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Reactions of β-(2-Furyl)propionyl Coenzyme A with "General" Fatty Acyl-CoA Dehydrogenase[†]

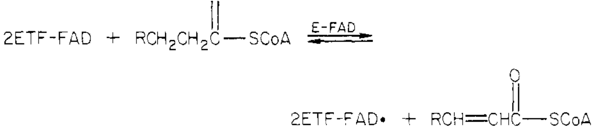
James T. McFarland,* Mei-Young Lee, James Reinsch, and Wanda Raven

ABSTRACT: We have prepared a new pseudosubstrate for the "general" acyl-CoA dehydrogenase, β-(2-furyl)propionyl-CoA (FPCoA). This substrate reacts with enzyme to yield *trans*-β-(2-furyl)acryloyl-CoA (FACoA) which absorbs maximally at 340 nm, the isosbestic point for oxidized and fully reduced flavin. FPCoA is a better substrate (lower K_m, higher V_{max}) than butyryl-CoA but not as good a substrate as octanoyl-CoA. By observing the rate of formation of FACoA and comparing it with the rate of formation of the semiquinone of electron-transfer flavoprotein (ETF), we have established a 2:1 stoichiometry for this reaction (see eq 1b). The reaction of FPCoA with acyl-CoA dehydrogenase is a biphasic first-order reaction when either flavin reduction or FACoA formation is observed. However, observation of FACoA production reveals a new oxygen-dependent production of enoyl-CoA product which is not reflected in the reaction profile of the FAD of fatty acyl-CoA dehydrogenase. This reaction requires oxygen, produces H_2O_2, and can therefore be characterized an "oxidase" reaction. The reaction is zero order and is linearly dependent upon enzyme concentration. The charge transfer product complex of FACoA and acyl-CoA dehydrogenase is not stable and completely dissociates, as evidenced by the complete disappearance of the charge transfer electronic band with a first-order rate constant of 2×10^{-2} s^{-1}. The zero-order rate constant characterizing the production of FACoA is also 2×10^{-2} s^{-1}. Therefore, oxygen reacts with reduced acyl-CoA dehydrogenase at the rate of dissociation of the charge transfer product complex. Another electron acceptor, crotonyl-CoA, reacts in a transhydrogenation reaction at this same rate of charge transfer product complex dissociation. It is significant that the normal electron acceptor, ETF, reacts with a much larger rate constant of $\simeq 30$ s^{-1}. This establishes the fact that normal electron transfer to ETF occurs within the charge transfer product complex. These data would seem to establish that the charge transfer product complex formed upon reduction of acyl-CoA dehydrogenase with saturated fatty acyl-CoA substrate is essential in assuring the transfer of electrons to ETF in preference to electron transfer to oxygen or enoyl-CoA substrate.

Fatty acyl-CoA dehydrogenase (pig liver, general enzyme) is an FAD-containing enzyme responsible for the oxidation of saturated fatty acyl-CoA esters and the transfer of electrons to a protein substrate, electron transfer flavoprotein (ETF) (Crane & Beinert, 1956), as shown in eq 1.

$$\text{E-FAD} + \text{RCH}_2\text{CH}_2\overset{\overset{\displaystyle O}{\|}}{C}\text{—SCoA} \rightleftharpoons \text{E}\begin{cases}\text{FADH}_2\\ \text{RCH}{=}\text{CH}\overset{\overset{\displaystyle O}{\|}}{C}\text{—SCoA}\end{cases}$$

(1a)



$$2\text{ETF-FAD} + \text{RCH}_2\text{CH}_2\overset{\overset{\displaystyle O}{\|}}{C}\text{—SCoA} \xrightleftharpoons{\text{E-FAD}} 2\text{ETF-FAD}\cdot + \text{RCH}{=}\text{CH}\overset{\overset{\displaystyle O}{\|}}{C}\text{—SCoA}$$

(1b)

The reaction shown in eq 1a shows biphasic kinetics and a very large primary isotope effect (Reinsch et al., 1980a). The reaction in Figure 1b also shows a primary isotope effect (J. Schmidt, J. Reinsch, and J. T. McFarland, unpublished results) and consists of the reaction of ETF to yield an anionic semiquinone at a rate which is sufficiently fast to be consistent with the overall process of β-oxidation (Reinsch et al., 1980b). In order to correlate the rate of C=C formation with flavin reduction, we have synthesized β-(2-furyl)propionyl-CoA (FPCoA), a pseudosubstrate, which produces a product, *trans*-β-(2-furyl)acryloyl-CoA (FACoA), absorbing at 340 nm. Since the flavin species involved in the enzyme reaction of Figure 1a have an isosbestic point at 340 nm, this substrate allows us to determine the absorbance change occurring during double-bond formation.

Experimental Procedures

Reagents and Enzyme. The enzyme and ETF purifications have been described previously (McKean et al., 1979).

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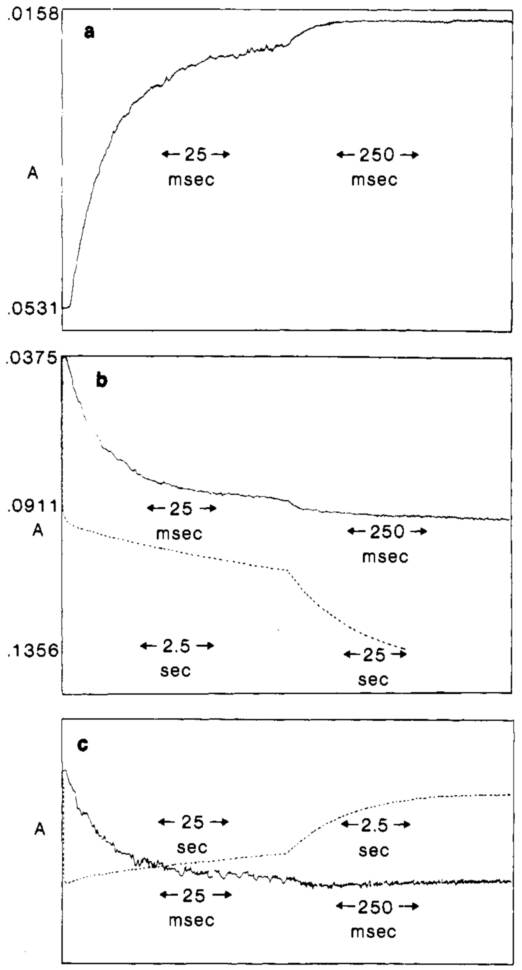


FIGURE 1: Reaction of FPCoA with enzyme. (a) Flavin absorbance (450 nm) change during the oxidation of FPCoA. (b) FACoA absorbance (340 nm) changes during the oxidation of FPCoA. (c) "Charge-transfer complex" absorbance (650 nm) change during the oxidation of FPCoA. Conditions: pH 8.5, 20 mM Tris; [FPCoA] = 24 μM; [enzyme] = 5.1 μM.

Substrates. Butyryl-CoA, octanoyl-CoA, and crotonyl-CoA were purchased from P-L Biochemicals and used without further purification. FPCoA was synthesized as described previously (Reinsch et al., 1980a). FACoA was synthesized from β-(2-furyl)acryloyl chloride (Bernhard et al., 1965) by adding CoASH to $\approx$20-fold excess of acyl chloride in pH 9.0 HCO_3^-/CO_3^{2-}. The pH was maintained by the addition of solid Na_2CO_3 to the reacting solution; reaction was allowed to proceed for 30 min to 1 h until no free CoASH could be detected with sodium nitroprusside. The reaction product was purified by high-performance liquid chromatography (HPLC) on a C-18 column (Waters, Inc.) using 50% $MeOH/H_2O$ (containing 0.01 M tetrabutylammonium phosphate) as an eluting solvent. Sulfonated 2,4-dichlorophenol was prepared as described previously (Barham & Trinder, 1972). 4-Aminoantipyrine was purchased from Aldrich Chemical and used without further purification.

Steady-State Kinetic Experiments. (*a*) *DCI Method.* The substrate FPCoA was mixed with 2 μM ETF and 3×10^{-5} M dichloroindophenol in its oxidized state. The reaction was initiated by the addition of $(1-5) \times 10^{-8}$ M acyl-CoA dehydrogenase. The reaction was monitored at 600 nm [$\Delta\epsilon$ = 20000 L/(M·cm)] (McKean et al., 1979); a Cary 16 or Cary 17 spectrometer was used to determine the initial velocity of the reaction. All experiments were carried out at 25 °C in pH 8.5 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer.

(*b*) *ETF Fluorescence Method.* The substrate FPCoA was mixed with ETF (2.6 μM) and introduced into the reservoir of a stopped-flow spectrometer where it was mixed with 0.25 μM dehydrogenase (Reinsch et al., 1980b). The reaction was monitored by fluorescence (ETF) and FACoA formation at 340 nm. Initial velocities were determined, and steady-state kinetic parameters were computed from the reciprocal plots. All experiments were carried out at 25 °C in pH 8.5 20 mM Tris buffer.

Kinetics of the Partial 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 450 (flavin reduction), 340 (double-bond formation), and 640 nm (charge transfer complex formation) were stored in the Northern Scientific 560 computer for retrieval and display on an X-Y recorder. The transient absorbance changes were analyzed by using $\Delta A(t) = \Delta A_1 e^{-k_1 t} + \Delta A_2 e^{-k_2 t}$ [for details, see Reinsch et al. (1980a)]. After the "transient" partial reaction, a slow zero-order process was observed at 340 nm but not at 450 nm. This process was analyzed by taking an initial velocity measurement. A dissociation rate for the charge-transfer complex could also be measured at 640 nm; this rate process was a single exponential and was calculated from the equation $\Delta A(t) = \Delta A e^{-kt}$.

Spectroscopy of Double-Bond Formation. In order to obtain the visible spectrum of the product of the "oxidase" reaction, we have studied the steady-state production of FACoA both aerobically and anaerobically. This experiment was carried out with [FPCoA] = 28 μM and [fatty acyl-CoA dehydrogenase] = 0.5 μM. For the anaerobic experiment, the solutions were subjected to vacuum degassing and argon flushing alternatively for 10–20 min. All experiments were at 25 °C.

Peroxide Analysis. Two procedures were used for peroxide determination. In the first, cytochrome *c* Fe^{3+} was reduced to cytochrome *c* Fe^{2+} by excess ascorbate. The ascorbate was removed by dialysis, and 100 μL of the cytochrome *c* Fe^{2+} (1 mg/mL) was added to the assay medium as an electron acceptor. The final H_2O_2 assay medium contained 1 μM acyl-CoA dehydrogenase, 100 μL of cytochrome *c* Fe^{2+}, and 10 μL of 1.0 mg/mL horseradish peroxidase (grade VI; Sigma Chemical Co.). The rate of H_2O_2 generation was determined from the absorbance change at 550 nm [$\Delta\epsilon$ = 19400 L/(M·cm)]. The second procedure involved the kinetic or static determination of H_2O_2 from the formation of the peroxidase-catalyzed coupling product between sulfonated 2,4-dichlorophenol and 4-aminoantipyrine. In the static assay, the FACoA formation was followed at 340 nm ([FPCoA] = 30–50 μM and [acyl-CoA dehydrogenase] = 1 μM), and H_2O_2 was determined at various time intervals from the 515-nm absorbance of coupling product formed upon addition of 5 μL of (1 mg/mL) peroxidase per mL of solution, 4.5×10^{-5} M 4-aminoantipyrine, and 3.4×10^{-3} M dichlorophenol. The $\Delta\epsilon$ = 22000 L/(M·cm) was determined from a Beer's law plot of A_{515} vs. [H_2O_2]. The peroxide concentration was determined by thiosulfate titration with starch/I_2 as an indicator. The assay outlined above could also be carried out in a kinetic mode by following the rate of H_2O_2 production determined from the initial velocity of the formation of H_2O_2 as a function of time.

Transhydrogenation Reaction. A stopped-flow kinetic experiment was carried out as described previously for the partial reaction and its subsequent oxidase reaction. Addition of 73.2 μM crotonyl-CoA as an electron acceptor yields a marked change in the kinetic profile. The reduction in the rate of production of FACoA as a function of reaction time (which

Table I: Kinetic Parameters from Reaction of FPCoA with Fatty Acyl-CoA Dehydrogenase[a,b]

[FPCoA] (M)	k_1 (450 nm) (s^{-1})	k_2 (450 nm) (s^{-1})	$\Delta A_1/\Delta A_{total}$ (450 nm)	k_1 (340 nm) (s^{-1})	k_2 (340 nm) (s^{-1})	$\Delta A_1/\Delta A_{total}$
4.8×10^{-6}	16.5	2.1	0.73	14.2	2.0	0.67
8.6×10^{-6}	19.4	2.3	0.76			
13.9×10^{-6}	29.5	4.9	0.70	24.3	5.8	0.71
53.4×10^{-6}	33.6	3.7	0.84	35.0	4.3	0.73

[a] See eq 1b. [b] Extrapolated parameters: K_{app} (450 nm), 8.5 μM; $k_{1,max}$ (450 nm), 39.7 s^{-1}; K_{app} (340 nm), 6.7 μM; $k_{1,max}$ (340 nm), 38.6 s^{-1}.

is very large in the oxidase reaction) is not observed with the crotonyl-CoA acceptor. In order to characterize the transhydrogenase reaction, we have carried out reversed-phase HPLC experiments using a C-18 column and an elution solution of 50% methanol/water with 0.01 M tetrabutylammonium phosphate added as a paired ion chromatography medium. We were able to achieve separation of butyryl-CoA, crotonyl-CoA, FPCoA, and FACoA. With 115 μM FPCoA, 73.2 μM crotonyl-CoA, and 2.5 μM enzyme, the 340-nm absorbance was recorded as a function of time, and 25-μL aliquots were removed and subjected to HPLC. The 260-nm absorbance was recorded for each chromatogram, and the concentration of reagent and product was determined from the area under the chromatographic peaks and the original concentrations.

Results

The steady-state kinetic parameters determined from the rate of reduction of ETF by β-(2-furyl)propionyl-CoA (reaction 1a) are $K_m = 9.1$ μM and $V_{max}/E_0 = 29.6\ s^{-1}$. This compares well to steady-state kinetic data collected by coupling the reaction of ETF with reduction of the reduction/oxidation dye, dichloroindophenol (DCI); this kinetic technique yields $K_m = 9.5$ μM and $V_{max}/E_0 = 13.6\ s^{-1}$. The 2:1 relationship between the V_{max}/E_0 values reflects the expected stoichiometry for the two-electron acceptor, DCI, and the one-electron acceptor, ETF. A single measurement of C=C formation at 340 nm (λ_{max} for FACoA) gave $V_{max}/E_0 = 14.7\ s^{-1}$ at [FPCoA] = 38 μM while measurement of ETF fluorescence change gave $V_{max}/E_0 = 27.7\ s^{-1}$ at the same substrate concentration. This confirms our previous suggestion that the overall stoichiometry of the ETF reaction is two ETF semiquinone molecules formed per double bond (Reinsch et al., 1980b). These steady-state parameters for FPCoA are comparable to the steady-state parameters of $K_m \approx 1$ μM and $V_{max}/E_0 = 15$–$30\ s^{-1}$ for octanoyl-CoA (Hauge, 1956; Hall & Kamin, 1975), the optimal substrate for the general fatty acyl-CoA dehydrogenase. The optimal substrate reacts nearly quantitatively with ≃85% bleaching of enzyme-bound flavin while poorer substrates such as butyryl-CoA ($K_m = 60$ μM and $V_{max}/E_0 = 15\ s^{-1}$) bleach only ≃60% of the FAD of dehydrogenase. Thus FPCoA reacts as a poorer substrate than octanoyl-CoA (looser binding and slower reaction rate constant) but as a better substrate than butyryl-CoA.

Figure 1 shows the behavior of FPCoA reacting aerobically with general fatty acyl-CoA dehydrogenase (reaction 1b). Notice that only ≃65% of the enzyme-bound flavin is bleached by excess substrate and that the reaction of flavin bleaching or C=C formation is biphasic with the same fractional reaction pattern in each of the two sequential exponential steps. Table I shows the kinetic parameters for the absorbance changes and first-order rate constants derived from the rapid kinetic experiment using the equation $\Delta A(t) = \Delta A_1 e^{-k_1 t} + \Delta A_2 e^{-k_2 t}$ [for details on the kinetic treatment, see Reinsch et al. (1980a)]. We were surprised to notice that although flavin bleaching was complete after the second of the two exponential processes (Figure 1), C=C formation continued until all of the FPCoA was converted to FACoA; the rate for this conversion could be treated as a zero-order process. The V_{max}/E_0 determined, using the assumption that the process was zero order and would follow Michaelis–Menten behavior, was linearly dependent upon enzyme concentration as shown in Table II. The electronic spectrum of product was indistinguishable from FACoA prepared from *trans*-β-(2-furyl)acrylic acid (Figure 2). The extinction coefficient for FACoA was determined by allowing the reaction of a known concentration of β-(2-furyl)propionyl CoA (based upon the extinction coefficient of CoA's adenine moiety) to proceed quantitatively to product. This extinction coefficient is ε = 22 964 L/(M·cm). When the extinction coefficient for the product determined as discussed above was used, the C=C stoichiometry of the specific dehydrogenase reaction of FPCoA and fatty acyl-CoA dehydrogenase (the two single exponential kinetic processes in which flavin is bleached) is consistently slightly less (≈20%) than the stoichiometry determined from flavin bleaching. This

Table II: Enzyme Concentration Dependence for "Oxidase" Reaction and for Dissociation of the Charge-Transfer Complex

[S] (μM)	[E] (M)	k_1 (650 nm) (s^{-1})	V (340 nm) [mol/(L·s)]	V/E_0 (340 nm) (s^{-1})
30	7.0×10^{-6}	1.3×10^{-2}	8.4×10^{-8}	1.2×10^{-2}
30	3.4×10^{-6}	1.1×10^{-2}	4.4×10^{-8}	1.3×10^{-2}
30	1.7×10^{-6}	1.0×10^{-2}	2.2×10^{-8}	1.3×10^{-2}
30	0.85×10^{-6}		0.9×10^{-8}	1.1×10^{-2}
5.3	2.8×10^{-6}	1.4×10^{-2}	3.3×10^{-8}	1.2×10^{-2}
49.3	2.8×10^{-6}	1.2×10^{-2}	3.5×10^{-8}	1.3×10^{-2}

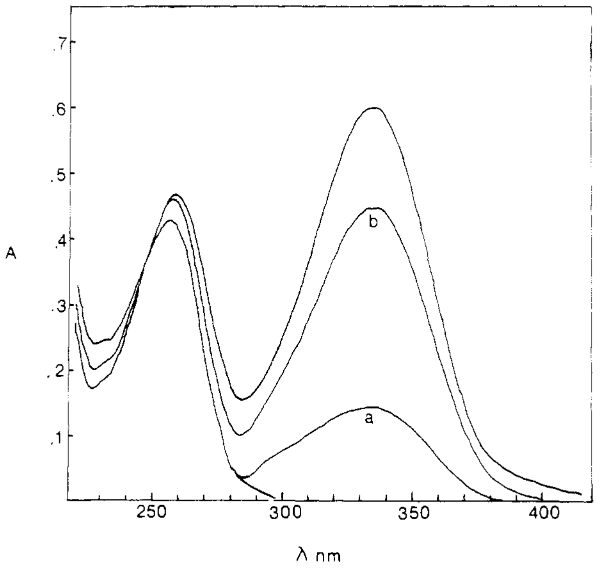


FIGURE 2: Spectrum of FACoA produced from enzymatic "oxidase" reaction of FPCoA; pH 8.5, 20 mM Tris, 25 °C; [FPCoA] = 27.7 μM and catalytic enzyme concentration ≃1 × 10^{-8} M. (a) and (b) are intermediate incubation times. ϵ_{335} = 22 964 L/(M·cm).

Table III: Kinetic Peroxide Assay for Oxidase Reaction

substrate	cytochrome *c* H_2O_2 assay, V_{max}/E_0 (s^{-1})	coupling reaction H_2O_2 assay, V_{max}/E_0 (s^{-1})	340-nm absorbance (s^{-1})[a]	k_{off} (s^{-1})
[octanoyl-CoA] = 21 μM		2×10^{-4}		3.1×10^{-3}
[FPCoA] = 29 μM	3×10^{-4}	4×10^{-4}	8×10^{-4}	1.2×10^{-2}
[butyryl-CoA] = 230 μM	2.8×10^{-3}	2.5×10^{-3}		2.0

[a] Note that the V_{max}/E_0 value for FPCoA is considerably less than that reported in Table II. The marked slowing of C=C production with time makes it impossible to obtain the initial velocity at these enzyme concentrations without using a rapid kinetic spectrometer.

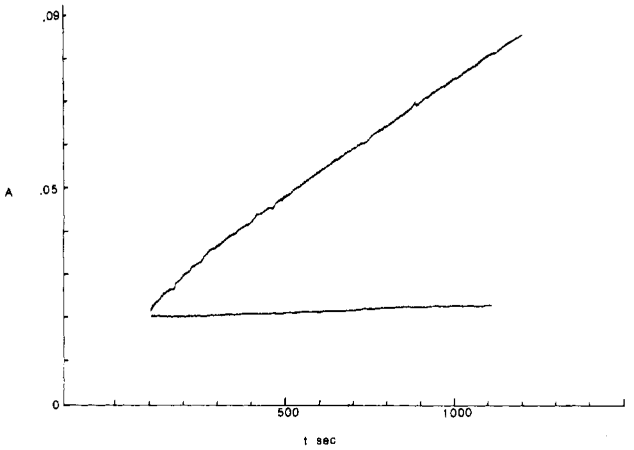


FIGURE 3: Time dependence of oxidase activity in the presence and absence of oxygen; pH 8.5, 20 mM Tris, 25 °C; [enzyme] = 1 μM; [FPCoA] = 30 μM. (Upper trace) Aerobic conditions. (Lower trace) Anaerobic conditions; solutions were degassed and purged with Ar. Glucose and glucose oxidase were added to remove residual oxygen.

difference, however, is sufficiently small that it may reflect a small perturbation in the extinction coefficient of enzyme-bound product compared to an aqueous solution of FACoA. Figure 1 shows formation and dissociation of a charge-transfer (CT) complex between reduced flavin and FACoA [for the spectrum of this CT complex, see Reinsch et al. (1980a)]. Notice again that the formation of the complex is biphasic but that dissociation consists of a single exponential process following first-order kinetics. Table II shows that this first-order rate constant is independent of both substrate and enzyme concentrations over the range investigated. Table II also establishes the excellent correlation between the rate constant for dissociation of charge-transfer complex and the zero-order rate constant for production of C=C.

One possible electron acceptor for this slow zero-order process related to formation of FACoA is O_2. In order to test this hypothesis, we have carried out the steady-state reaction in the presence and absence of O_2 (Figure 3). Notice that the values of V_{max}/E_0 reported in Table III for this experiment are well below those shown for the stopped-flow rapid kinetic experiments in Table II. We have chosen not to use the stopped-flow kinetic spectrometer in these experiments because of the difficulty of maintaining the anaerobicity of our samples. Figure 1b and Figure 4 (right-hand frame) show that the time course for C=C formation slows markedly so that the rate of C=C formation after 30 s decreases by $\simeq$30-fold. The V_{max} values listed in Table III are much smaller than those reported in the stopped-flow spectrometer because of the $\simeq$30-s mixing dead time in our introducing the reactants into the spectrometer. If O_2 is the electron acceptor during the zero-order process for C=C formation (i.e., if there is a slow oxidase activity of the general fatty acyl-CoA dehydrogenase), the second product of reaction must be H_2O_2 or O_2^-. In order to determine the O_2 reduction product, we have carried out two different H_2O_2 assays. Table III shows the agreement between the rate of C=C formation and H_2O_2 production by using a kinetic assay involving peroxide oxidation of cytochrome *c* Fe^{2+} catalyzed by peroxidase. We also have performed a static H_2O_2 assay depending on the chemical oxidative coupling reaction of 2,4-dichlorophenol and aminoantipyrine (Barham & Trinder, 1972). It appears qualitatively that H_2O_2 is the primary reduction product of O_2; however, we can account for only 43-53% of the H_2O_2 which presumably is formed during the time course of our oxidase reaction, based upon analysis of double-bond formation. We have also carried out the peroxide assays in the presence of superoxide dismutase, SoD,

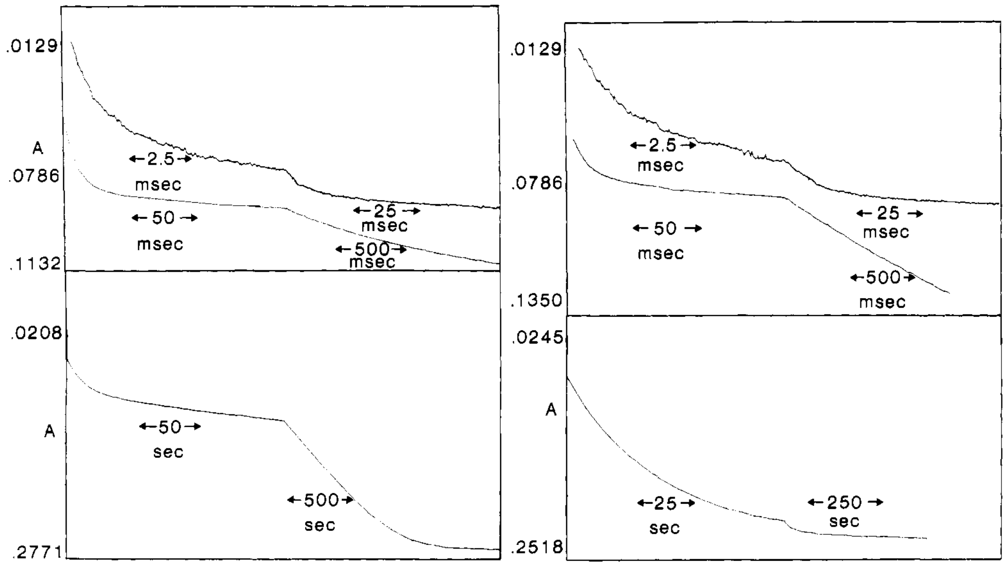


FIGURE 4: Oxidase and transhydrogenase reaction of FPCoA. (Left-hand frames) Conditions: pH 8.5, 20 mM Tris, 25 °C; [FPCoA] = 11.2 μM; [enzyme] = 5.0 μM. (Right-hand frames) Conditions: [FPCoA] = 11.2 μM; [enzyme] = 5.0 μM; [crotonyl-CoA] = 20.6 μM.

Table IV: Reaction of FPCoA and Crotonyl-CoA: "Transhydrogenation Reaction"

time of reaction (min)	$[\text{FACoA}]_{340\text{nm}}$ (μM)	$[\text{FACoA}]_{\text{HPLC}}$ (μM)	[crotonyl-CoA]$_{\text{HPLC}}$ (μM)	[butyryl-CoA]$_{\text{HPLC}}$ (μM)	$[\text{FPCoA}]_{\text{HPLC}}$ (μM)
20.2	6.6	5.8	65.8	5.7	109.7
90.4	27.0	24.3	49.3	22.2	91.2
152.8	42.0	39.3	35.8	35.8	76.5
226	55.0	53.3	21.2	52.0	63.5
322	64.6	61.2	10.7	60.8	54.2
390	68.6	65.8	8.6	62.9	50.0

in order to determine if the H_2O_2 stoichiometry is low relative to C=C as the result of the formation of O_2^-. The addition of superoxide dismutase results in production of only a bit more H_2O_2 (2.5 μM with SoD and 2.2 μM without SoD), showing that O_2^- makes up only a small amount of the reduced O_2 products.

In order to further investigate the correlation between oxidase activity and the rate of charge transfer complex dissociation for various product complexes, we have investigated the rate of H_2O_2 production and CT complex dissociation for butyryl-CoA and octanoyl-CoA. Our studies indicate that although the FACoA CT complex undergoes complete dissociation, both crotonyl-CoA and octenoyl-CoA undergo only 30–60% dissociation. As a result, titration experiments with both substrates result in formation of some stable charge transfer product complex while titration with FPCoA yields no stable product complex. The dissociation rate constant for CT complex is qualitatively proportional to the rate of H_2O_2 evolution for the three substrates. That is, the order of peroxidase activity, butyryl-CoA > FPCoA > octanoyl-CoA, reflects the same order for the rate of the charge transfer product complex dissociation. However, for butyryl-CoA, the rate constant for H_2O_2 evolution is substantially smaller than the rate constant for CT product dissociation (Table III).

Figure 4 shows an example of another electron acceptor which can accept electrons at the rate of product charge transfer complex dissociation. V/E_0 for this reaction at a saturating concentration of crotonyl-CoA = 20.6 μM is $V/E_0 = 1.3 \times 10^{-2}\ \text{s}^{-1}$. In this experiment, crotonyl-CoA is added as an electron acceptor in a "transhydrogenation" reaction. The oxidase reaction is shown in Figure 4 for comparison. Notice that C=C formation during the transhydrogenation reaction does not slow markedly with time and that the reaction does not proceed to completion as the oxidase reaction does. An explanation for the markedly different time courses for the two reactions involves the formation and breakup of an intermediate during the oxidase reaction but not during the transhydrogenase reaction. It has been shown that dihydroflavin reacts with O_2 to form a C-4a hydroperoxide (Yamasaki & Yamano, 1973); accumulation of such a product whose breakup was rate limiting would explain the rapid slowing of C=C formation and the less than quantitative production of H_2O_2. The latter would result from the accumulation of H_2O_2 in the C-4a hydroperoxide intermediate. Figure 5 shows a high-performance liquid chromatogram of the products and reactants of the reaction of FPCoA and crotonyl-CoA catalyzed by acyl-CoA dehydrogenase. Note that crotonyl-CoA has been transformed into the transhydrogenation product, butyryl-CoA. From the quantitative data in Table IV, we can show that within experimental error the concentration of product FACoA observed from the 340-nm absorbance is identical with that calculated from the HPLC chromatograms using 260-nm absorbance of CoA and the area under the chromatographic peaks to calculate concentration. Furthermore, the last data point which represents chemical equilibrium can be used to estimate $\Delta G_0 = -1.41$ kcal/mol based upon the equilibrium constant for the transhydrogenation reaction shown in eq 2.

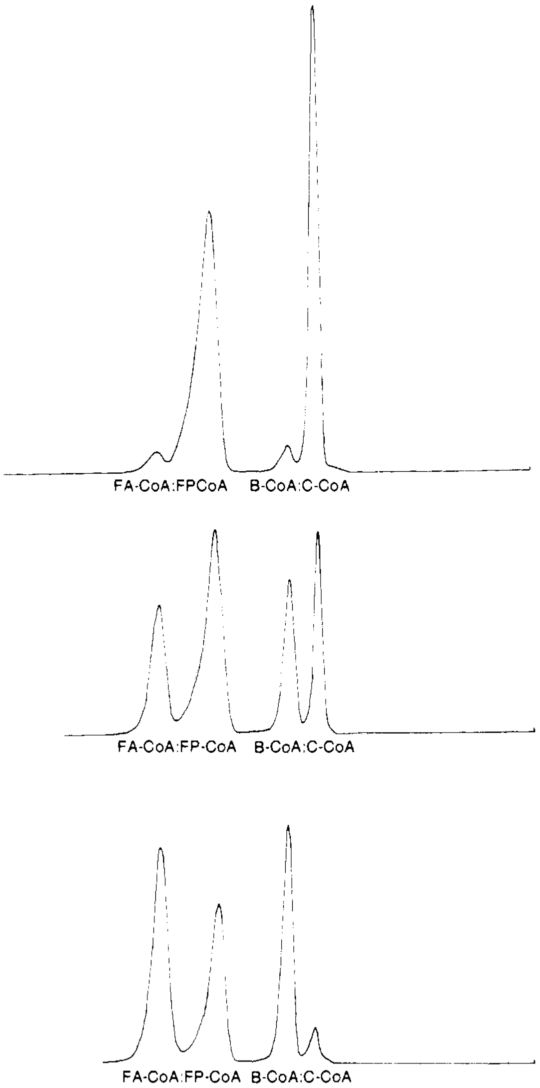
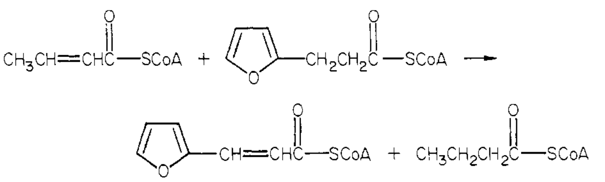


FIGURE 5: Time course of the reaction of crotonyl-CoA and FPCoA. [FPCoA] = 115.5 μM; [crotonyl-CoA] = 73.2 μM; pH 8.5, 20 mM Tris, 25 °C. (Upper frame) Time: 20.2 min. (Middle frame) Time: 152.8 min. (Lower frame) Time: 389.8 min.



(2)

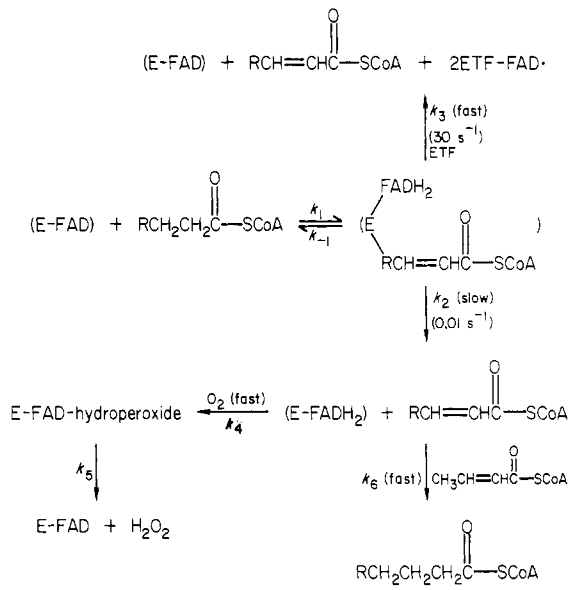
In conclusion, we have found that fatty acyl-CoA dehydrogenase catalyzes two unusual reactions at the rate at which the charge-transfer complex of reduced enzyme and FACoA dissociates. The first of these is an oxidase reaction producing H_2O_2, while the second is a transhydrogenase reaction in which crotonyl-CoA is reduced to butyryl-CoA. The fact that these reactions proceed only as rapidly as product charge-transfer complex dissociates stands in marked contrast to the rapid reaction of ETF with FPCoA. This indicates that the charge-transfer complex is essential for the specificity of transfer of electrons to ETF and that electron transfer clearly proceeds through this complex.

Discussion

The results shown in Figure 1 and Table I establish that the biphasic kinetics for flavin bleaching in the FAD of fatty acyl-CoA dehydrogenase upon substrate reaction is also observed for the production of C═C with our chromophoric pseudosubstrate. Furthermore, the relative stoichiometry of the reaction in each of the two steps is the same on the basis of the use of the normal extinction coefficient change for FAD bleaching (Hall & Kamin, 1975) and ϵ_{340} for FACoA. There are two possible explanations for the biphasic behavior; the first is that it results from subunit interaction within the tetrameric enzyme, and the second is that it results from intermediate formation or the reverse kinetic step of C═C reduction during approach to chemical equilibrium. We are currently performing kinetic investigations which will distinguish between these alternatives.

One striking observation has come from our investigation of the reaction of our new pseudosubstrate with fatty acyl-CoA dehydrogenase; it is possible to determine that fatty acyl-CoA dehydrogenase acts as a slow oxidase catalyst producing *trans*-FACoA and H_2O_2. Furthermore, the oxidase activity takes place at a rate equal to the rate for charge transfer product complex dissociation. It has been recognized for many years (Massey & Hemmerich, 1980) that reduced flavoproteins can react with molecular oxygen to yield oxidized flavin and H_2O_2 or O_2^-. However, it appears that the fatty acyl-CoA dehydrogenase CT complex formed during reaction protects the reduced flavin and allows it to transfer electrons only to ETF and subsequently to the electron transport chain. As the CT complex dissociates, the reduced flavin of dehydrogenase reacts with O_2 at the same rate as complex dissociation (octanoyl-CoA or FPCoA) or at a slower rate (butyryl-CoA). With the latter substrate, it seems likely that reaction with O_2 has become rate limiting. Equation 3 outlines our mechanistic proposal.

Since ETF reacts with a rate constant of 30 s^{-1}, the ETF reaction must proceed through the charge transfer product complex, and no dissociation of product is necessary for this reaction to take place. This stands in marked contrast to the reaction with other electron acceptors such as O_2 or crotonyl-CoA which require dissociation of enoyl-CoA in order for reaction to occur. It is appealing to suggest that the concentration of fully oxidized ETF controls the ultimate fate of reducing equivalents of fatty acids in the mitochondrion, with electrons flowing through the electron-transfer chain, when sufficient ETF is present. In the absence of oxidized ETF, small electron acceptors react with reduced acyl-CoA dehydrogenase at the rate at which product dissociates from the charge-transfer complex, allowing excess reducing power to be expended rather than being converted directly to ATP



(3)

through the process of electron transfer and oxidative phosphorylation. The formation of CT product complex thus mitigates against oxidase or transhydrogenase activity for the dehydrogenase enzyme and assures that electrons are profitably transferred to the ET chain. The CT product complex plays a central and essential role in catalysis of transfer of electrons from fatty acyl-CoA to the ET chain.

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