ETF purifications have been described previously (McKean et al., 1979; Coudron & Frerman, 1982).

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Reagents obtained commercially were used without further purification. 2,2-Bifuroyl (furil), deuterated malonic acid, and 4-aminoantipyrine were purchased from Aldrich Chemical. Octanoyl-CoA was purchased from P-L Biochemicals, and peroxidase (type VI) was purchased from Sigma. Sulfonated 2,4-dichlorophenol was prepared as described previously (Barham & Trinder, 1972). FPCoA was synthesized as described previously (Reinsch et al., 1980a).

Preparation of Tetradeuterated FPCoA. The preparation of tetradeuterated FPCoA involved a series of reactions. The first reaction was the formation of deuterated 2-furaldehyde from 2,2-bifuroyl (25 mmol) and D_2O (6.5 mL) in the presence of dioxane (15.5 mL) and KCN (2.0 g). A side product of the reaction was the anion of 2-furoic acid which was separated from deuterated 2-furaldehyde by precipitation of the acid with concentrated H_2SO_4 (3.8 mL) in ice (35 g). A second reaction was carried out between deuterated 2-furaldehyde (25 mmol) and deuterated malonic acid (26 mmol) in the presence of pyridine (4.4 mL) and piperidine (0.053 mL). The reaction mixture was heated on a steam bath for 2.5 h and then refluxed gently for 30 min and vigorously for 15 min. The product of this reaction (deuterated FA acid) was precipitated with a 9:1 mixture of H_2O/H_2SO_4 (2.5 mL). The third reaction was the reduction (with deuterium) of deuterated FA acid. Deuterated FA acid (1 g) reacted with D_2O (5.3 mL) in the presence of Na_2O (0.23 g) and NaHg (21.4 g) to form tetradeuterated FP acid. A chemical ionization mass spectrograph showed the expected parent ion of tetradeuterated FP acid. At this point, deuterated FP acid was esterified with coenzyme as described previously (Reinsch et al., 1980a).

Peroxide Analysis. FPCoA reacted with dissolved oxygen from the atmosphere in the presence of fatty acyl-CoA oxidase (6.4×10^{-8} M) to form equimolar amounts of FACoA and peroxide. Formation of FACoA was determined from the absorbance reading at 340 nm by using a Cary 16 spectrometer. Peroxide was determined from the amount of colored coupling product formed from the reaction of sulfonated 2,4-dichlorophenol (3.4×10^{-3} M), 4-aminoantipyrine (4.5×10^{-5} M), and the peroxide formed in the oxidation of FPCoA. This reaction was catalyzed by peroxidase (5 μL of 1 mg/mL buffer solution). Formation of the coupling product was determined spectrophotometrically from the change of absorbance at 515 nm [$\Delta\epsilon$ = 22 000 L/(mol·cm)] (Barham & Trinder, 1972). These reactions were carried out at pH 6.0 and 8.0; imidazole (20 mM) and tris(hydroxymethyl)aminomethane (Tris) (20 mM) buffers were used, respectively.

pH Dependence. (A) Steady-State Kinetic Experiments with Acyl-CoA Oxidase. The rate of oxidation of FPCoA to FACoA was followed at 340 nm with a Cary 16 spectrometer. The concentration of FPCoA substrate was varied from 0.7 to 7.2 μM. The concentration of fatty acyl-CoA oxidase was approximately 10^{-9} M, being slightly higher at lower pH values so that measurable kinetic traces could be obtained. In any event, oxidase concentrations were always sufficiently low to assure steady-state conditions. Acetate (pH 5.0), imidazole (pH 6.0 and 7.0), and Tris buffers (pH 8.0, 8.2, and 9.0) were used at a constant ionic strength (μ = 0.020 M, T = 25 °C) to assure constant electrostatic interactions between enzyme and substrate. Imidazole was employed instead of phosphate in the oxidase pH experiment because phosphate was a steady-state enzyme inhibitor.

(B) Steady-State Kinetic Experiment with Acyl-CoA Dehydrogenase. All experiments were carried out on a Durrum stopped-flow spectrometer. Enzyme was combined with ETF and mixed in the stopped-flow spectrometer with substrate (FPCoA). Typical concentrations were [enzyme] = 0.25 μM, [ETF] = 2.5 μM, and [substrate] = $^1/_5K_m - 2K_m$. The enzyme was maintained at a sufficiently high concentration to permit observation of kinetics without significant reoxidation of reduced ETF. Phosphate was employed as a buffer at pH 6–7 and Tris at pH 7–9. Ionic strength was maintained at μ = 20 mM (T = 25 °C).

Kinetics of the Reductive Half-Reaction. FPCoA and fatty acyl-CoA dehydrogenase were introduced into the driving syringes of a Durrum stopped-flow spectrometer. The solutions were mixed, and the absorbance changes at 340 nm (double-bond formation) were measured. This reaction consists of two single experimental kinetic processes which can be fit to the expression $\Delta A(t) = \Delta A_1 e^{-k_1 t} + \Delta A_2 e^{-k_2 t}$ [for details on the kinetic treatment, see Reinsch et al. (1980a)]. The dissociation rate for the charge-transfer complex was measured at 640 nm; this rate process was a single exponential and was calculated from the equation $\Delta A(t) = \Delta A e^{-kt}$. Approximate concentrations used were [enzyme] = 6 μM and [FPCoA] = 30 μM. Phosphate was employed as a buffer at pH 6.7 and Tris at pH 9.0 (T = 25 °C).

Anaerobic Experiments. The reactions of FPCoA and octanoyl-CoA with fatty acyl-CoA oxidase were carried out in an anaerobic cell. This cell consisted of a regular quartz, 10-mm path length, 1-mL spectrophotometric cell to which a side arm and a side pouch were attached. The side pouch had a large surface area to facilitate degassing and eliminate the need to stir the enzyme solution. The substrate was placed into the side arm and the enzyme into the side pouch during the degassing of the cell. Vacuum was applied several times with nitrogen purging after each application. Substrate and enzyme were sufficiently far apart so that there was no danger of mixing while degassing. The enzyme and substrate solutions were always within the same closed system so that there was no need to open the system to add the substrate after degassing. Once the system was degassed and purged with nitrogen, spectra of the enzyme were obtained before and after mixing with substrate.

Finally when the cell was opened to air, disappearance of the charge-transfer (CT) complex of FPCoA [λ_{max} = 600 nm, ϵ = 1000 L/(mol·cm)], reoxidation of enzyme [λ_{max} = 450 nm, ϵ = 15 200 L/(mol·cm)], and formation of a carbon–carbon double bond [λ_{max} = 340 nm, ϵ = 22 964 L/(mol·cm)] were monitored at approximately 3-min intervals. Typical concentrations used were the following: acyl-CoA oxidase, approximately 12 μM; substrate (octanoyl-CoA or FPCoA), at least 3 or 4 times the concentration of enzyme (to ensure complete saturation of enzyme with substrate).

Results

We have studied the reaction of ETF with FPCoA catalyzed by fatty acyl-CoA dehydrogenase. In these investigations, we have employed stopped-flow kinetic techniques in order to avoid the reaction of reduced ETF with oxygen; i.e., at the short times of the stopped-flow time scale, there was no observed oxidation of reduced ETF. Figure 1 shows the pH dependence of the turnover number and the Michaelis constant for the dehydrogenase-catalyzed reaction under steady-state conditions. These data were collected from observations based upon the double bonds produced during reaction and are very similar to the pH data collected from observing the reduction of ETF with butyryl-CoA (Schmidt et al., 1981). Both sets of data show a pH dependence in which the turnover number increases with increasing pH while the pH dependence of the apparent binding constant indicates that binding is tighter at acidic pH values. Independent studies using crotonyl-CoA as

Table I: Isotope Effects upon the Steady-State Oxidation of FPCoA Catalyzed by Dehydrogenase and Oxidase

enzyme	pH	FPCoA (H)		FPCoA (D)		
		K_M (μM)	V_{max}/E_0 (s^{-1})	K_M (μM)	V_{max}/E_0 (s^{-1})	$V_{max}(H)/V_{max}(D)$
dehydrogenase	6.7	1.0 ± 0.5	0.93 ± 0.09	1.0 ± 0.5	0.25 ± 0.005	3.7
	8.5	7.0 ± 1.5	20.5 ± 1.5	6.7 ± 2.0	3.3 ± 0.2	6.2
oxidase	6.0	3.4 ± 0.6	0.97 ± 0.11	3.3 ± 0.5	0.33 ± 0.02	2.9
	8.9	23.5 ± 1.4	10.7 ± 2.2	19.6 ± 4.4	3.97 ± 0.39	2.7

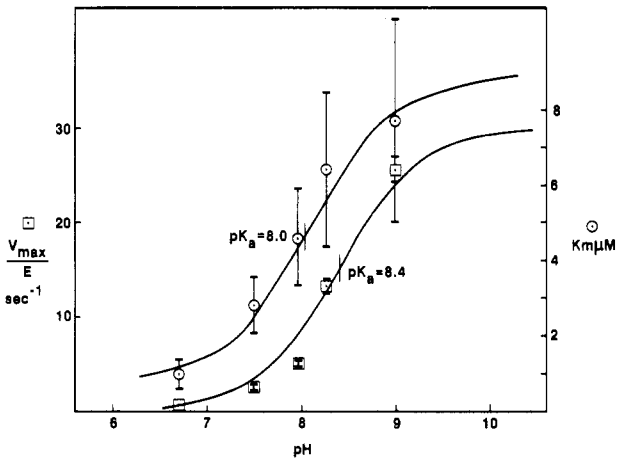


FIGURE 1: pH dependence of steady-state parameters for the reaction of FPCoA with ETF (2.5 μM) catalyzed by fatty acyl-CoA dehydrogenase (0.25 μM). The reactions were carried out at 25 °C in 20 mM ionic strength solutions. Error bars represent standard deviations; pK values are "best fits" from linear regression analysis.

an inhibitor indicate that the K_I also shows similar pH behavior to that of K_m for substrates (Reinsch et al., 1983). The primary difference between the pH dependence of the reaction of butyryl-CoA and FPCoA is that the best-fit pK_a values for the present data with FPCoA are 8.0 for K_m and 8.4 for the turnover number. The values for butyryl-CoA are pK_a = 7.5 for the pH dependence of both K_m and the turnover number (Schmidt et al., 1981; Reinsch et al., 1983). In order to interpret the pH dependence data in terms of a chemical mechanism, it is necessary to show that the chemical step of hydride transfer from C-3 or proton transfer from C-2 is rate limiting throughout the pH range. Table I shows the isotope effect for the reduction of ETF at two pH values using tetradeuterated FPCoA as substrate. Notice that there are primary isotope effects throughout the pH range. We have previously suggested that catalysis proceeds through C-2 ionization followed by "hydride transfer" (Schmidt et al., 1981); the isotope effect we measure is the result of removal of the C-2 and C-3 protons. There exist mechanistic possibilities under which our measured isotope effects might not represent a mechanism involving rate-determining hydrogen transfer from C-3. This is the result of our measurement of isotope effects on the tetradeuterated FPCoA rather than on the specifically C-2 and C-3 deuterated substrates. For example, if the movement of the C-2 and C-3 hydrogens were concerted, then our observed isotope effects would be equal to the product of those two isotope effects as well as the smaller secondary isotope effects. Carbon–acid proton exchange in the D-amino acid oxidase reaction is associated with an isotope effect k_H/k_D = 1.9 (Walsh et al., 1973), near the maximum value observed in other carbon ionization reactions (Cram et al., 1961). If we were to remove this isotope effect from our observed values, we would predict C-3 isotope effects of at least 1.5–3.0. Although the latter is clearly primary, the former is not. At present, it is possible to conclude only that the transfer of hydrogen from C-2 and C-3 in the fatty acyl-CoA

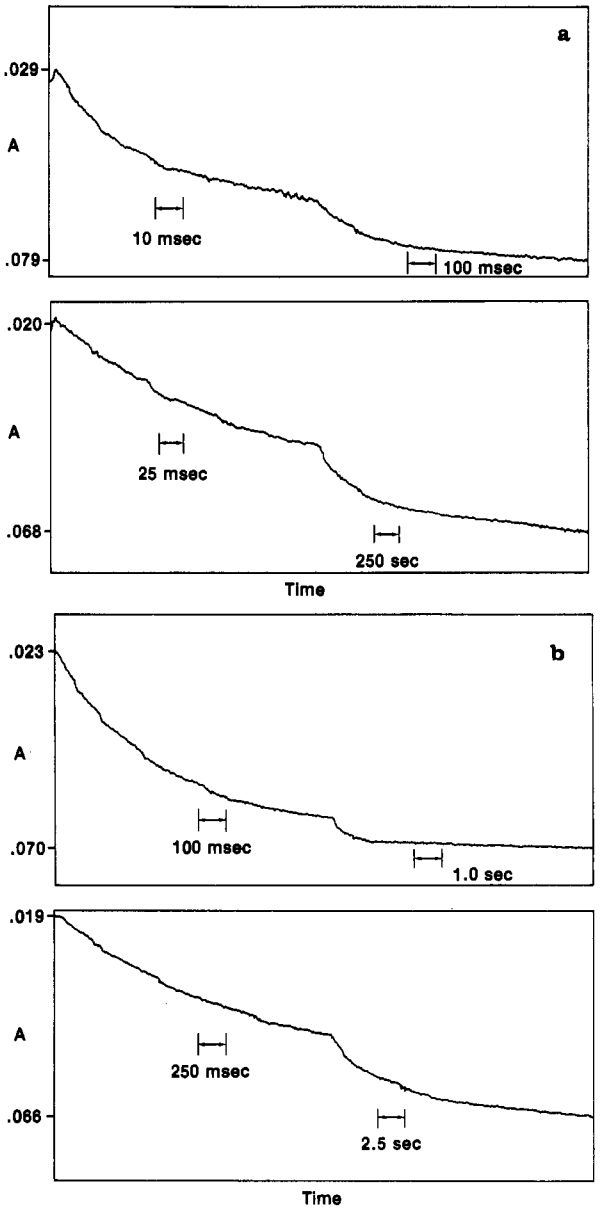


FIGURE 2: Isotope effect on the reductive half-reaction of FPCoA (26 μM) and dehydrogenase (5 μM) at pH 9.0 and 6.7. (a) pH 9.0, 340-nm FACoA production (upper trace H, lower trace D); (b) pH 6.7, 340-nm FACoA production (upper trace H, lower trace D).

dehydrogenase and fatty acyl-CoA oxidase catalyzed reactions is at least partially rate limiting, and this fact permits us to interpret the pH dependence in terms of a chemical mechanism. A recent study on the isotope effect of the reduction of crotonyl-CoA by the deuterated 5-deaza-FAD hydroquinone of dehydrogenase (Ghisla et al., 1984) confirms our previous suggestion (Reinsch et al., 1980) that there is a primary isotope effect on the transfer of the β-hydrogen of substrate. These data support our contention that the pH dependence of the complete reaction is consistent with removal of the α-proton of the substrate followed by rate-limiting bond breaking of the C–H bond at the β-carbon to form either H· or H:⁻.

Table II: pH Dependence and Isotope Effects of the Reductive Half-Reaction of Dehydrogenase

	k_1 (s^{-1})			k_2 (s^{-1})		
pH	FPCoA (H)	FPCoA (D)	H/D	FPCoA (H)	FPCoA (D)	H/D
9	49.6	6.8	7.3	6.6	2.1	3.1
6.7	6.1	0.68	9.0	1.9	0.10	19

Panels a and b of Figure 2 show kinetic traces of the production of FACoA during the reductive half-reaction of FPCoA and fatty acyl-CoA dehydrogenase at acidic pH (pH 6.7) and basic pH (pH 9.0), respectively, with FPCoA and tetradeuterio-FPCoA. This reaction consists of two single exponential kinetic processes which can be fit to the expression $\Delta A(t) = \Delta A_1 e^{-k_1 t} + \Delta A_2^{-k_2 t}$ (Reinsch et al., 1980a). The chemical significance of these two kinetic processes is unclear; the present investigation of the isotope effects on these reactions shows that both steps involve the bond breaking of the C–H bonds at C-2 and C-3 since both show large primary isotope effects. The most likely explanation of the two kinetic steps observed on the reductive half-reaction is that there are two "classes" of enzyme sites in dynamic equilibrium with substrate. The dissociation of the product charge-transfer complex shows no isotope effect. Similarly, we observe no isotope effect upon the fatty acyl-CoA dehydrogenase catalyzed oxidase reaction of FPCoA. These observations are consistent with our earlier suggestion [based upon rate equivalence (McFarland et al., 1982b)] that the oxidase reaction occurs only after dissociation of FACoA from the charge-transfer product complex. We have compared the rate constants and deuterium isotope effects for the reductive half-reaction at two pH values (Table II). The method employed for determination of the kinetic parameters is described under Experimental Procedures. Since these experiments are carried out at substrate saturation, the second step accounts for only 15% of the absorbance change; thus, the second step kinetic parameters are much less accurate than those associated with the first step. These data indicate that the reductive half-reaction is not rate limiting for the overall ETF reaction; the rates of the former reaction are significantly faster than the latter. Furthermore, Table II indicates that the isotope effect at both pH values is much larger for the reductive half-reaction than for the complete reaction. This observation is similar to that for the oxidation of butyryl-CoA and is consistent with a mechanism of substrate binding in which ETF binds only to the binary enzyme–butyryl-CoA complex and reaction takes place through an ETF ternary complex (Reinsch et al., 1983). Another test of this hypothesis is the examination of the kinetic profile of the complete reaction involving ETF for a transient "burst" of double-bond production due to the reductive half-reaction. As seen in Table II, the reductive half-reaction at pH 6.7 is much faster than the complete reaction. If the reductive half-reaction precedes the rate-limiting step, a pre-steady-state burst of FACoA should be observed. As can be seen in Figure 3b, no such burst is observed. Figure 3a shows the time course of the reduction of ETF; since no ETF would be reduced during the half-reaction, no pre-steady-state reaction could be observed during ETF reduction. The similarity of the time course of the two reactions indicates that there is no product burst. These facts establish that the details of the catalytic events of reaction of substrate and ETF do not involve the reductive half-reaction so that no comparison of rate constants or isotope effects of the reductive half-reaction with those parameters for the complete reaction is appropriate in a consideration of the

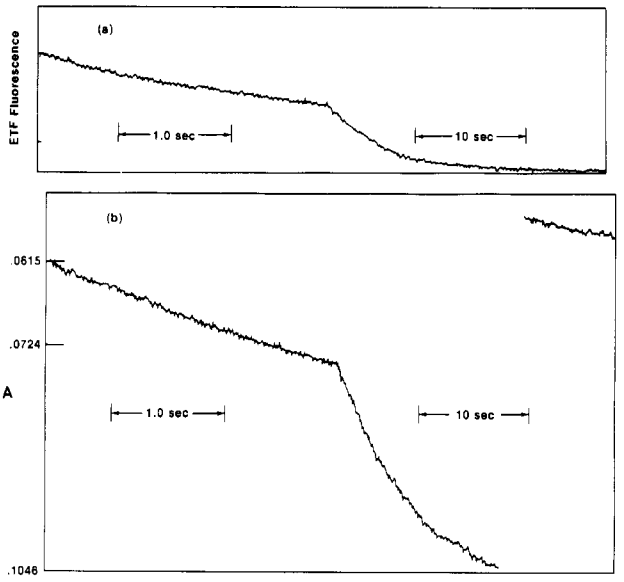


FIGURE 3: Time course of the reaction of FPCoA (11.3 μM) with ETF (1.3 μM) catalyzed by fatty acyl-CoA dehydrogenase (0.32 μM). Reaction was carried out at 25 °C, pH 6.7, in 20 mM ionic strength phosphate buffer. (a) Kinetic trace of the fluorescence decrease during reduction of ETF; (b) kinetic trace of the absorbance increase of 340 nm during FACoA formation.

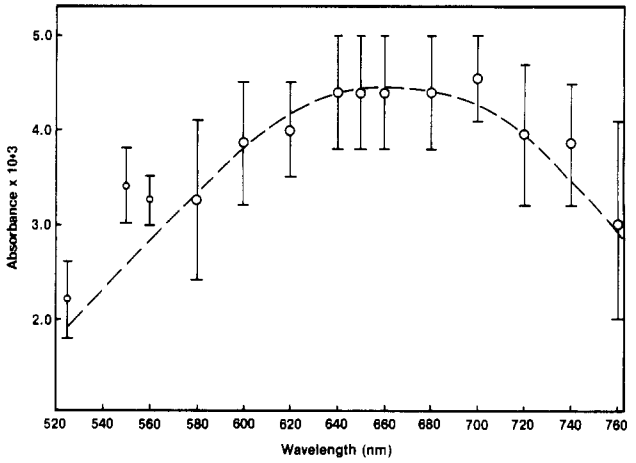


FIGURE 4: Stopped-flow spectrometer trace of the charge-transfer complex formed from the reaction of FPCoA (27 μM) and dehydrogenase (4.6 μM). Reaction was carried out in Tris buffer (pH 8.5, 20 mM) at 25 °C. ϵ_{650} = 1000 L/(mol-cm) for the charge-transfer complex.

mechanism of the complete reaction.

Figure 4 shows the electronic spectrum of the product charge-transfer complex of dehydrogenase. Notice that maximum absorbance of the long-wavelength band, 650 nm, is considerably shifted from that for butyryl-CoA, providing some indication that this is a charge-transfer band rather than a band associated with a blue semiquinone of flavin since its wavelength maximum is substrate dependent. These data were collected by employing the stopped-flow spectrophotometer as a single-beam spectrometer. This is necessary because the charge-transfer intermediate is kinetically unstable and the spectrum can be observed only at the short times available in the stopped-flow spectrophotometer.

In order to compare and contrast the function of fatty acyl-CoA oxidase with the dehydrogenase, we have studied the pH and isotope dependence of the reaction of FPCoA with the oxidase under steady-state kinetic conditions. Figure 5 shows the pH dependence of the steady-state parameters K_m

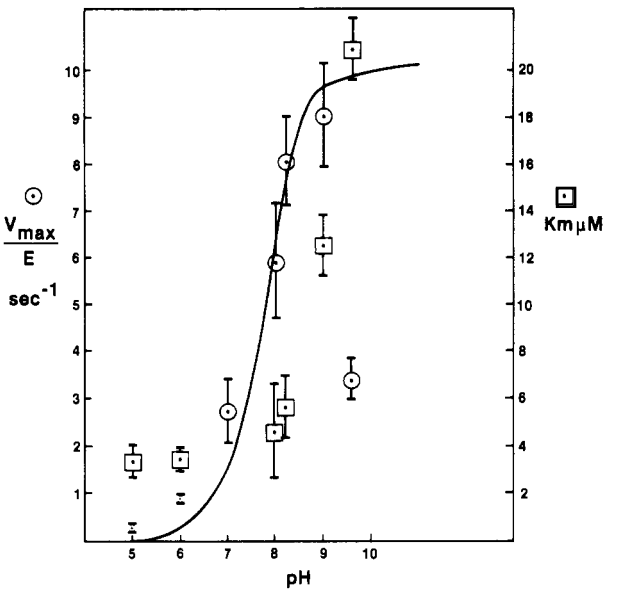


FIGURE 5: pH dependence of the reaction of O_2 with FPCoA (1–20 μM) catalyzed by fatty acyl-CoA oxidase (0.5–2.0 μM). Reactions were carried out at 25 °C at constant ionic strength. Error bars are standard deviations; pK for velocity (7.9) is the "best-fit" value.

and V/E for the reaction of FPCoA with O_2 catalyzed by fatty acyl-CoA oxidase. In order to quantitate the reaction products, we have compared the amount of H_2O_2 and FACoA formed at two different pH values. At pH 8.0, 20 μM FPCoA was converted to 20 μM FACoA, and during the oxidation, 18.7 μM H_2O_2 was produced. At pH 6.0, 37 μM FPCoA was converted to 37 μM FACoA, producing 33.2 μM H_2O_2 in the reaction. The major product of O_2 reduction is H_2O_2 as should be the case for an oxidase enzyme; use of the chromophoric substrate permits demonstration of this fact in a particularly graphic manner.

Notice that the pH dependence for the oxidase-catalyzed reaction is qualitatively similar to the pH dependence for the dehydrogenase-catalyzed reaction, with the Michaelis constant decreasing at more acidic pH values and the turnover number increasing at more basic pH values. The quantitative aspects of the comparison show some differences; if one pK_a is chosen to roughly fit the observed behavior, it is clear that the pK_a observed for the turnover number is more basic for the dehydrogenase. Likewise, it appears that the pK_a observed in the pH dependence of the Michaelis constant is more basic for the oxidase. In order to determine if the chemical step of "hydride" transfer is rate limiting for the oxidase, we have determined the isotope effect upon the reaction of tetradeuterio-FPCoA and O_2 catalyzed by oxidase. The isotope effects are primary at both pH values studied (Table I), leading to the conclusion that the rate-limiting step is associated with the chemical step of reduction of flavin. The data support the notion that both enzymes share a common mechanism for β-oxidation of FPCoA up to the reaction of the reduced enzyme–product charge-transfer complex.

Figure 6 shows the spectrum of the product charge-transfer complex formed during the reductive half-reaction between FPCoA and the oxidase. Notice that the extinction coefficient is the same as that for the comparable complex with dehydrogenase but that the wavelength maximum is 640 nm for dehydrogenase and 600 nm for the oxidase. Furthermore, the complex of oxidase is kinetically stable while that for dehydrogenase is labile (McFarland et al., 1982b). Figure 6 also shows the O_2 reaction of the product of the reductive half-reaction of oxidase and FPCoA; note the correlation between

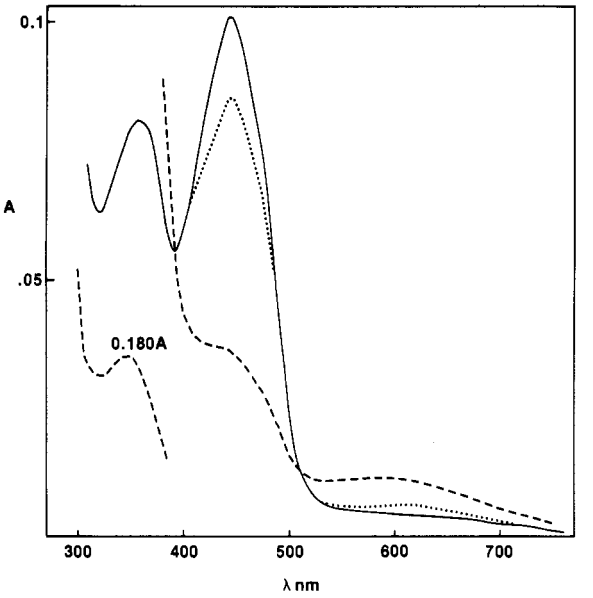


FIGURE 6: Charge-transfer complex from the reaction of FPCoA (19 μM) and oxidase (6.3 μM): oxidized enzyme (solid line); charge-transfer product complex (dashed line); charge-transfer product complex plus O_2 (dotted line). Reaction was carried out at 25 °C in phosphate buffer (20 mM, pH 7.0). ϵ_{600} = 1000 L/(mol·cm) for the charge-transfer complex.

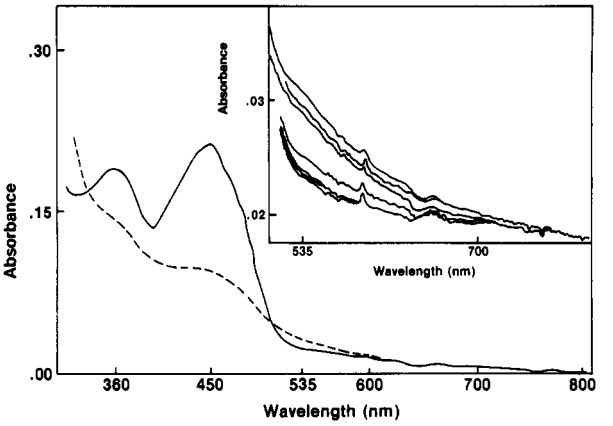


FIGURE 7: Charge-transfer complex from the reaction of octanoyl-CoA (154 μM) and oxidase (16 μM): oxidized enzyme (solid line); charge-transfer product complex (dashed line). Reaction was carried out at 25 °C in phosphate buffer (20 mM, pH 7.0). Insert shows loss of absorbance at 535 nm as O_2 is allowed to react with reduced enzyme. $\epsilon_{535} \simeq$ 500 L/(mol·cm) for the charge-transfer complex.

the extent of reoxidation of flavin and the disappearance of the product charge-transfer complex. This observation supports the conclusion that electron transfer to O_2 takes place through the charge-transfer complex. The rapid reaction of oxidase charge-transfer complex with O_2 stands in marked contrast to the observation that the dehydrogenase charge-transfer complex must dissociate completely before O_2 can react with reduced enzyme. In the same vein, ETF reacts directly and rapidly with the product charge-transfer complex of dehydrogenase but will not accept electrons from oxidase over a 1-h time interval. Figure 7 shows the product charge-transfer complex of oxidase and octenoyl-CoA. We have found both this complex and that formed from FPCoA to be extremely reactive to O_2, and this has required special measures, outlined under Experimental Procedures, to ensure anaerobicity. The extreme O_2 sensitivity may explain why other researchers have failed to observe a long-wavelength band associated with the substrate reduction of oxidase (Jiang & Thorpe, 1983). We discovered in early experiments on the

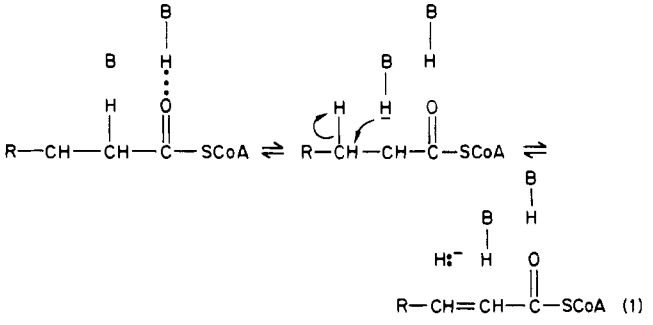
oxidase that conventional methods for excluding O_2 including degassing followed by N_2 purging in the presence of glucose and glucose oxidase in an ordinary cuvette fitted for anaerobic titrations resulted in turnover of our pseudosubstrate, i.e., greater than 3–4 equiv of FACoA formed per FAD reduced. After adopting the anaerobic cell described under Experimental Procedures, we observed the following stoichiometries for FACoA formation and FAD reduction in the two enzyme systems. The difference extinction coefficient for dehydrogenase reduction is based upon the electronic spectrum of electrochemically reduced dehydrogenase while that for oxidase is based upon the dithionite reduction product (Jiang & Thorpe, 1983).

enzyme	[FPCoA] (μM)	C=C produced (μM)	total flavin (μM)	flavin reduced (μM)
dehydrogenase	41	8.5	9.8	10.4
oxidase	63	18.2	14.6	13.0

After each "specific" reaction, there is an O_2-catalyzed turnover due to the leak rate of O_2 into the cuvette. This is not substantial for the dehydrogenase reaction, but the leak rate for the oxidase reaction is 8.3×10^{-12} mol/s. The reason for the difference in observed leak rate for the two reactions is the kinetic slowness of the reaction of dehydrogenase with O_2. Note that the specific reaction for the dehydrogenase can be regarded as an active-site titration in which all of the flavin is reduced and the amount of FACoA produced is approximately equal to the dehydrogenase concentration. In the oxidase reaction, notice that the reduction of enzyme is not complete as evidenced by the residual 450-nm oxidized flavin peak remaining after reduction; this is in contrast to the situation with dehydrogenase in which complete reduction of flavin is observed at a lower concentration of FPCoA than that used in the oxidase reaction. This observation is typical of a large number of runs and indicates that the half-cell reduction potential of oxidase is more negative than that for dehydrogenase. The excess of double-bond formation over flavin reduction for oxidase is an indication that we have not been successful in reducing residual O_2 concentration below 5 μM and that this residual O_2 gives some turnover of substrate.

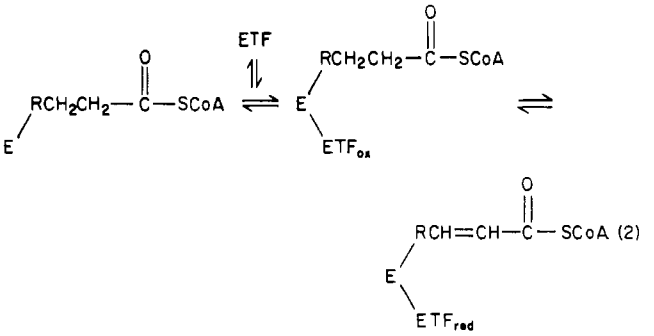
Discussion

As mentioned previously, our purpose in this investigation is to determine the functional difference between two enzymes involved in β-oxidation of fatty acids and to relate these functional differences to structural features of the enyzmes. In carrying out this correlation, we have compared the pH dependencies and isotope effects of a single pseudosubstrate with each enzyme. Figure 1 shows the pH dependence of the two steady-state parameters V/E and K_m for the reaction of fatty acyl-CoA with ETF catalyzed by fatty acyl-CoA dehydrogenase. We have observed a similar pH profile with butyryl-CoA and have interpreted this behavior as consistent with the following mechanism:



On the basis of data of the complete reaction of substrate and ETF catalyzed by acyl-CoA dehydrogenase summarized under Results, an attractive mechanism is that there is a preequilibrium removal of the C-2 proton (eq 1) followed by the elimination of hydride from C-3. The latter step is associated with a primary deuterium isotope effect and shows pH dependence reflecting the dependence of the rate upon formation of the kinetically important anion intermediate. It is possible that for some substrates, these two steps will also occur in a concerted fashion, perhaps explaining the large isotope effect of $k_H/k_D = 19$ for the overall reaction of ETF and perdeuteriobutyryl-CoA (Schmidt et al., 1981).

Previous work on the order of substrate binding has suggested that acyl-CoA and ETF observe an ordered binding mechanism (McKean et al., 1979); as noted under Results, the data in Figures 2 and 3 and Table II establish that the reductive half-reaction of FPCoA is not involved in the complete reaction of FPCoA and ETF. The following reactions summarize the mechanism of the combination of substrates and enzyme consistent with the data:

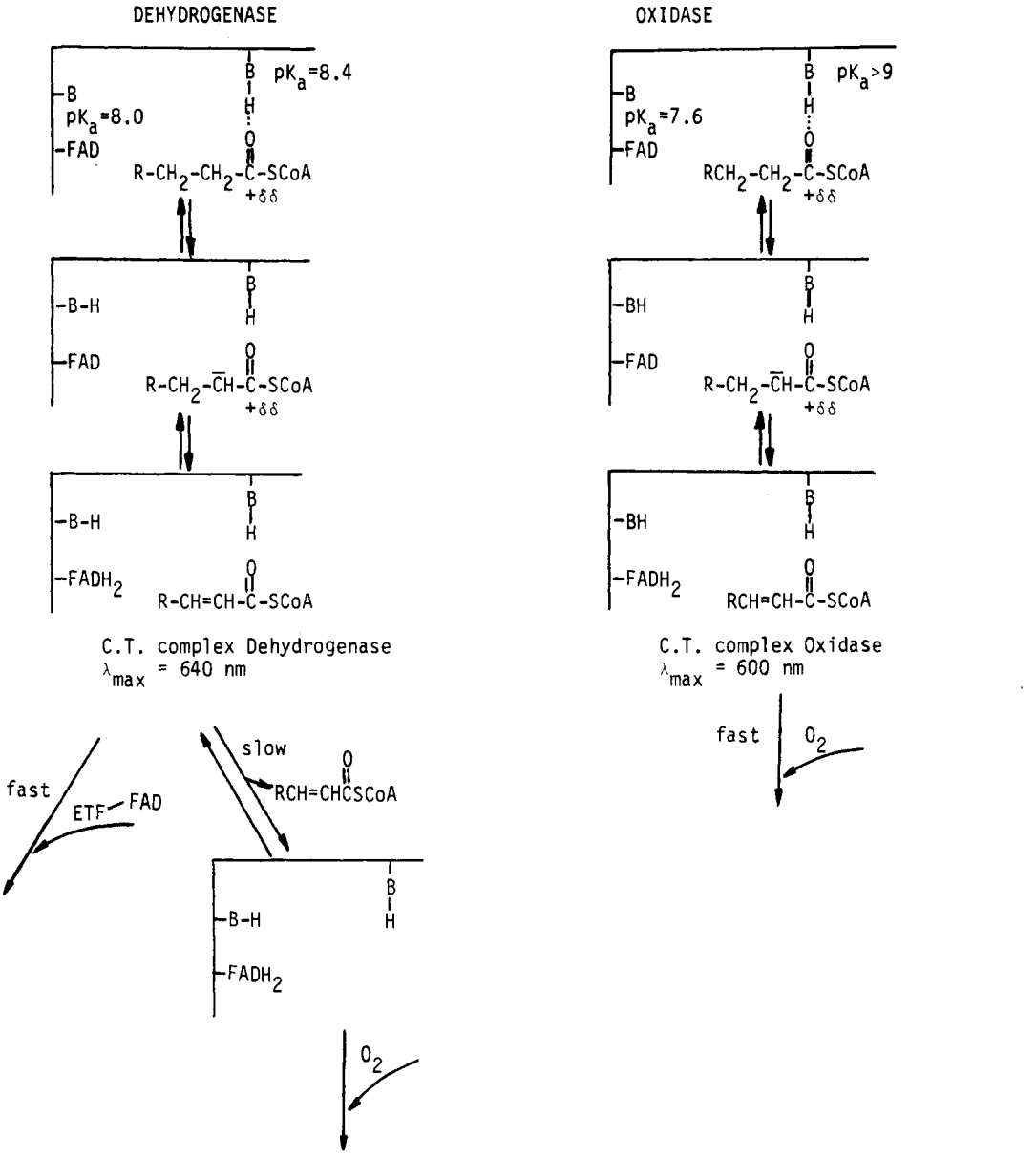


The data cited under Results indicate that the reductive half-reaction does not take place within the ternary complex in eq 2 at the rate that is observed in the absence of ETF; therefore, the details of kinetic constants and isotope effects upon the reductive half-reaction do not apply to eq 2. The reason for the kinetic difference in the reduction of flavin and oxidation of FPCoA between reductive half-reaction and complete reaction is not clear at present. One possibility is that the reaction in the presence of ETF proceeds through semiquinone while the reductive half-reaction does not.

Previous experiments with dehydrogenase and FPCoA indicated that the reduced flavin enoyl-CoA charge-transfer complex is kinetically labile and reacts readily with ETF but does not react either with O_2 or with an alternate enoyl-CoA. This would suggest that this intermediate is the key in controlling the function of the β-oxidation enzymes. Accordingly, we have determined the transient absorbance spectrum of the product charge-transfer complex; this spectrum is shown in Figure 4. The spectrum represents a point by point determination on a single-beam stopped-flow spectrometer. As mentioned previously, the λ_{max} of the charge-transfer band with FPCoA is considerably shifted from that for butyryl-CoA, giving some indication that this band is a substrate-dependent charge-transfer band and not a blue neutral semiquinone of flavin.

Figure 5 shows the pH dependence of the reaction of FPCoA with O_2 catalyzed by fatty acyl-CoA oxidase; as shown under Results, the product of the O_2 reaction is H_2O_2. Notice the qualitative similarity of the pH dependence of the oxidase and dehydrogenase reactions. Both show apparent base catalysis of the reaction, and both show tighter binding of substrate [and in the case of dehydrogenase inhibitor (Reinsch et al., 1983)] at acidic pH. Up to the product charge-transfer

Scheme I



complex, both enzymes exhibit data consistent with the mechanism of eq 1. Our first hypothesis in explaining the functional difference between the two enzymes was that oxidase forms no stable charge-transfer product complex. Indeed, experiments with octanoyl-CoA in the absence of O_2 show only a hint of a long-wavelength absorption band (Jiang & Thorpe, 1983). We have found that ordinary experiments designed to eliminate O_2 from aqueous solution do not eliminate turnover of FPCoA; i.e., we observe production of more than 1 equiv of FACoA per enzyme active site. We have developed a reaction cell which does permit the observation of the reductive half-reaction of oxidase without significant turnover with O_2. This cell is described under Experimental Procedures. Figure 6 shows the spectrum of the product charge-transfer complex with FACoA. The first difference seen with respect to the equivalent complex with dehydrogenase is that the value of λ_{max} is 600 nm while that for the dehydrogenase is 640 nm. Furthermore, the complex of oxidase is stable and does not dissociate like that of dehydrogenase. Figure 7 also shows the O_2 reactivity of the complex with oxidase. Note the correlation of the decrease of charge-transfer band with the increase of reoxidized flavin. Qualitatively, this would seem to indicate that O_2 reacts directly with the charge-transfer product complex. Keep in mind that the complex of dehydrogenase reacts only with ETF; dissociation must occur before reduced dehydrogenase will react with either O_2 or enoyl-CoA (McFarland et al., 1982a). Figure 7 shows the product of the reaction of octanoyl-CoA with oxidase, demonstrating that the production of complex is not specific to our pseudosubstrate; again, note the substrate dependence of λ_{max}, indicating that this is most likely a charge-transfer complex. We have tested the chemical reactivity of the charge-transfer complex formed from the reductive half-reaction of FPCoA and oxidase. In an experiment similar to that shown for the reaction of O_2 with complex (Figure 6), ETF showed no reaction over a period of 1 h; on the other hand, crotonyl-CoA reacted immediately to produce oxidized enzyme. The chemical characteristics of the two charge-transfer complexes can be summarized as follows:

dehydrogenase	oxidase
kinetically unstable, $k = 10^{-2}$	kinetically stable
reacts with ETF	reacts with O_2 and enoyl-CoA
does not react with O_2 or enoyl-CoA	does not react with ETF

It should be noted that the function of these enzymes is controlled by the reactivity of the charge-transfer complexes. Each complex reacts specifically with its electron acceptor but does not cross-react.

We have previously reported structural studies on these two enzymes carried out by resonance Raman spectroscopy. The Raman results are consistent with the conclusion that there is extensive hydrogen bonding between FAD and dehydrogenase at the active site but that there is little or no hydrogen bonding between FAD and oxidase. If one considers each hydrogen bond with protein as a dipole and takes into account the dipole stabilization of a charged intermediate (Warshel, 1978), then one would expect the stabilization of the charge-transfer electronic excited state to result in lower energy for this transition for the dehydrogenase. Indeed, a comparison of Figures 4 and 6 indicates that the energy of the charge-transfer band for dehydrogenase is lower; this observation is consistent with the stabilization of the electronic excited state of dehydrogenase relative to oxidase. Furthermore, the observation that E_0' is more negative for oxidase is also consistent with the Raman structural data. Hydrogen bonding between the dehydrogenase and FAD at the active site would result in electron delocalization from the FAD (Watanbe et al., 1982) and would therefore increase the oxidation potential over that of the oxidase-bound FAD. In keeping with this prediction, the data on the FPCoA reaction indicate that the FAD of dehydrogenase is thermodynamically a better oxidizing agent than that of oxidase.

In summary, Scheme I shows the reactions of and functional differences between fatty acyl-CoA dehydrogenase and fatty acyl-CoA oxidase.

Registry No. FPCoA, 75368-14-8; tetradeuterio-FPCoA, 95693-72-4; deuterated FA acid, 95673-96-4; tetradeuterated FP acid, 95673-97-5; 2,2-bifuroyl, 492-94-4; deuterated 2-furaldehyde, 42220-86-0; deuterated malonic acid, 813-56-9; fatty acyl-CoA dehydrogenase, 9027-65-0; fatty acyl-CoA oxidase, 61116-22-1; octanoyl-CoA, 1264-52-4; deuterium, 7782-39-0.

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