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Stabilization of the Red Semiquinone Form of Pig Kidney General Acyl-CoA Dehydrogenase by Acyl Coenzyme A Derivatives†

John P. Mizzer and Colin Thorpe*

ABSTRACT: Pig kidney general acyl-CoA dehydrogenase forms the blue neutral radical on dithionite or photochemical reduction [Thorpe, C., Matthews, R. G., & Williams, C. H. (1979) *Biochemistry 18*, 331–337] in accord with its classification as a flavoprotein dehydrogenase. However, dithionite reduction of the enzyme in the presence of crotonyl coenzyme A (crotonyl-CoA) or octenoyl-CoA generates the red radical anion as the predominant species at pH 7.6. Crotonyl-CoA binds preferentially to the red radical form, depressing the apparent p*K* by at least 2.5 pH units to a value of 7.3. Butyryl-, octanoyl-, and palmitoyl-CoA induce very similar spectral changes to those induced by enoyl-CoA derivatives when added anaerobically to the blue semiquinone enzyme. In contrast, the competitive inhibitors acetoacetyl-CoA and heptadecyl-SCoA do not markedly perturb the spectrum of the neutral flavosemiquinone species. The stability of the enzyme radical complexes with either crotonyl- or octanoyl-CoA suggests that there is not effective intraflavin transfer of reducing equivalents between subunits. Perturbation of the spectrum of the one-electron-reduced enzyme by ligands may complicate interpretation of the reaction between substrate complexes of the general acyl-CoA dehydrogenase and electron-transferring flavoprotein.

Mammalian acyl-CoA dehydrogenases catalyze the first step of β oxidation with the insertion of a trans double bond between C-2 and C-3 of their fatty acyl thioester substrates. Three classes of mammalian enzymes have been identified with overlapping substrate specificities for short (Green et al., 1954), medium (Crane et al., 1956; Hall & Kamin, 1975; Thorpe et al., 1979), and long acyl chains (Hauge, 1956; Hall et al., 1976). The enzyme with a broad specificity profile for me-

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dium-chain substrates has received the most attention and is termed the general acyl-CoA dehydrogenase. Turnover of the general acyl-CoA dehydrogenase is initiated on binding an appropriate saturated thioester, e.g., octanoyl coenzyme A (octanoyl-CoA), with the subsequent appearance of a long-wavelength-absorbing species (Crane et al., 1956; Thorpe et al., 1979; Hall et al., 1979). This band was originally ascribed to a semiquinone (Beinert, 1957), but in light of a body of evidence from butyryl-CoA dehydrogenase and other flavoproteins, it probably represents a charge-transfer complex between reduced flavin as the donor and enoyl-CoA as the acceptor (Engel & Massey, 1971; Massey & Ghisla, 1974). This reduced enzyme·octenoyl-CoA complex is an extremely tight one (Steyn-Parvé & Beinert, 1958; Thorpe et al., 1981). The physiological oxidant of this charge-transfer complex is electron-transferring flavoprotein (ETF)[1] (Crane et al., 1956; Crane & Beinert, 1956). On the basis of rapid reaction studies, ETF is reduced within the ternary complex first to the red anion semiquinone state and then more slowly to the dihydroflavin form (Hall & Lambeth, 1980; Reinsch et al., 1980). Thus, it would be anticipated that the semiquinone form of acyl-CoA dehydrogenase is catalytically important in the oxidative half-reaction.

We have previously reported the results of dithionite titrations and photochemical reduction of the pig kidney general acyl-CoA dehydrogenase (Thorpe et al., 1979, 1980). In all cases, acyl-CoA dehydrogenases appear to stabilize the blue neutral flavosemiquinone form as would be expected for a flavoprotein dehydrogenase (Massey & Hemmerich, 1980). This paper reports the surprising finding that crotonyl-CoA binding stabilizes the red radical form of the pig kidney enzyme by a depression of the apparent pK by at least 2.5 pH units.

Experimental Procedures

Materials. Pig kidney general acyl-CoA dehydrogenase was prepared as described by Thorpe et al. (1979). Butyryl-, octanoyl-, palmitoyl-, crotonyl-, and acetoacetyl-CoA were from P-L Biochemicals. *S*-Heptadecyl-CoA was prepared as described by Ciardelli et al. (1981) and was the gift of Dr. Theodor Wieland. 3,10-Dimethyl-5-deazaisoalloxazine and lumiflavin 3-acetate were gifts from Dr. V. Massey. Sodium dithionite (low-iron grade) was from Fisher, and methyl viologen was from Sigma. Octenoyl-CoA was synthesized via the mixed anhydride method (Bernert & Sprecher, 1977).

Methods. Unless otherwise stated, all buffers contained 0.3 mM EDTA; quoted pH values refer to 25 °C. Concentrations of pig kidney general acyl-CoA dehydrogenase are calculated by using an ϵ_{446} value of 15.4 mM^{-1} cm^{-1} for enzyme-bound flavin (Thorpe et al., 1979). Anaerobic spectrophotometric experiments were performed in 1-mL capacity anaerobic cuvettes using the techniques summarized by Williams et al. (1979). Visible and UV spectra were recorded with a Cary 219 spectrophotometer.

Reduction Methods. Several methods were tried during attempts to generate the flavosemiquinone state quantitatively from oxidized pig kidney general acyl-CoA dehydrogenase. Photoreduction at pH 7.6 with 25 mM oxalate using either 2.9 μM deazaflavin (Massey & Hemmerich, 1978) or 1.3 μM lumiflavin 3-acetate gave maximal apparent ϵ_{560} values of 2.2 and 0.2 mM^{-1} cm^{-1}, respectively. Anaerobic titration of the enzyme with one-electron-reduced methyl viologen, prepared by dithionite reduction, led to the formation of the two-electron-reduced enzyme with no significant accumulation of radical. Ferricyanide back-titration of the fully reduced enzyme gives the highest yields of semiquinone to date (Thorpe et al., 1979).

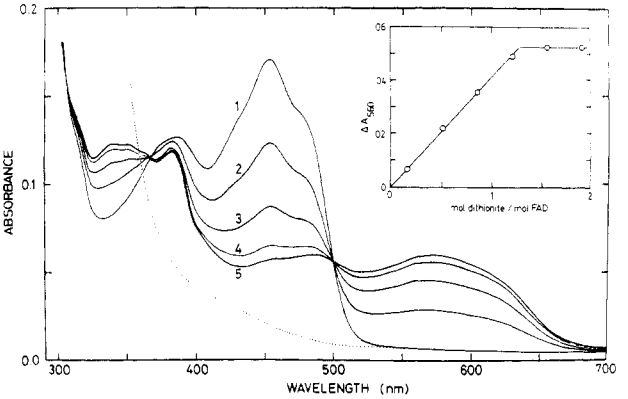


FIGURE 1: Dithionite reduction of pig kidney general acyl-CoA dehydrogenase at pH 6.7 in the presence of crotonyl-CoA. An anaerobic solution of enzyme (11.8 μM enzyme-bound FAD in 0.75 mL of 10 mM potassium phosphate buffer, pH 6.7, 25 °C, containing 190 μM crotonyl-CoA) was titrated with a solution of 3.09 mM dithionite (see Methods). Curve 1, 0; 2, 0.52; 3, 0.88; 4, 1.23; 5, 1.59 mol of dithionite/mol of FAD. Intermediate spectra have been omitted for clarity. The dotted spectrum was recorded after addition of excess dithionite. The inset shows absorbance at 560 nm vs. mol of dithionite/mol of FAD.

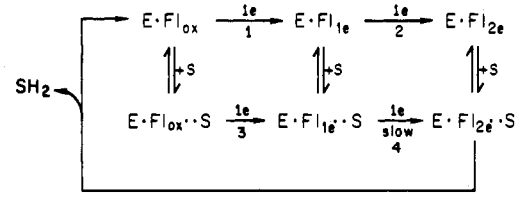
Calculations. A microcomputer was used to calculate K_D values (given total enzyme and ligand concentrations, and fractional saturation data) and to generate data for fitting a theoretical ionization curve to the inset in Figure 3.

Results and Discussion

Spectrophotometric titrations of the pig kidney general acyl-CoA dehydrogenase have been previously shown to yield appreciable levels of the blue flavosemiquinone species on the addition of 0.5 equiv of dithionite/mol of FAD (Thorpe et al., 1979). Dithionite titrations have been repeated at pH 6.7, 9.6 (data not shown), and 8.6 (see below). The course of these titrations is very similar to that observed at pH 7.6, with a maximal yield of blue semiquinone corresponding to apparent ϵ_{560} values of 2.6, 2.8, and 2.9 mM^{-1} cm^{-1} at pH values of 6.7, 8.6, and 9.6, respectively. These results are consistent with the generalization that flavoprotein dehydrogenases stabilize the blue, neutral, flavosemiquinone form (Massey & Palmer, 1966; Massey & Hemmerich, 1980). Figure 1 shows a dithionite titration of general acyl-CoA dehydrogenase at pH 6.7 in the presence of 190 μM crotonyl-CoA. The distinct shoulder at 480 nm in curve 1 reflects the perturbation of the spectrum of the native enzyme on binding crotonyl-CoA (K_D = 18 μM, not shown). Clearly a much higher yield of the blue semiquinone species is obtained (observed ϵ_{560} = 4.9 mM^{-1} cm^{-1}) than with comparable titrations performed in the absence of enoyl-CoA. Although the spectrum of the flavosemiquinone form shown in Figure 1 is similar to that observed in the absence of crotonyl-CoA, the presence of this ligand induces a red shift of approximately 8 nm in the λ_{max} of the long-wavelength band. An isosbestic point at 500 nm is preserved until maximal semiquinone formation is attained, in contrast to the behavior of the free enzyme, where increasing levels of two-electron-reduced enzyme complicate the titration (Thorpe et al., 1979). The flavosemiquinone is generated rapidly after each addition of dithionite, and a plot of A_{560} vs. equivalents of dithionite is shown in the inset to Figure 1. Maximal semiquinone formation requires 1.3 mol of dithionite in marked contrast to the 0.5 mol expected for titration of the free enzyme

[1] Abbreviations used: ETF, electron-transferring flavoprotein; E·Fl_{ox}, E·Fl_{1e}, and E·Fl_{2e}, oxidized, one-electron-reduced, and two-electron-reduced enzyme; GAD, general acyl-CoA dehydrogenase; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide.

Scheme I



(Thorpe et al., 1979). Further additions of dithionite (see inset, Figure 1) are consumed by the system as evident by following the absorbance of the solution at 320 nm. However, no significant changes in the visible spectrum of the solution occur until a concentration of dithionite equivalent to that of the crotonyl-CoA originally present has been added. The dotted line in Figure 1 is the final spectrum produced by excess dithionite.

The results obtained in this work are consistent with Scheme I, which is discussed at this stage for clarity. The designation E·Fl$_{1e}$ is adopted here since Scheme I applies to the formation of both neutral and anion flavosemiquinone species (see below).

The single horizontal arrows (reactions 1 and 2) at the top of the scheme indicate reduction of the enzyme by dithionite. As observed with many other flavoproteins, the disproportionation reaction:

$$2E\cdot Fl_{1e} \rightleftharpoons E\cdot Fl_{ox} + E\cdot Fl_{2e}$$

is very slow with the pig kidney enzyme (Thorpe et al., 1979; V. Massey and C. Thorpe, unpublished experiments), and thus the products of reductive titrations in the absence of mediator are kinetically determined. Thus, the yield of semiquinone is markedly dependent on the method of reduction (Thorpe et al., 1979; see below). A kinetic analysis of the reduction of general acyl-CoA dehydrogenase by dithionite has not been undertaken, but for simplicity, the reaction is depicted as involving two successive one-electron steps (Lambeth & Palmer, 1973). The maximal yield of about 50% flavosemiquinone obtained during dithionite titrations of the free enzyme (Thorpe et al., 1979) suggests that reaction 2 is significant relative to reaction 1. Since crotonyl-CoA can reoxidized E·Fl$_{2e}$ (but *not* E·Fl$_{1e}$), it serves to regenerate oxidized enzyme which can then undergo another round of dithionite reduction.

Thus, a high proportion of the enzyme would eventually be sequestered in the semiquinone form, no longer capable of reoxidization by the product. The stoichiometry of 1.3 molecules of dithionite consumed before a maximal level of semiquinone is reached is consistent with this explanation. The differences between the spectra of semiquinone generated in the presence or absence of crotonyl-CoA suggest that the semiquinone is at least partially saturated with thioester under the conditions shown in Figure 1. This is also suggested by examination of the rate of dithionite reduction of the flavosemiquinone in the presence or absence of crotonyl-CoA. While reduction of the free flavosemiquinone by dithionite at pH 6.7 is complete before absorbance measurements can be made, as judged by either the loss of semiquinone absorbance at 560 nm or the decay of dithionite absorbance at 320 nm, the oxidation of aliquots of dithionite added in the plateau region (inset to Figure 1) is much slower, taking several minutes for completion. Thus, bound crotonyl-CoA hinders further reduction of the flavosemiquinone by dithionite (see Scheme I). A control experiment, performed in the absence of enzyme, ruled out the possibility that nonenzymatic reduction of crotonyl-CoA was significant over the time scale of these experiments.

It should be noted that the E·Fl$_{1e}$··S species (Scheme I) could be generated either on complexation of E·Fl$_{1e}$ by S or by one-electron reduction of the E·Fl$_{ox}$··S complex (reaction 3). The relative importance of these two processes is not yet known.

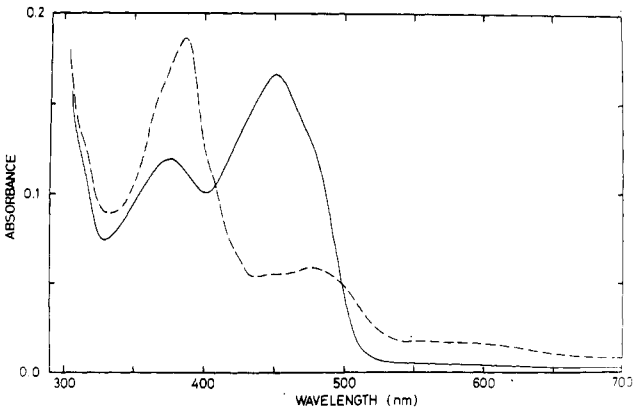


FIGURE 2: Dithionite titration of general acyl-CoA dehydrogenase at pH 8.6 in buffer containing crotonyl-CoA. The enzyme (11.6 μM in 0.74 mL of 50 mM Tris buffer, pH 8.6, containing 177 μM crotonyl-CoA) was deoxygenated and the spectrum recorded before (—) and after (---) the addition of 2.0 mol of dithionite/mol of enzyme FAD.

In striking contrast to Figure 1, a dithionite reduction performed at pH 8.6 yields large levels of the red flavosemiquinone species (Figure 2). The spectrum at maximal semiquinone formation exhibits a λ_{max} of 385 nm and an apparent extinction coefficient of 16.0 mM^{-1} cm^{-1} (Figure 2). Despite the clear resemblance of the spectrum to that of a red semiquinone, a weak featureless long-wavelength tail extends beyond 700 nm (Figure 2) whereas the red semiquinone of glucose oxidase does not absorb significantly at wavelengths greater than 600 nm (Massey & Palmer, 1966). The band shown in Figure 2 does not represent turbidity since it disappears on reoxidation of the enzyme at the end of the experiment. Recently, a similar long-wavelength absorbance in the spectrum of complexes of the red radical of lactate oxidase with pyruvate has been observed (Choong & Massey, 1980) and ascribed to a charge-transfer interaction between the semiquinone as donor and the α-keto acid as acceptor. The long-wavelength band in Figure 2 could thus represent an analogous interaction between the flavin and the enoyl-CoA moiety.

A dithionite titration performed at pH 7.6 in the presence of 155 μM crotonyl-CoA revealed a mixture of blue and red forms, and thus it was of interest to determine the apparent pK for the interconversion of these species. The blue semiquinone was generated in 10 mM phosphate buffer, pH 6.7, containing 190 μM crotonyl-CoA, and the pH of the solution was raised by addition of aliquots of an anaerobic solution of 2 M Tris buffer, pH 9.1. Figure 3 shows that the decline in the absorbance at 560 nm is accompanied by the appearance of the red semiquinone spectrum. These changes were complete before absorbance measurements could be made. The inset plots the A_{385} data vs. the pH of the solution. The solid line is a curve calculated by assuming that the conversion of blue to red semiquinone forms involves release of 1.7 protons per flavin (see legend, Figure 3). A theoretical curve, representing a single proton ionization with the same midpoint (pK = 7.3, not shown), is clearly a worse fit to the data.

Thus, crotonyl-CoA appears to stabilize the red semiquinone form of general acyl-CoA dehydrogenase, and this is most directly tested by adding the enoyl-CoA to preformed blue semiquinone at pH 8.6. This experiment would also allow estimation of the apparent dissociation constant for crotonyl-CoA. Unfortunately, we have been unable to generate the

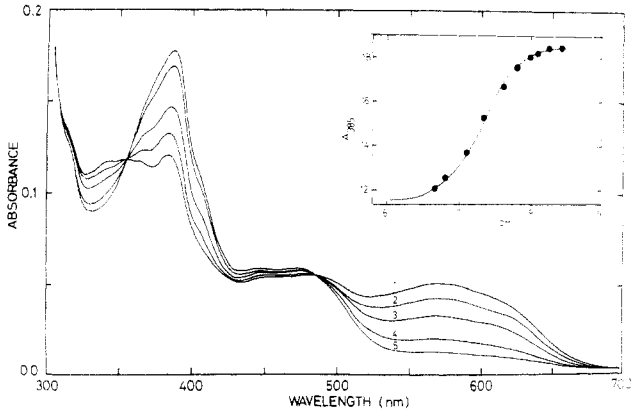


FIGURE 3: pH titration of the flavosemiquinone-crotonyl-CoA complex. An anaerobic solution of enzyme (11.8 μM in 10 mM phosphate buffer, pH 6.7, containing 190 μM crotonyl-CoA) was titrated to maximal blue semiquinone formation as described in Figure 1. The spectrum was recorded (curve 1), and then the pH of the solution was raised by the addition of deoxygenated Tris buffer (2 M, pH 9.1). Curves 1–5 correspond to pH values of 6.8, 7.10, 7.34, 7.80, and 8.44, respectively, determined from a control titration. The final pH of the solution agreed with that expected from control experiments. The inset is a plot of the absorbance at 385 nm, corrected for dilution, vs. pH. The solid line is calculated for a process involving the release of 1.7 H^+ with a midpoint of pH 7.3.

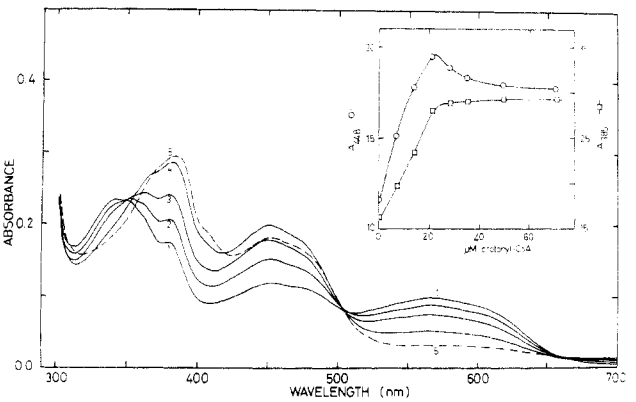


FIGURE 4: Titration of flavosemiquinone at pH 8.6 with crotonyl-CoA. Oxidized dehydrogenase (23.6 μM in 0.78 mL of 50 mM Tris-HCl, pH 8.6, 25 °C) was fully reduced anaerobically with 2.95 mM dithionite and then back-titrated with an anaerobic solution of 0.97 mM ferricyanide to yield curve 1. This enzyme was then titrated with an anaerobic solution of 5.8 mM crotonyl-CoA: curves 2–5, 7.2, 14.3, 21.4, and 50 μM crotonyl-CoA, respectively. The inset plots absorbances at 385 and 448 nm, corrected for dilution, vs. concentration of crotonyl-CoA. With the assumption of simple binding, a 90% completion of A_{385} changes at 24 μM crotonyl-CoA requires a K_D of 3×10^{-7} M (see Methods).

blue radical form of general acyl-CoA dehydrogenase quantitatively, and the consequent presence of dihydroflavin and oxidized flavin forms complicated interpretation of these experiments. The best yields of semiquinone to date are obtained after back-titration of the fully reduced enzyme with ferricyanide (Thorpe et al., 1979). (Several other methods which have been tried are described under Methods.) The spectrum of the semiquinone form of uncomplexed general acyl-CoA dehydrogenase at pH 8.6 is shown in curve 1 of Figure 4. Comparison of these data with those of titrations at pH 9.6, 7.6, and 6.7 suggests that little, if any, red semiquinone form is present at pH values below 9.6. Spectra 2–5 represent the anaerobic additions of crotonyl-CoA to this solution, and the inset displays absorbance values at 448 and 385 nm vs. concentration of thioester added. Clearly, crotonyl-CoA is inducing the appearance of the red radical as judged by the rise in absorbance at 385 nm (see inset) and the concomitant decrease in the long-wavelength band. The rise in the 450-nm

Table I: Interaction of Acyl-CoA Derivatives and Analogues with Semiquinone Forms of Pig Kidney General Acyl-CoA Dehydrogenase

compound (L)	procedure	predominant semiquinone form observed
crotonyl-CoA	E·FAD_{ox} + 155 μM L + dithionite, pH 7.6, 8.6[a]	red
crotonyl-CoA	E·FAD_{1e} + 177 μM L, pH 8.6	red
octenoyl-CoA	E·FAD_{ox} + 100 μM L + dithionite, pH 7.6	red
butyryl-CoA	E·FAD_{1e} + 114 μM L, pH 8.6	red
octanoyl-CoA	E·FAD_{1e} + 65 μM L, pH 8.6	red
palmitoyl-CoA	E·FAD_{1e} + 126 μM L, pH 8.6	red
acetoacetyl-CoA	E·FAD_{ox} + 137 μM L + dithionite, pH 8.6	none observed
	E·FAD_{1e} + 74 μM L, pH 8.6	blue
heptadecyl-SCoA	E·FAD_{ox} + 52 μM L + dithionite, pH 8.6	blue
heptadecyl-SCoA	E·FAD_{1e} + 38 μM L, pH 8.6	blue

[a] pH 8.6 buffer was 50 mM Tris-HCl; pH 7.6 buffer was 100 mM potassium phosphate.

absorbance probably reflects reoxidation of the E·Fl_{2e} species by crotonyl-CoA (see above; Thorpe et al., 1979). The subsequent decline in A_{450} probably reflects perturbation of the oxidized enzyme on the addition of further crotonyl-CoA. The generation of an isosbestic point at 660 nm again suggests that the rise in absorbance at 700 nm is not due to turbidity (see above). This new band does not represent a charge-transfer interaction between crotonyl-CoA and E·Fl_{2e} since a complex of this composition is not observed in static experiments with the pig kidney dehydrogenase (Thorpe et al., 1979; J. P. Mizzer and C. Thorpe, unpublished experiments). Although it is not possible to determine an accurate dissociation constant for the crotonyl-CoA·enzyme semiquinone complex from this data, the 385-nm absorbance changes are nearly complete after the addition of 1 equiv of crotonyl-CoA (see inset), suggesting that an upper estimate for the apparent K_D would be in the range of 1 μM (see Methods).

The experiments described above show that crotonyl-CoA binding lowers the apparent pK of the blue flavosemiquinone enzyme species by at least 2.5 pH units, thereby inducing formation of appreciable levels of the red radical above pH 7. Thus, it was of interest to test whether this effect could also be observed with ligands other than crotonyl-CoA. As shown in Table I, octenoyl-CoA also induces red semiquinone formation, and the ratio of red to blue forms at pH 7.6 is very similar to that observed in a comparable experiment using crotonyl-CoA. Surprisingly, the phenomenon is not restricted to enoyl-CoA ligands, since the saturated acyl thioesters butyryl-, octanoyl-, and palmitoyl-CoA all induce red radical formation when added to the preformed blue semiquinone enzyme at pH 8.6 (Table I). As would be expected, the flavosemiquinone in these substrate complexes does not undergo further reduction to the dihydroflavin level, since this would generate a corresponding acyl-CoA radical species. The resistance of the red radical toward one-electron reduction in the E·FAD_{1e}·SH_2 complex, or toward a corresponding oxidation in the E·FAD_{1e}·S species (Figure 2), underscores the extreme slowness of semiquinone disproportionation in this enzyme. In addition, it suggests that there is no effective intraflavin redox communication between the four subunits. Thus, disproportionation would generate E·Fl_{ox} which would be rapidly reduced by octanoyl-CoA to the characteristic E·Fl_{2e}·octenoyl-CoA complex (Crane et al., 1956; Thorpe et al., 1979). Conversely, the dihydroflavin form derived from a radical disproportionation reaction would be rapidly reox-

idized by crotonyl-CoA to E·Fl_{ox} (Thorpe et al., 1979; J. P. Mizzer and C. Thorpe, unpublished experiments).

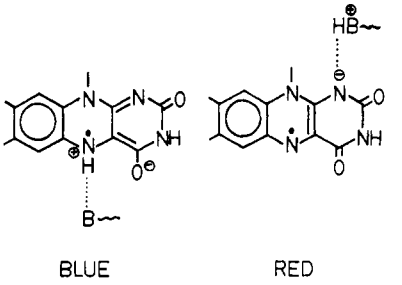
Acetoacetyl-CoA has been shown to bind tightly to acyl-CoA dehydrogenases from a variety of sources (Engel & Massey, 1971; McKean et al., 1979; C. Thorpe, unpublished experiments), with the formation of a long-wavelength band ascribed to a charge-transfer interaction between the enolate form of the ligand as donor and oxidized flavin as acceptor (Engel & Massey, 1971; Massey & Ghisla, 1974; McKean et al., 1979). Acetoacetyl-CoA does not induce conversion of blue to red radical states at pH 8.6 (Table I). In addition, insignificant amounts of either semiquinone were generated on dithionite reduction of the oxidized enzyme·acetoacetyl-CoA complex (Table I). The long-chain thioether analogue heptadecyl-SCoA has been shown to bind extremely tightly to pig kidney general acyl-CoA dehydrogenase but is incapable of enzyme-catalyzed dehydrogenation (Thorpe et al., 1981). Reduction of the enzyme·thioether complex yields the characteristic blue semiquinone spectrum (maximal observed ϵ_{560} = 2.0 mM^{-1} cm^{-1}) with no indication of the red form. Similarly, an anaerobic titration of the blue radical with the thioether analogue at pH 8.6 gave clear indications of complex formation (e.g., with an approximately 8-nm red shift in the position of the long-wavelength band), but without detectable conversion to the red semiquinone. This is in marked contrast to the long-chain substrate palmitoyl-CoA which stabilizes the red radical (Table I).

Massey and co-workers have emphasized the general correlation between the ability of a flavoprotein to stabilize the red semiquinone form and the ease with which sulfite attacks the N-5 position to form flavin adducts (Massey et al., 1969). As would be expected, pig kidney general acyl-CoA dehydrogenase does not form detectable levels of such an adduct using 0.2 M sulfite in 100 mM phosphate buffer, pH 7.6. Since crotonyl-CoA binding induces formation of the red radical form of this dehydrogenase, it was of interest to repeat the experiments in the presence of crotonyl-CoA. No adduct formation was detected.

The occurrence of the red radical form in flavoprotein dehydrogenases appears to be rare. However, NADH:cytochrome b_5 reductase exhibits the red rather than the blue radical forms when NAD^+ is added to partially reduced enzyme (Iyanagi, 1977). The present case is of great interest because, like glucose oxidase (Massey & Palmer, 1966), the levels of the two forms can be readily manipulated since the pK of the interconversion lies well within the range of stability of the protein. The formation of red radical induced by enoyl-CoA and corresponding saturated derivatives raises questions concerning the mechanism of stabilization of this species and the possible relevance of this phenomenon to catalysis in the acyl-CoA dehydrogenases. These points are addressed below.

The N-5 locus on the isoalloxazine ring seems the most likely position for entry of reducing equivalents derived from dehydrogenation of C–H substrates (Bright & Porter, 1975; Hemmerich, 1976; Ghisla et al., 1979; Walsh, 1980; Bruice, 1980) although the precise mechanism of this reaction is disputed. Thus, in the acyl-CoA dehydrogenases, a close approach between C-2 and/or C-3 of the substrate and the N-5 position would be anticipated. Massey & Hemmerich (1980) have suggested that the N-5 position of the isoalloxazine ring is also involved in the stabilization of the blue radical state via formation of a H bond between the N-5 H and a protein acceptor (Scheme II). In contrast, the red anion form would be stabilized by an electrostatic interaction between the negatively charged N-1–C-2(=O) locus and a suitable protonated residue on the protein. Thus, from the above arguments, any ligand which disrupts the H-bonding interaction at N-5 might be expected to shift the equilibrium in favor of the red radical. Obviously, this explanation does not readily account for the inability of heptadecyl-SCoA and acetoacetyl-CoA to induce red radical formation. It is not yet clear what aspects of the ligand–protein interaction are important in lowering the pK of the blue semiquinone species.

Scheme II



It should be noted that there are other examples in which the ionization state of a flavin radical is influenced by the presence of a ligand. For example, the addition of the product glucono-δ-lactone to the blue flavin radical of glucose oxidase at pH 6.3 affects the appearance of a red radical spectrum (Massey et al., 1966). Conversely, addition of the negatively charged benzoate moiety to the anion radical of D-amino acid oxidase causes the appearance of a blue semiquinone spectrum (Yagi, 1975). Salicylate similarly promotes blue radical formation in salicylate hydroxylase (Yasuda et al., 1967).

A number of studies have addressed the mechanism of reoxidation of acyl-CoA dehydrogenase·substrate complexes by the physiological electron acceptor, ETF (Crane & Beinert, 1956; Hall & Lambeth, 1980; Reinsch et al., 1980). Hall & Lambeth (1980) observed biphasic absorbance changes at 375 nm (a wavelength selected to monitor the red radical anion of ETF). The initial rapid ($\sim 6 \times 10^6$ M^{-1} s^{-1}) absorbance increase was ascribed to generation of the ETF radical via a corresponding one-electron oxidation of the dehydrogenase. The subsequent decrease in the 375-nm absorbance was about 10-fold slower and was attributed to generation of the ETF dihydroflavin. For the purposes of discussion, this sequence of reactions involving the ternary complex of dehydrogenase, thioester, and ETF will be represented.

$$[GAD(Fl_{2e})\cdot S]\cdot[ETF(Fl_{ox})] \xrightarrow{\text{fast}} [GAD(Fl_{1e})\cdot S]\cdot[ETF(Fl_{1e})]$$

$$[GAD(Fl_{1e})\cdot S]\cdot[ETF(Fl_{1e})] \xrightarrow{\text{slower}} [GAD(Fl_{ox})\cdot S]\cdot[ETF(Fl_{2e})]$$

A surprising finding of the stopped-flow absorbance studies of Hall & Lambeth (1980) was that one-electron reduction of ETF was not accompanied by the expected appearance of a long-wavelength absorbance due to the blue semiquinone of the dehydrogenase. These authors concluded that this species was not involved in turnover. Our studies offer a plausible explanation for their results. At pH 7.6, about 75% of general acyl-CoA dehydrogenase semiquinone is present as the red radical when complexed with either crotonyl- or octenoyl-CoA. The apparent extinction coefficient of such a mixture of red and blue forms is about 1.8 mM^{-1} cm^{-1} at 600 nm (data calculated from Figures 1 and 2) compared to 2.3 mM^{-1} cm^{-1} for the E·FL_{2e}·octenoyl-CoA complex (Thorpe et al., 1979). Since neither the free radical nor the oxidized forms of ETF absorb significantly at 600 nm, an overall decline of absorbance

at this wavelength would be anticipated. This is precisely the result obtained by Hall & Lambeth (1980).

This study has demonstrated, in several types of static titration experiments, the thermodynamic stabilization afforded the red radical form of the enzyme on binding enoyl-CoA derivatives and acyl-CoA substrates. Rapid reaction studies will be required to confirm whether a similar behavior occurs within the ternary complex as has been suggested here. Certainly, the results of earlier stopped-flow studies should be reviewed with this possibility in mind, particularly the validity of following the extent of ETF radical anion production at 375 nm.

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