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Articles

Alternate Electron Acceptors for Medium-Chain Acyl-CoA Dehydrogenase: Use of Ferricenium Salts[†]

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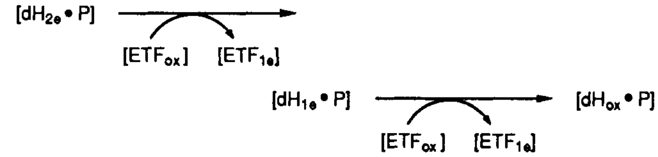
ABSTRACT: Medium-chain acyl-CoA dehydrogenase reduced with octanoyl-CoA is reoxidized in two one-electron steps by two molecules of the physiological oxidant, electron transferring flavoprotein (ETF). The organometallic oxidant ferricenium hexafluorophosphate ($Fc^+PF_6^-$) is an excellent alternative oxidant of the dehydrogenase and mimics a number of the features shown by ETF. Reoxidation of octanoyl-CoA-reduced enzyme (200 μM $Fc^+PF_6^-$ in 100 mM Hepes buffer, pH 7.6, 1 °C) occurs in two one-electron steps with pseudo-first-order rate constants of 40 s^{-1} and about 200 s^{-1} for k_1 and k_2, respectively. The reaction is comparatively insensitive to ionic strength, and evidence of rate saturation is encountered at high ferricenium ion concentration. As observed with ETF, the free two-electron-reduced dehydrogenase is a much poorer kinetic reductant of $Fc^+PF_6^-$, with rate constants of 3 s^{-1} and 0.3 s^{-1} (for k_1 and k_2, respectively) using 200 μM $Fc^+PF_6^-$. In addition to the enoyl-CoA product formed during the dehydrogenation of octanoyl-CoA, binding a number of redox-inert acyl-CoA analogues (notably 3-thia- and 3-oxaoctanoyl-CoA) significantly accelerates electron transfer from the dehydrogenase to $Fc^+PF_6^-$. Those ligands most effective at accelerating electron transfer favor deprotonation of reduced flavin species in the acyl-CoA dehydrogenase. Thus this rate enhancement may reflect the anticipated kinetic superiority of anionic flavin forms as reductants in outer-sphere electron-transfer processes. Evidence consistent with the presence of two distinct loci for redox communication with the bound flavin in the acyl-CoA dehydrogenase is presented.

Several mitochondrial FAD-linked dehydrogenases involved in fatty acid oxidation, amino acid catabolism, and one-carbon metabolism deliver reducing equivalents to electron transferring flavoprotein (ETF;[1] Crane & Beinert, 1956), a heterodimeric protein carrying a single flavin prosthetic group (Furuta et al., 1981; Gorelick et al., 1982; McKean et al., 1983; Husain & Steenkamp, 1983). ETF, in turn, discharges reducing equivalents to the respiratory chain via a third flavoprotein, ETF-QO oxidoreductase (Ruzicka & Beinert, 1977; Beckmann & Frerman, 1985; Ramsay et al., 1987). Of the primary dehydrogenases servicing ETF, the medium-chain acyl-CoA dehydrogenases have received the most attention. Reduction of the dehydrogenase by a preferred substrate such as octanoyl-CoA (SH_2) leads to the rapid formation of a purple charge-transfer complex between two-electron-reduced flavin and bound enoyl-CoA product ($dH_{2e}{\cdot}P$; Beinert, 1963; Massey & Ghisla, 1974; Hall et al., 1979; McFarland et al., 1982; Thorpe & Massey, 1983; Schopfer et al., 1988; Lau & Thorpe, 1988):

$$dH_{ox} + SH_2 \rightleftharpoons [dH_{ox}{\cdot}SH_2] \rightleftharpoons [dH_{2e}{\cdot}P]$$

This air-stable species reacts with ETF yielding the red ETF semiquinone at rates compatible with overall catalysis (Hall & Lambeth, 1980; Reinsch et al., 1980; Gorelick et al., 1985). Further studies showed that $dH_{2e}{\cdot}P$ was reoxidized in two rapid one-electron steps by 2 molecules of oxidized ETF with half-times of about 30 ms for equimolar reactants (10 μM, 1 °C, pH 7.6; Gorelick et al., 1985):



Such interflavin redox reactions appear to involve obligatory one-electron steps (Gorelick & Thorpe, 1986) and probably proceed by an outer-sphere mechanism from the comparatively buried dehydrogenase flavin (Thorpe & Massey, 1983) to the exposed dimethylbenzene edge of the prosthetic group in ETF (Gorelick & Thorpe, 1986). The subsequent reduction of ETF_{1e} to ETF_{2e} by either semiquinone or dihydroflavin forms of the dehydrogenase is much slower ($t_{1/2}$ of about 1 s) and is not catalytically significant (Hall & Lambeth, 1980; Reinsch et al., 1980; Gorelick et al., 1985).

The importance of bound enoyl-CoA product in accelerating interflavin electron transfer between dehydrogenase and ETF is shown by the some 30-fold slower rates when photochemically reduced enzyme was used (Gorelick et al., 1985). The molecular basis for this kinetic modulation is however unclear, particularly since product binding renders the dehydrogenase a thermodynamically poorer reductant of ETF (Gorelick et al., 1985; Stankovich & Soltysik, 1987). Possibly, product

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[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; dH_{ox}, dH_{1e}, and dH_{2e}, oxidized, semiquinone, and two-electron-reduced forms of acyl-CoA dehydrogenase without regard to protonation state of the flavin prosthetic group; ETF, electron transferring flavoprotein; $Fc^+PF_6^-$, ferricenium hexafluorophosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; P, *trans*-2-octenoyl-CoA product; PMS, phenazine methosulfate.

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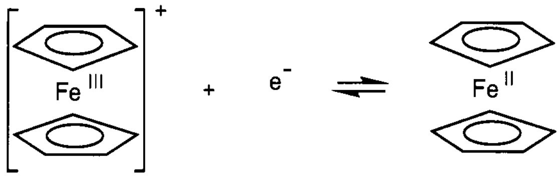
binding may induce conformational changes allowing a closer, or more effective, alignment of redox centers between dehydrogenase and ETF (Gorelick et al., 1985). A second suggestion is that the anionic flavin species stabilized by product binding (Mizzer & Thorpe, 1981; Thorpe & Massey, 1983) are more facile reductants than their neutral counterparts in the free dehydrogenase (Mizzer & Thorpe, 1982; Gorelick et al., 1985). A possible theoretical basis for this effect is presented below.

The present paper shows that the enhanced reactivity of product-complexed acyl-CoA dehydrogenase toward ETF is also manifest with several artificial redox dyes, and thus the effect is not limited to physiological partners. During this work we discovered that ferricenium salts ($Fc^+PF_6^-$) are excellent acceptors for the acyl-CoA dehydrogenase (Lehman et al., 1990), reacting some 40-fold faster than ferricyanide and some 150-fold faster than the widely employed mediator of the acyl-CoA dehydrogenases, phenazine methosulfate. Although widely employed as oxidants for heme, copper, and Fe/S containing proteins, the use of ferricenium derivatives with flavoproteins has been largely restricted to electroanalytical applications [e.g., Cass et al. (1985) and Lange et al. (1985)]. Finally, we show that a number of redox-inactive acyl-CoA derivatives can accelerate electron transfer from the reduced dehydrogenase to $Fc^+PF_6^-$, and thus ligand-induced modulation of kinetic reactivity is not limited to enoyl-CoA product. A molecular basis for this effect is proposed.



($Fc^+PF_6^-$) (Ferrocene)

MATERIALS AND METHODS

Materials. Ferrocene, 1,1′-dimethylferrocene, and $NaPF_6$ were purchased from Aldrich. CoASH (lithium salt), acetoacetyl-CoA, octanoyl-CoA, glucose, glucose oxidase, 2,6-dichlorophenolindophenol, phenazine methosulfate, crotonase, and octyl-Sepharose CL-4B were obtained from Sigma. Potassium ferricyanide was from Fisher. Octanoic acid, *trans*-2-octenoic acid, and 2-octynoic acid were obtained from Pfaltz and Bauer. Sodium dithionite was from the Virginia Smelting Co., Portsmouth, VA. 5-Deazariboflavin was a gift from Dr. Sandro Ghisla.

Synthesis of Acyl-CoA Thioesters. All CoA thioesters were prepared from the corresponding free acids by the mixed anhydride procedure (Bernert & Sprecher, 1977) and purified as in Powell et al. (1987). 3-Thia- and oxaoctanoyl-CoA were synthesized and purified as described earlier (Lau et al., 1988), and 3-ketooctanoyl-CoA was prepared by hydration of 2-octynoyl-CoA (Thorpe, 1986). The thioether octyl-CoA was prepared as in Powell et al. (1987).

Preparation of Ferricenium Salts. Ferrocene was converted to $Fc^+PF_6^-$ with minor modifications to the method of Neuss (1985). Ferrocene (0.5 g) was dissolved in 10 mL of concentrated H_2SO_4, incubated for 1 h at room temperature, and poured carefully into 150 mL of water. The blue solution was filtered through two layers of Whatman No. 1 filter paper, mixed with 10 mL of a saturated aqueous solution of $NaPF_6$, and cooled for 30 min on ice. The resulting blue crystals were collected by filtration and washed with cold water. $Fc^+PF_6^-$ was further purified by recrystallization from water and then dried and stored in a desiccator. Concentrated stock solutions of $Fc^+PF_6^-$ (about 10 mM) were prepared daily in 10 mM HCl and standardized spectrophotometrically at 617 nm by using an extinction coefficient of 410 mM^{-1} cm^{-1} (Carney et al., 1984). 1,1′-Dimethylferricenium hexafluorophosphate was prepared as above.

General. Medium-chain acyl-CoA dehydrogenase from pig kidney was purified as described previously by Thorpe (1981) with an additional octyl-Sepharose step to remove traces of contaminating enoyl-CoA hydratase activity (Lau et al., 1986). Enzyme covalently modified with 2-octynoyl-CoA was prepared by using a 10-fold excess of inhibitor in 50 mM phosphate buffer, pH 7.6 (Freund et al., 1985). The inactivated enzyme was washed in 100 mM Hepes, pH 7.6, using Centricon PM10 microconcentrators to remove excess inhibitor. Enzyme reconstituted with 1,5-dihydrodeaza-FAD was prepared as in Ghisla et al. (1984). ETF from pig kidney was purified as previously reported (Gorelick et al., 1982). Static absorbance measurements were made on Cary 219 or Hewlett Packard 8452A diode-array spectrophotometers. All spectral experiments were performed in 100 mM Hepes buffer, pH 7.6, unless otherwise stated. Flavoproteins were standardized spectrophotometrically in terms of the concentration of bound FAD [acyl-CoA dehydrogenase, 15.4 mM^{-1} cm^{-1} at 446 nm, Thorpe et al. (1979); and ETF, 13.3 mM^{-1} cm^{-1} at 436 nm, Gorelick et al. (1982)].

Anaerobic Procedures. General anaerobic techniques and apparatus were as described previously (Gorelick et al., 1985). Although the acyl-CoA dehydrogenase reduced with octanoyl-CoA is stable for hours in air (Beinert, 1963; Thorpe et al., 1979), autoxidation of the charge-transfer complex is catalyzed by the presence of oxidized dehydrogenase (Lehman and Thorpe, unpublished results). Experiments were therefore routinely performed anaerobically. Redox dyes in 100 mM Hepes buffer, pH 7.6, were deoxygenated by bubbling with oxygen-free nitrogen for at least 20 min. Photoreduction of acyl-CoA dehydrogenase used 3 μM 5-deazariboflavin in 100 mM buffer containing 5 mM EDTA (Massey & Hemmerich, 1978; Thorpe et al., 1979). Where appropriate, photoreduction was conducted in the presence of the indicated concentrations of acyl-CoA analogues (see Results). The semiquinone form of the acyl-CoA dehydrogenase·octenoyl-CoA complex was prepared by mixing 10 μM enzyme with a 5-fold excess of octenoyl-CoA. Photoreduction was monitored spectrophotometrically to ensure a reasonable yield of radical, while avoiding overreduction to the two-electron-reduced charge-transfer form. Control experiments in the stopped-flow spectrophotometer showed that EDTA had no measurable effect on the reaction between reduced acyl-CoA dehydrogenase and $Fc^+PF_6^-$.

Assays. Routine assays followed the reduction of 200 μM ferricenium ion at 300 or 617 nm (extinction coefficient changes of 4.3 and 0.41 mM^{-1} cm^{-1}, respectively; Lehman et al., 1990) in Hepes buffer, pH 7.6, at 25 °C, containing 50 μM octanoyl-CoA after the addition of 30 nM enzyme. Assays at 1 °C were performed by using the stopped-flow spectrophotometer at 617 nm at a final concentration of 100–500 nM dehydrogenase. Particular care must be taken when using highly absorbing solutions of redox acceptors not to confuse deviations in the linearity of the spectrophotometer with apparent saturation or inhibition in kinetic traces (Dommes & Kunau, 1976). When these precautions are observed, the ferricenium assay follows Michaelis–Menten kinetics with no apparent product inhibition at both 300 and 617 nm (Lehman & Thorpe, 1990).

Table I: Extinction Coefficients for Medium-Chain Acyl-CoA Dehydrogenase, Ferrocene, and Ferricenium Hexafluorophosphate[a]

λ (nm)	$dH_{ox}{\cdot}P$	$dH_{1e}{\cdot}P$	$dH_{2e}{\cdot}P$	dH_{ox}	dH_{1e}	dH_{2e}	Fc^0	$Fc^+PF_6^-$
570	0.0	1.4	3.8	0.1	5.3	0.2	0.0	0.28
450	14.1	6.0	2.9	15.4	4.8	0.9	0.33	0.15
390	10.4	14.9	3.9				0.16	0.28

[a] Extinction coefficients are in mM^{-1} cm^{-1}. Values for medium-chain acyl-CoA dehydrogenase are from Gorelick et al. (1985).

Stopped-Flow Measurements. Reactions were followed at 1 °C in a Kinetics Instruments stopped-flow spectrophotometer equipped with a 2-cm absorbance cell with peripherals and software for data acquisition and fitting from Online Instruments Systems. Prior to anaerobic experiments, the driving syringes and the flow cell were treated with an anaerobic solution containing 50 mM glucose and 0.5 mg/mL glucose oxidase in 50 mM KP_i, pH 7.6, for 1 day. The syringes were then flushed with anaerobic water, followed by a 1-h treatment with an anaerobic solution of 10 mM sodium dithionite in 50 mM sodium pyrophosphate buffer, pH 8.4. The syringes were again rinsed with deoxygenated water and then equilibrated with anaerobic reagents from the tonometers.

Absorbance data were analyzed by using nonlinear regression fits to an equation for two sequential pseudo-first-order processes (Frost & Pearson, 1961) modified to include the absorbance contribution of each species (Table I). Neither the ferricenium ion nor ferrocene itself absorbs strongly in the visible region (Lehman & Thorpe, 1990), and thus they make minor contributions to the overall changes seen (Table I). In some cases, values of k_1 and k_2 were selected manually for examination of the resulting fits to the data.

In addition to $Fc^+PF_6^-$, a limited series of experiments were run using 200 μM DCIP, potassium ferricyanide, and phenazine methosulfate as potential oxidants of the acyl-CoA dehydrogenase. Reoxidation was followed at 450 nm where these dyes have minimal absorbance.

RESULTS

Steady-State Kinetics and Static Titrations. Steady-state kinetics using $Fc^+PF_6^-$ as an electron acceptor for octanoyl-CoA-reduced medium chain length acyl-CoA dehydrogenase gave a V_{max} of 1400 min^{-1} and a K_m of 55 μM at 25 °C (Lehman et al., 1990; see Materials and Methods). The enzyme obeyed Michaelis–Menten kinetics up to at least 1 mM ferricenium (see Materials and Methods). At 1 °C, the temperature used for subsequent stopped-flow experiments, the turnover number obtained with 200 μM ferricenium was 27% of that observed at 25 °C (see Materials and Methods).

Titrations of reduced acyl-CoA dehydrogenase with ferricenium are shown in Figure 1. The enzyme was treated with substoichiometric amounts of octanoyl-CoA to avoid the possibility of turnover in this experiment. As expected, substrate-reduced enzyme is completely reoxidized by 2 equiv of this obligatory one-electron oxidant (panel A). Spectra recorded at intermediate stages of the titration are consistent with the accumulation of only small levels of the red anionic semiquinone during the conversion of the characteristic purple reduced enzyme•enoyl-CoA complex (curve 1) to the corresponding oxidized enzyme•product species (curve 4). Since disproportionation reactions in the acyl-CoA dehydrogenase occur very slowly (Thorpe et a., 1979), the low yield of semiquinone observed in Figure 1A suggests that the initial one-electron oxidation of the dihydroflavin is slower than the subsequent conversion of radical to oxidized flavin. Rapid reaction studies support this conclusion (see below).

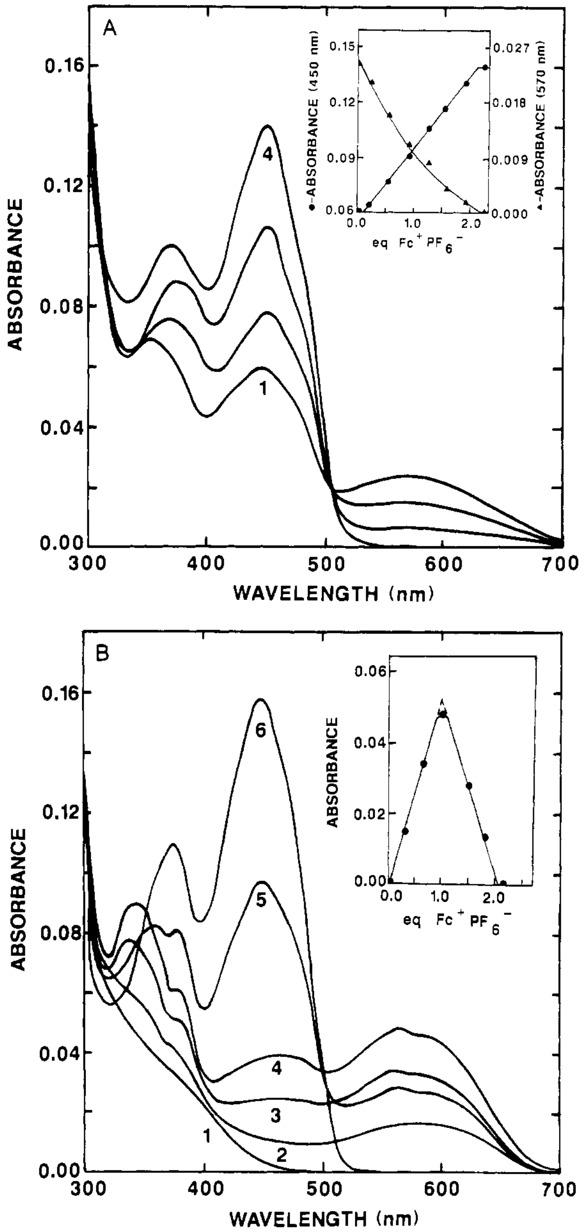


FIGURE 1: Anaerobic reoxidation of substrate and photochemically reduced medium-chain acyl-CoA dehydrogenase by ferricenium hexafluorophosphate. Titrations were performed in 0.8 mL of 100 mM Hepes buffer, pH 7.6, at 25 °C in anaerobic cuvettes (see Materials and Methods). Substrate-reduced enzyme was prepared by the addition of 0.83 equiv (10 μM) of octanoyl-CoA to 12 μM dehydrogenase (panel A). Curves 1–4: After the addition of 0, 0.6, 1.2, and 2.4 equiv of $Fc^+PF_6^-$, respectively. Panel B shows reoxidation of 10 μM photoreduced enzyme (see Materials and Methods) by 0, 0.33, 0.66, 1.0, 1.45, and 2.4 equiv of $Fc^+PF_6^-$ (curves 1–6, respectively). In both panels, intermediate curves have been omitted for clarity, and the insets show absorbance values recorded after each addition of titrant. Equivalents of oxidant are based on the initial concentration of reduced enzyme.

Photochemically reduced enzyme is reoxidized through the blue neutral semiquinone state (Figure 1B) as would be expected for uncomplexed enzyme (Thorpe et al., 1979). The extrapolation in the inset suggests an extinction coefficient of 5.3 mM^{-1} cm^{-1} at 570 nm for this species (see Materials and Methods). The high yield of radical attained in these titrations of free reduced enzyme suggests that now formation of the semiquinone is considerably faster than its decay to oxidized flavin (see below). Further differences in behavior between free and complexed acyl-CoA dehydrogenase will be elaborated later.

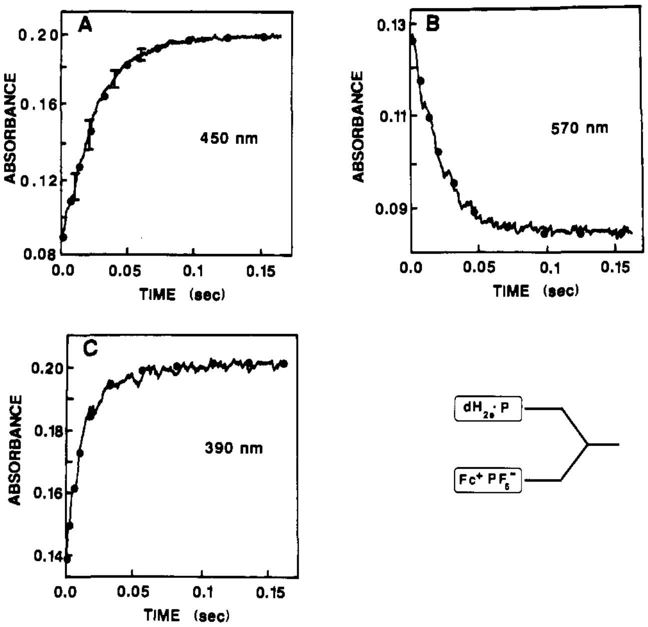


FIGURE 2: Traces of absorbance vs time for the reaction of substrate-reduced medium-chain acyl-CoA dehydrogenase and ferricenium hexafluorophosphate. Oxidized enzyme (12 μM) was reduced with substoichiometric amounts of octanoyl-CoA (10 μM) in an anaerobic tonometer and mixed with an equal volume of deoxygenated 400 μM $Fc^+PF_6^-$ in a stopped-flow spectrophotometer to give final concentrations of 5 μM reduced enzyme and 200 μM $Fc^+PF_6^-$ (2-cm path length cell). Both solutions contained 100 mM Hepes buffer, pH 7.6, at 1 °C, and traces were recorded at 450, 570, and 390 nm (panels A–C, respectively). The solid circles are data generated for rate constants of 40 and 200 s^{-1} for k_1 and k_2, respectively. The lower and upper ranges indicated by the bars in panel A show the comparatively small effect of increasing k_2 from 100 to 500 s^{-1}, respectively.

Rapid Reaction Studies of the Reoxidation of Octanoyl-CoA-Reduced Acyl-CoA Dehydrogenase. Reoxidation of the reduced enzyme·octenoyl-CoA complex (5 μM after mixing in the stopped-flow spectrophotometer) by 200 μM ferricenium ion was studied in 100 mM Hepes buffer, pH 7.6, at 1 °C at a variety of wavelengths with 450, 570, and 390 nm as shown in panels A, B, and C, respectively, of Figure 2. Reoxidation occurs in two successive one-electron steps as shown:

$$dH_{2e}\cdot\text{octenoyl-CoA} + [Fe^{III}(Cp)_2]^+ \xrightarrow{k_1} dH_{1e}\cdot\text{octenoyl-CoA} + [Fe^{II}(Cp)_2] \quad (1)$$

$$dH_{1e}\cdot\text{octenoyl-CoA} + [Fe^{III}(Cp)_2]^+ \xrightarrow{k_2} dH_{ox}\cdot\text{octenoyl-CoA} + [Fe^{II}(Cp)_2] \quad (2)$$

Since the ferricenium ion is a strong thermodynamic oxidant ($E^{\circ\prime}$ = +0.38 V versus the standard hydrogen electrode; Pladziewicki et al., 1985), the reactions depicted above are essentially irreversible under the conditions studied.

The solid circles shown in Figure 2 are simulations of these two sequential pseudo-first-order reactions (k_1 = 40 s^{-1} and k_2 = 200 s^{-1}: see Materials and Methods) using the extinction coefficients shown in Table I. Data at 450 nm are dominated by a comparatively large increase in flavin absorbance associated with k_2 (see Table I). Absorbance changes at 570 nm (panel B) reflect the disappearance of the purple-colored charge-transfer complex (dH_{2e}·octenoyl-CoA; see above). Little semiquinone absorbance is evident in panel C since k_2 is some 5-fold faster than k_1. It should be noted that although k_1 can be relatively well determined in these experiments, the faster second phase cannot be established as precisely. This is illustrated by the bars in panel A, which show the comparatively small effect on the overall fit of varying k_2 5-fold (from 100 to 500 s^{-1}) while keeping k_1 at 40 s^{-1}.

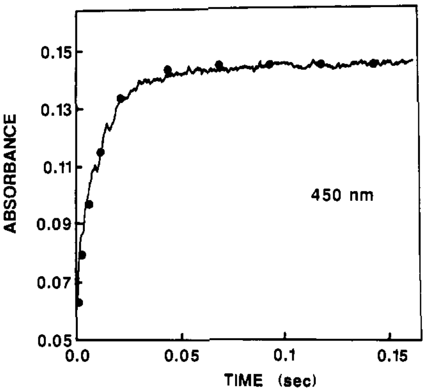


FIGURE 3: Reoxidation of octenoyl-CoA·dehydrogenase semiquinone complex (dH_{1e}·octenoyl-CoA) by ferricenium hexafluorophosphate. dH_{1e}·octenoyl-CoA was prepared as described under Materials and Methods and mixed anaerobically in the stopped-flow spectrophotometer to give 5 μM enzyme and 200 μM $Fc^+PF_6^-$ in 100 mM Hepes buffer, pH 7.6, at 1 °C. The solid circles correspond to a fit of the absorbance changes at 450 nm using k_1 = 100 s^{-1}.

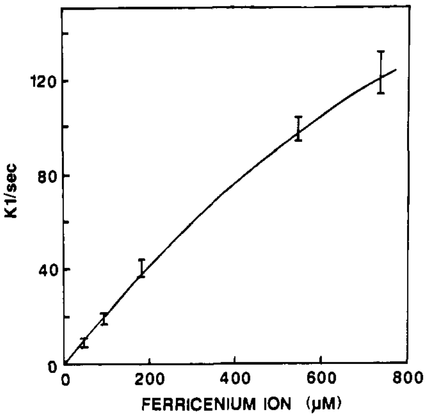


FIGURE 4: Concentration dependence of the apparent k_1 values for the reoxidation of substrate-reduced acyl-CoA dehydrogenase by ferricenium hexafluorophosphate. The conditions of Figure 2 were used but with final concentrations of 50–740 μM $Fc^+PF_6^-$ after mixing in the stopped-flow spectrophotometer. The apparent pseudo-first-order values for k_1 (3 determinations at 450 nm) were obtained as under Materials and Methods and plotted as a function of oxidant concentration. The curve is a nonlinear least-squares fit to a limiting apparent rate constant of 450 s^{-1} and a dissociation constant of 2 mM.

A limited number of experiments with 200 μM 1,1′-dimethylferricenium hexafluorophosphate showed slightly slower reoxidation rates than encountered with $Fc^+PF_6^-$ (k_1 and k_2 values of 13 and 50 s^{-1} compared to 40 and 200 s^{-1}, respectively). The dimethyl derivative is a somewhat weaker oxidant and, e.g., reacts some 4-fold slower with horse heart cytochrome *c* (Carney et al., 1984).

In principle, reoxidation of the dH_{1e}·P form of the enzyme by $Fc^+PF_6^-$ (step k_2) can be measured directly after photoreduction of the dehydrogenase·octenoyl-CoA complex to the corresponding semiquinone species in an anaerobic tonometer (Mizzer & Thorpe, 1981; see Materials and Methods; Figure 3). Reoxidation of the dH_{1e}·octenoyl-CoA complex by 200 μM ferricenium yields an apparent rate constant of about 100 s^{-1}. The reason why this rate is some 2-fold slower than the k_2 value estimated from Figure 2 is not understood. At least part of the discrepancy may arise from difficulties in estimating k_2 from Figure 2 (see above).

Figure 4 shows the dependence of the slower phase, k_1, on ferricenium concentration. Control experiments showed that the PF_6^- ion per se was not inhibitory when 1 mM $Na^+PF_6^-$ was added to 200 μM $Fc^+PF_6^-$. Hence, the slight curvature

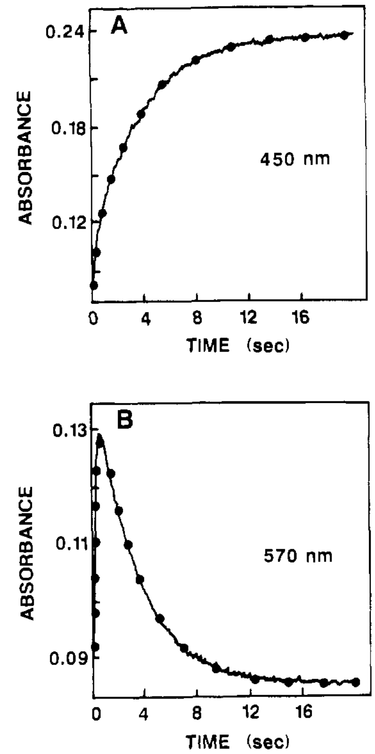


FIGURE 5: Reoxidation of photoreduced medium-chain acyl-CoA dehydrogenase by ferricenium hexafluorophosphate. Final concentrations after anaerobic mixing were 5 μM photoreduced acyl-CoA dehydrogenase (see Materials and Methods) and 200 μM $Fc^+PF_6^-$ in 100 mM Hepes buffer, pH 7.6, at 1 °C. Reoxidation was followed at 450 and 570 nm (panels A and B, respectively), and the circles are best fits for k_1 of 3.0 s^{-1} at both wavelengths and 0.27 and 0.34 s^{-1} at 450 and 570 nm, respectively.

observed up to 740 μM $Fc^+PF_6^-$ in Figure 4 is consistent with a rather weak encounter complex between reduced enzyme and the ferricenium ion prior to electron transfer (K_d apparent about 2 mM; see Discussion). Neglecting this curvature, the slope of the line at low ferricenium concentrations yields an apparent bimolecular rate constant of about 2.2×10^5 M^{-1} s^{-1} for the slow phase. Although the apparent pseudo-first-order k_2 values cannot be accurately determined from these data (see above), they also appear to increase markedly as the ferricenium concentration is raised and correspond to a bimolecular rate constant of about 10^6 s^{-1} (data not shown).

Reoxidation of Photoreduced Dehydrogenase. Reoxidation of two-electron-reduced acyl-CoA dehydrogenase prepared photochemically (see Materials and Methods) shows a different course, with the appearance of the blue semiquinone (Figure 5A; 570 nm) followed by its slower decline to yield oxidized enzyme. These two reactions are depicted as

$$dH_{2e} + [Fe^{III}(Cp)_2]^+ \xrightarrow{k_1} dH_{1e} + [Fe^{II}(Cp)_2] \quad (3)$$

$$dH_{1e} + [Fe^{III}(Cp)_2]^+ \xrightarrow{k_2} dH_{1e} + [Fe^{II}(Cp)_2] \quad (4)$$

The circles are simulations of 570- and 450-nm absorbance changes using an apparent k_1 value of 3 s^{-1} and a k_2 value some 10-fold slower (see legend, Figure 5). Clearly, reoxidation is slower with the free enzyme, requiring about 14 s for completion (using 200 μM $Fc^+PF_6^-$; Figure 5) compared to about 0.08 s under otherwise similar conditions in Figure 2. This drastic slowing of the overall reaction reflects an approximate 13-fold decrease in k_1 between substrate and chemically reduced forms, with an approximate 700-fold slowing of comparable k_2 values. The kinetic superiority of the substrate-reduced enzyme is all the more impressive when one considers that enoyl-CoA binding renders the reduced dehydrogenase a substantially poorer thermodynamic reductant than the free dehydrogenase (Gorelick et al., 1985; Stankovich & Soltysik, 1987). Similar rate enhancements in the presence of product were encountered with the physiological acceptor ETF (Gorelick et al., 1985), and their molecular basis will be considered later in this paper.

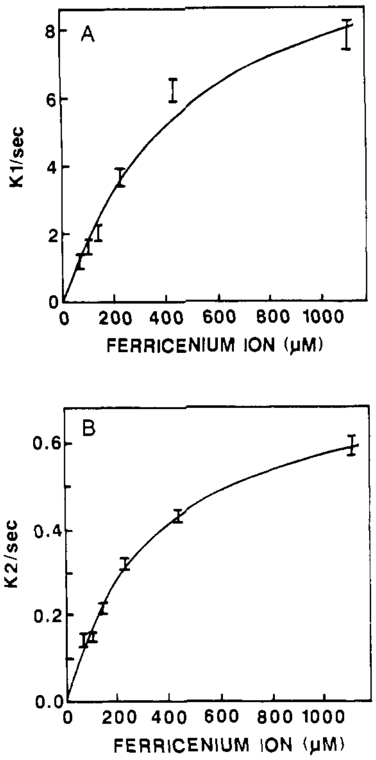


FIGURE 6: Reoxidation of photochemically reduced acyl-CoA dehydrogenase by ferricenium hexafluorophosphate. Final concentrations after anaerobic mixing in the stopped flow were 5 μM dehydrogenase (photoreduced to the dihydroflavin form as in Methods) and 50–1100 μM $Fc^+PF_6^-$ in 100 mM Hepes, pH 7.6, at 1 °C. Values for k_1 and k_2 were determined for 3 shots at 450 nm and plotted in panels A and B, respectively. The curves are drawn assuming limiting rates of 10 and 0.8 s^{-1} and apparent K_d values of 500 μM and 350 μM for k_1 and k_2, respectively.

Figure 6 presents the concentration dependence of the apparent pseudo-first-order rate constants k_1 and k_2 for the free reduced dehydrogenase up to 1.1 mM ferricenium ion concentration. Again, evidence for rate saturation is shown by the curvature in these plots, yielding apparent K_d values of 500 and 350 μM for k_1 and k_2, respectively.

Comparison with Other Electron Acceptors. Three other electron acceptors which have been widely used with flavoproteins (ferricyanide, phenazine methosulfate, and 2,6-dichlorophenolindophenol) were tested as potential oxidants of the medium-chain acyl-CoA dehydrogenase. Comparison of the half-times for reoxidation using 200 μM of each oxidant and 5 μM of either substrate-reduced or photochemically reduced enzyme is presented in Table II. $Fc^+PF_6^-$ reoxidizes the substrate-reduced enzyme about 40-fold faster than the next best oxidant (ferricyanide) and is some 35 000-fold faster than DCIP. As might be expected for such a diverse series of oxidants, there is no correlation between redox potential and rate of electron transfer (Table II). $Fc^+PF_6^-$, ferricyanide, and PMS all react more rapidly with the substrate-reduced dehydrogenase (by about 5- to 50-fold in terms of half-time), and in this respect they resemble the physiological oxidant ETF (Gorelick et al., 1985). DCIP is a striking exception, reacting

Table II: Comparison of the Half-Times for Oxidation of Medium-Chain Acyl-CoA Dehydrogenase with a Variety of Acceptors[a]

oxidant	$E°'$ (V)	$t_{1/2}$(substrate) (s)	$t_{1/2}$(free) (s)	$t_{1/2}$(free)/ $t_{1/2}$(substrate)
$Fc^+PF_6^-$	0.38	0.02	1	50
PMS	0.08	3	14	4.7
$K_3Fe(CN)_6$	0.42	0.8	15	18.7
DCIP	0.22	700[b]	0.15	2.1×10^{-4}

[a] Except where noted, reactions run in the stopped-flow spectrophotometer at 1 °C using a final concentration of 5 μM of either substrate-reduced or free reduced acyl-CoA dehydrogenase and 200 μM of the acceptors shown. Reactions were followed at 450 nm and are compared by half-times as indicated. [b] Reaction recorded anaerobically on a Cary 219 spectrophotometer at 7 °C due to the long half-time of the reaction.

Table III: Half-Times for Reoxidation of Two-Electron-Reduced Medium-Chain Acyl-CoA Dehydrogenase in the Presence of Various Ligands[a]

ligand	$t_{1/2}$ (s)	$t_{1/2}$(free)/ $t_{1/2}$(ligand)	wavelength of detection (nm)
free	1	1	450
octenoyl-CoA	0.02	50	450
3-thia-C_8-CoA	0.03	33	415
3-oxa-C_8-CoA	0.05	20	450
3-keto-C_8-CoA	0.12	8	440
acetoacetyl-CoA	0.4	2.5	440
octyl-CoA	0.16	6.3	456
2-octynoyl-CoA	0.45	2.3	450

[a] Ligands were prepared as described under Material and Methods. Enzyme (10 μM) was treated with a 5-fold excess of ligand, photochemically reduced as under Materials and Methods, and mixed with an equal volume of 400 μM $Fc^+PF_6^-$ in the stopped-flow spectrophotometer at 1 °C in 100 mM Hepes, pH 7.6.

about 5000-fold faster with the free enzyme. In fact, DCIP shows a surprising 7- to 100-fold higher reactivity with the free reduced enzyme than the other oxidants (see Discussion).

Modulation of the Reactivity of Reduced Acyl-CoA Dehydrogenase by CoA Derivatives. Several other ligands at a concentration of 50 μM were found to accelerate the reoxidation of photochemically reduced dehydrogenase by 200 μM ferricenium ion. Table III shows that both 3-thia- and 3-oxaoctanoyl-CoA (Lau et al., 1988) were almost as effective as *trans*-2-octenoyl-CoA, whereas acetoacetyl-CoA, 3-ketooctanoyl-CoA, and the thioether octyl-SCoA showed smaller rate enhancements. Clearly, this effect is not unique to substrate reduced enzyme (see Discussion). Enzyme covalently modified by the mechanism-based inhibitor 2-octynoyl-CoA (Powell & Thorpe, 1988) is about 2-fold more reactive than free reduced enzyme.

Ionic Strength Effects. The zwitterionic buffer, Hepes, used in this study has a low ionic strength at pH 7.6 (p*K* 7.55; Good et al., 1966); however, the addition of 200 mM NaCl decreased k_1 by only 30% at 200 μM $Fc^+PF_6^-$. No significant effect was seen in the standard assay upon the addition of 100 mM NaCl (see Materials and Methods), presumably since turnover is not limited by the rate of electron transfer per se (Lehman and Thorpe, unpublished results). The small decrease in k_1 noted above is consistent with the interaction of the monopositive ferricenium ion with a negatively charged surface on the dehydrogenase believed to be the site interacting with ETF (Beckmann & Frerman, 1983; Frerman et al., 1980). Thus reoxidation by the triply anionic acceptor, ferricyanide, at a similar locus on the dehydrogenase would be expected to show an increase in rate with increasing ionic strength due to electrostatic screening. This is not the case: 200 mM NaCl effects a 2-fold decrease in overall rate of reoxidation judged by the half-time for recovery of oxidized flavin absorbance (see Discussion).

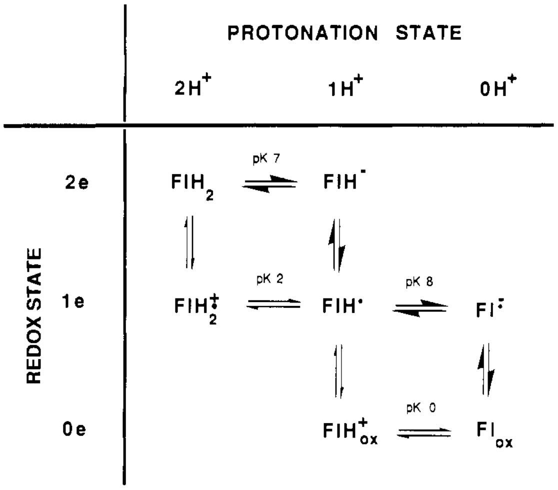
DISCUSSION

This study shows that the ferricenium ion is a facile oxidant of substrate-reduced medium-chain acyl-CoA dehydrogenase, enabling a sensitive assay for the acyl-CoA dehydrogenases to be developed (Lehman & Thorpe, 1990). The turnover number in this assay of 5 s^{-1} (at 1 °C with 200 μM $Fc^+PF_6^-$) is clearly not seriously limited by k_1 (40 s^{-1}) or k_2 (about 200 s^{-1}) in the oxidative half-reaction. Similarly, the initial reduction of the enzyme by octanoyl-CoA is rapid at 1 °C (about 300 s^{-1}: Lau et al., 1989; Gorelick et al., 1985) and not significantly limiting in overall turnover. In fact, the major limitation in turnover appears to be dissociation of the tightly bound product, *trans*-2-octenoyl-CoA, from the reoxidized enzyme (K_d about 90 nM; Powell et al., 1987).

$Fc^+PF_6^-$, phenazine methosulfate, and ferricyanide all show enhanced reactivity toward substrate-reduced enzyme compared to that generated photochemically (Table II). Hence this modulation of kinetic reactivity is not peculiar to the physiological acceptor, ETF, and we wish to ultimately understand the molecular basis for this effect. One obvious suggestion is that a conformational change induced upon binding CoA ligands allows a closer approach, or a more favorable alignment of redox centers. Such explanations are plausible but do not readily account for the 20-fold range of effectiveness shown by various ligands (Table III). Further, it is perhaps surprising that the physiological acceptor ETF shares this stimulation with a range of much smaller structurally diverse nonprotein oxidants.

A second explanation rests on the correlation that those ligands found to be most effective in accelerating electron transfer, e.g., *trans*-2-octenoyl-CoA, 3-thiaoctanoyl-CoA, and 3-oxaoctanoyl-CoA, have been found to induce ionization of the blue neutral semiquinone, yielding the red anion radical (Mizzer & Thorpe, 1981; Lehman and Thorpe, unpublished results). Indeed, a link between protonation state and kinetic reactivity might be expected from consideration of the mechanism of outer-sphere electron-transfer reactions (Marcus & Sutin, 1985; Scott et al., 1985; Wherland & Gray, 1977; Tollin et al., 1986). Since electron transfer per se is so much faster than any internuclear motion of reactants or solvent, adjustments of, e.g., bond angles, lengths, protonation state, and solvation need to be made to the system prior to electron transfer if the activation energy of the overall process is to be minimized (Marcus & Sutin, 1985). Scheme I considers various redox and protonic states of free flavin for illustration. Oxidation of the neutral dihydroflavin (Scheme I; top left) would yield the unfavorable protonated semiquinone (FlH_2^+; p*K* 2) as the immediate product of one-electron oxidation. A more favorable pathway kinetically would involve an initial facile deprotonation of the dihydroflavin (p*K* 7) yielding FlH_{2e}^- (horizontal arrow, Scheme I) followed by one-electron transfer to generate the blue neutral semiquinone directly. Similarly, the blue neutral radical would be expected to be a slower one-electron reductant than the red anionic semiquinone because it would again result in an oxidized flavin in an unfavorable protonic state (FlH_{ox}^+; p*K* 0) rather than yielding normal oxidized flavin directly. Applying microscopic reversibility, one would then predict that the anionic flavin would be the immediate product of outer-sphere one-electron reduction of oxidized flavin even if the blue neutral semiquinone is the form favored statically. This is precisely the behavior observed during pulse radiolysis of riboflavin binding protein

Scheme I: Thermodynamic Cycle Illustrating Selected Protonation and Redox States of Free Flavin[a]



[a] Bold arrows suggest a preferred route for one-electron-transfer reactions (see text). p*K* values are taken from Muller (1983).

(Klapper & Faraggi, 1983) and flavodoxin (Anderson et al., 1987) with the appearance of a transient red semiquinone which relaxes to the thermodynamically favored blue neutral species. Experimental evidence for the enhanced reactivity of flavin anionic semiquinones over the neutral species toward inorganic electron acceptors and several redox proteins comes from the studies of Tollin and Cusanovich and their colleagues (Cusanovich et al., 1985; Przysiecki et al., 1985; Vaish & Tollin, 1971).

This simple analysis, developed by using free flavin as an example, represents the extreme case, and it seems likely that the requirement for frank proton transfer could be avoided in some flavoproteins by the provision of strong hydrogen bonds to sites whose p*K* is linked to redox state. Nevertheless, it appears likely that the proton is an important factor in the redox reactivity of the acyl-CoA dehydrogenases, and the intrinsically higher reactivity and lower redox potential of anionic flavin species (Edmondson & Tollin, 1983) is consistent with their presence in product-complexed dehydrogenase (see above).

There appear to be at least two distinct loci for input and output of reducing equivalents in the acyl-CoA dehydrogenases (Figure 7). One involves the substrate site, which provides access to the *re* face of the flavin nucleus at the N-5 position for both thioester substrates and chemical reductants such as borohydride (Ghisla et al., 1984; Manstein et al., 1986). This site remains occupied by an enoyl-CoA derivative during turnover to ensure efficient reoxidation of the dehydrogenase (see above; Gorelick et al., 1985). Clearly, a ternary complex mechanism (McKean et al., 1979) requires a second distinct site used by ETF (Figure 7). A body of evidence suggests that this locus is negatively charged so as to interact electrostatically with an approaching complementary positively charged surface on ETF (Frerman et al., 1980; Beckmann & Frerman, 1983).

A number of redox reagents appear unable to communicate effectively with the bound flavin via the ETF site and may therefore have to compete with tightly binding CoA derivatives for access to the flavin ring (Figure 7). The effect is most conspicuous with DCIP (Table II). The suppression of reactivity toward DCIP in the presence of CoA thioesters was first noted by Beinert and Page (1956) and is exemplified by the 20 000-fold slowing of reoxidation in the presence of octenoyl-CoA (Table II). Although the effect is less dramatic, 100 μM of the redox-inactive thioether, octyl-SCoA, lowers the reactivity of the free reduced enzyme toward DCIP by some 8-fold (data not shown). Further, enzyme modified covalently with 2-octynoyl-CoA followed by photoreduction (see Materials and Methods) is essentially unreactive toward 200 μM DCIP although it is rapidly reoxidized by ETF (Freund et al., 1985). Since DCIP is generally regarded as a two-electron oxidant, it might be expected to be ineffective at the ETF site because redox communication through this locus involves obligatory one-electron steps (Gorelick & Thorpe, 1986).

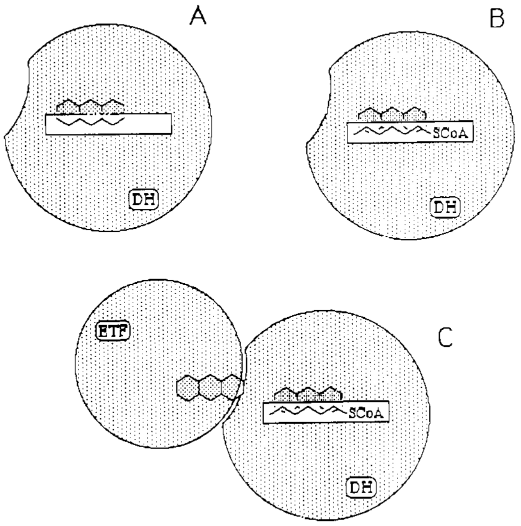


FIGURE 7: Schematic representation of acyl-CoA dehydrogenase and electron transferring flavoprotein. Only one subunit of the tetrameric dehydrogenase and of the heterodimeric ETF is shown for clarity. (A) Free dehydrogenase; (B) after binding acyl-CoA substrate; (C) after formation of the ternary complex between (B) and ETF.

In addition to DCIP, the suppression of reactivity of the enzyme by bound ligands is also encountered with dithionite as a reductant of the oxidized dehydrogenase, e.g., with CoA thioesters (Beinert & Page, 1957), with CoA persulfide (Engel & Massey, 1971; Williamson et al., 1982), and with 2-octynoyl-CoA-treated enzyme (Freund et al., 1985). Dithionite has been described as an inner-sphere reductant (Wherland & Gray, 1977) and as such would require close approach for effective delivery of reducing equivalents to the flavin. The results obtained with DCIP and dithionite strongly suggest that ligand binding can profoundly restrict van der Waals access of certain external reagents to the flavin ring. Whether such effects are caused by some form of direct steric blocking (Figure 7) or via an associated conformational change is not yet known.

While second-order kinetics are frequently encountered in small molecule/protein redox reactions (Scott et al., 1985), evidence for rate saturation of the sort observed in Figures 4 and 6 has been obtained, e.g., with plastocyanin (Sykes, 1985), azurin (Lappin et al., 1979a,b), Chromatium HIPIP (Adzamli et al., 1981), cytochrome b_5 (Chapman et al., 1984), and cytochrome *c* (Cho et al., 1988) with certain inorganic oxidants. Generally these results have been interpreted in terms of the formation of precursor complexes, and our data are consistent with relatively weakly interacting species of this type. We do not know whether the ferricenium ion accepts electrons from a single locus on the surface of the dehydrogenase within the ETF binding site, or whether additional sites prove kinetically significant. The existence of more than one electron-transfer site for nonphysiological acceptors has been suggested in a number of metalloenzymes, e.g., cytochrome *c* (Cho et al., 1988; Cheddar et al., 1989) and plastocyanin (Lappin et al., 1979a,b; Pladziewicz & Brenner, 1987).

In conclusion, the ferricenium ion is a facile one-electron oxidant for medium-chain acyl-CoA dehydrogenase. Both the ferricenium ion and ETF show enhanced reactivity toward the reduced dehydrogenase in the presence of enoyl-CoA product, showing that this effect is not unique to the physiological reactants. The protonic state of the bound flavin is likely to play an important role in modulating the kinetic reactivity of the bound flavin in the acyl-CoA dehydrogenases. Finally, additional evidence is presented for the existence of at least two potential sites for redox communication with the bound flavin prosthetic group in these enzymes.

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Equilibrium Binding of Thrombin to Recombinant Human Thrombomodulin: Effect of Hirudin, Fibrinogen, Factor Va, and Peptide Analogues[†]

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ABSTRACT: Thrombomodulin is an endothelial cell surface receptor for thrombin that acts as a physiological anticoagulant. The properties of recombinant human thrombomodulin were studied in COS-7, CHO, CV-1, and K562 cell lines. Thrombomodulin was expressed on the cell surface as shown by the acquisition of thrombin-dependent protein C activation. Like native thrombomodulin, recombinant thrombomodulin contained N-linked oligosaccharides, had M_r ~100 000, and was inhibited or immunoprecipitated by anti-thrombomodulin antibodies. Binding studies demonstrated that nonrecombinant thrombomodulin expressed by A549 carcinoma cells and recombinant thrombomodulin expressed by CV-1 and K562 cells had similar K_d's for thrombin of 1.3 nM, 3.3 nM, and 4.7 nM, respectively. The K_d for DIP-thrombin binding to recombinant thrombomodulin on CV-1(18A) cells was identical with that of thrombin. Increasing concentrations of hirudin or fibrinogen progressively inhibited the binding of ^{125}I-DIP-thrombin, while factor Va did not inhibit binding. Three synthetic peptides were tested for ability to inhibit DIP-thrombin binding. Both the hirudin peptide Hir$^{53-64}$ and the thrombomodulin fifth-EGF-domain peptide Tm$^{426-444}$ displaced DIP-thrombin from thrombomodulin, but the factor V peptide FacV$^{30-43}$ which is similar in composition and charge to Hir$^{53-64}$ showed no binding inhibition. The data exclude the significant formation of a ternary complex consisting of thrombin, thrombomodulin, and hirudin. These studies are consistent with a model in which thrombomodulin, hirudin, and fibrinogen compete for binding to DIP-thrombin at the same site.

Thrombomodulin is an endothelial cell surface receptor for the blood clotting serine protease thrombin (Esmon & Owen, 1981). The thrombin-thrombomodulin complex activates protein C at least 1000-fold more rapidly than thrombin alone (Esmon et al., 1982). Activated protein C (APC)[1] degrades clotting factors Va and VIIIa (Walker et al., 1979; Vehar & Davie, 1980) and thereby inhibits the further production of thrombin. This and other activities of thrombomodulin make it an important natural anticoagulant (Esmon, C. T., 1987; Esmon, N. L., 1987).

Thrombomodulin was first isolated from rabbit lung (Esmon et al., 1982), and human thrombomodulin was subsequently purified from lung (Maruyama et al., 1985) and placenta (Salem et al., 1984a). Human thrombomodulin has M_r ~ 100 000 and consists of a single polypeptide (Salem et al., 1984a). The nucleotide sequence of human thrombomodulin

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[1] Abbreviations: APC, activated protein C; DIP, diisopropyl fluorophosphate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HUVE, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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