Flavin Analogue Studies of Pig Kidney General Acyl-CoA Dehydrogenase†

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ABSTRACT: The apoprotein of pig kidney general acyl-CoA dehydrogenase has been reconstituted with eight ring-modified flavin adenine dinucleotide (FAD) analogues to probe the environment of the bound flavin and the interaction of the enzyme with acyl-CoA derivatives. Addition of 8-Cl-, 7-Br-, and 2-thio-FAD yields holoenzymes that can be reduced by octanoyl-CoA and that exhibit appreciable enzymatic activity in an assay system utilizing phenazine methosulfate as a mediator. In contrast, reconstitution of the enzyme with analogues that have more negative standard redox potentials than FAD, 1-deaza-, 5-deaza-, 6-OH-, 8-OH-, and 8-mercapto-FAD, generates derivatives that are inactive and not significantly reduced by thioester substrates. In the reverse direction, the 5-deaza-$FADH_2$ dehydrogenase is rapidly reoxidized by crotonyl-CoA, suggesting that the highly unfavored 5-deazaflavosemiquinone is not an obligatory intermediate in this reaction. Experiments with 8-Cl- and 8-mercapto-FAD–enzymes suggest that the 8-position of the isoalloxazine ring is not freely accessible to solvent. General acyl-CoA dehydrogenase apoprotein preferentially binds the yellow neutral form of 6-OH-FAD, by about 40-fold, over the corresponding green anion. However, binding either acyl-CoA substrates or products profoundly stabilizes this anionic flavin species. Correspondingly, deprotonation of the blue neutral semiquinone of the native dehydrogenase is induced on binding these ligands, leading to a marked stabilization of the anionic radical form. Further, although both 8-OH- and 8-mercapto-FAD anions are bound weakly to the apoenzyme, dissociation of these benzoquinoid species is prevented by the addition of octanoyl-CoA or crotonyl-CoA. These data suggest that the binding of acyl-CoA substrates or products to the dehydrogenase effects changes in the flavin environment that stabilize a negative charge developed at the N-1—C-2=O locus of the isoalloxazine ring.

Recently, there has been a resurgence of interest in the acyl-CoA dehydrogenases and in particular in the general acyl-CoA dehydrogenase that participates in mammalian fatty acid oxidation. This mitochondrial flavoprotein was first studied by Beinert and co-workers (Crane et al., 1956; Beinert, 1963) and exhibits a broad substrate specificity with optimal activity toward medium chain length acyl-CoA thioesters (Crane et al., 1956; Hall & Kamin, 1975; Thorpe et al., 1979). These substrates induce rapid bleaching of the flavin prosthetic group (Beinert, 1957; Hall et al., 1979), with the appearance of a long-wavelength band ascribed to the formation of a charge-transfer complex between reduced flavin as the donor and the bound enoyl-CoA as acceptor (Engel & Massey, 1971; Massey & Ghisla, 1974). Substrate-reduced enzyme is then reoxidized by a second flavoprotein, electron-transferring flavoprotein, which mediates the transfer of reducing equivalents to the electron-transport chain (Crane & Beinert, 1956; Ruzicka & Beinert, 1977).

Recently, we have developed a convenient method for the preparation of pig kidney general acyl-CoA dehydrogenase apoprotein (Mayer & Thorpe, 1981), in order to exploit the use of flavin analogues in the study of this enzyme. The work reported in this paper underscores the usefulness of this approach, providing new insights into the environment of the flavin binding site and into the interaction between acyl-CoA derivatives and the enzyme.

Experimental Procedures

Materials. Pig kidney general acyl-CoA dehydrogenase was purified and stored as described previously (Thorpe et al., 1979; Thorpe, 1981). Methyl methanethiolsulfonate was a gift from Dr. Jules Shafer, University of Michigan. Acetoacetyl-, butyryl-, crotonyl-, octanoyl-, and palmitoyl-CoA were from P-L Biochemicals. *S*-Heptadecyl-CoA was prepared as described earlier (Ciardelli et al., 1981) and was a gift of Dr. Theodor Wieland, Heidelberg. Thiophenol and sodium sulfide stock solutions were prepared as described earlier (Schopfer et al., 1981). FAD[1] analogues were obtained from the corresponding riboflavin analogues by the flavokinase/FAD-synthetase system of *Brevibacterium ammoniagenes* as described by Spencer et al. (1976). 8-Chlororiboflavin was a gift from Dr. John Lambooy, University of Maryland; 7-bromoriboflavin was from Dr. Stephen Mayhew, University of Dublin; 2-thio-, 5-deaza-, and 6-hydroxyriboflavins were from Dr. Peter Hemmerich, University of Konstanz; 8-hydroxyriboflavin was from Dr. Sandro Ghisla, Konstanz; and 1-deazariboflavin was from Dr. E. F. Rogers, Merck Sharp & Dohme Research Laboratories, Rahway, NJ. 8-Mercapto-FAD was prepared from 8-chloro-FAD by incubation with 5 mM sodium sulfide (Massey et al., 1979).

Methods. Visible and UV spectra were recorded on Cary 17 or 219 spectrophotometers. Most experiments were carried out in 50 mM potassium phosphate buffer, pH 7.6, at 4 °C to minimize problems due to turbidity. The cell compartments were flushed with dry air to avoid fogging. Concentrations of native enzyme are expressed in terms of bound flavin, using the experimentally determined extinction coefficient at 446 nm of 15.4 mM^{-1} cm^{-1} [a value about 1.3-fold greater than that of free FAD at 448 nm (Thorpe et al., 1979)]. We have found that the extinction coefficients of the free FAD analogues used here are increased correspondingly when bound to the apoenzyme.

Apoenzyme was prepared by a recent variation of the acid–ammonium sulfate procedure, which employs a charcoal slurry to adsorb the released prosthetic group (Mayer & Thorpe, 1981). Apoprotein was quantitated by using an extinction coefficient of 63 000 M^{-1} cm^{-1} at 280 nm (Mayer,

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[1] Abbreviations: FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid.

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Table I: Properties of General Acyl-CoA Dehydrogenase Substituted with FAD Analogues

FAD analogue	E_0' (mV)[a]	activity (%)[j]	long-wavelength band maximum (nm) with octanoyl-CoA	long-wavelength band maximum (nm) with acetoacetyl-CoA
FAD	−208[b]	100	570	550
8-Cl	−152[c]	8	550	570
7-Br	−154[d]	8	536	580
2-thio	−126[e]	7	ND[k]	ND
5-deaza	−311,[e] −273[f]	0	[l]	[l]
1-deaza	−280[e]	0	[l]	[l]
6-OH	−265[g]	0	ND	ND
8-OH	−335[h]	0	[l]	[l]
8-SH	−290[i]	0	[l]	[l]

[a] Redox potentials for two-electron couples taken from the indicated reference. [b] Draper & Ingraham, 1968. [c] Moore et al., 1978. [d] Abramovitz & Massey, 1976. [e] Walsh et al., 1978. [f] Stankovich & Massey, 1976. [g] P. Hemmerich, unpublished data. [h] Ghisla & Mayhew, 1980. [i] Moore et al., 1979. [j] Activity in standard assay (see Experimental Procedures) minus the 1% residual activity of the apoprotein. [k] ND, not determined (since peak position is obscured by flavin absorbance peak). [l] No significant long-wavelength band.

1981) and showed a residual activity of about 1%. Solutions of apoprotein were stable for several days at 4 °C without significant loss of reconstitutability.

Reconstitutions with oxidized analogues were performed at 4 or 25 °C for 1–2 h in 50 mM phosphate buffer containing 0.3 mM EDTA with 25–36 μM apoprotein subunits and 1–2 equiv of flavin analogue. Unbound flavin was removed by gel filtration or dialysis vs. the same buffer. Reconstituted enzymes were stored at 4 °C. Most of them, except for the 8-hydroxy- and 8-mercapto-FAD forms, were stable to storage at −20 °C for periods of greater than 1 year. For the preparation of the 5-deaza-$FADH_2$–enzyme, 27 μM apoprotein was incubated at 25 °C for 10 min with 24 μM 5-deaza-$FADH_2$ freshly reduced with a few grains of sodium borohydride. Binding of the reduced analogue is accompanied by a considerable increase in flavin fluorescence. The reduced enzyme was dialyzed for 1 day at 4 °C. The fluorescence values quoted in the text are compared to those of an equivalent concentration of freshly reduced 5-deaza-$FADH_2$. Enzyme assays were performed as described previously, by using phenazine methosulfate to mediate the transfer of reducing equivalents from the dehydrogenase to 2,6-dichlorophenolindophenol (Thorpe, 1981).

Results and Discussion

8-Chloro-FAD–Enzyme. Reconstitution of pig kidney general acyl-CoA dehydrogenase apoprotein with 8-Cl-FAD (see Methods) yields the spectrum shown in Figure 1 (solid line, panel A). The spectrum is similar, in its general features, to that of the native enzyme (panel B; Thorpe et al., 1979), with little resolution of the two major absorbance peaks. The 8-Cl enzyme is rapidly reduced by 1 equiv of octanoyl-CoA (Figure 1A, inset), with extensive bleaching at 440 nm and the appearance of a long-wavelength band (λ_{max} = 550 nm). This band has been ascribed, in the native enzyme (panel B, Figure 1), to a charge-transfer interaction between reduced flavin as the donor and the bound enoyl-CoA as the acceptor (Engel & Massey, 1971; Massey & Ghisla, 1974). The 20-nm blue shift observed on replacement of FAD by the 8-Cl derivative is consistent with this ascription, since the latter has an oxidation reduction potential some 60 mV more positive than that of FAD (Table I). From the end point in the

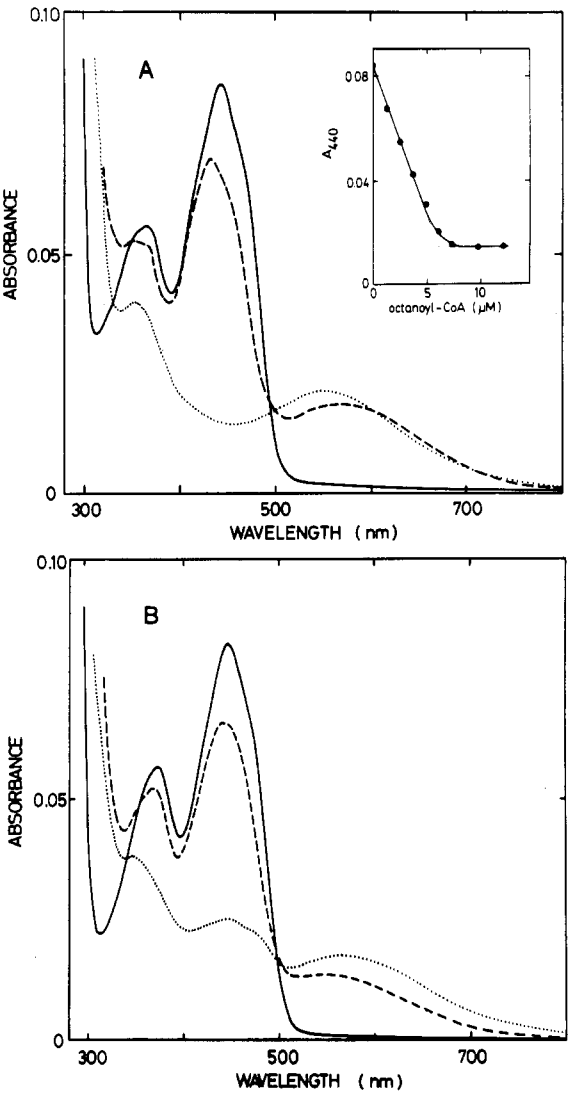


FIGURE 1: Effect of octanoyl- and acetoacetyl-CoA on the visible spectra of the 8-Cl-FAD general acyl-CoA dehydrogenase and of the native enzyme. (Panel A) The spectrum of 6.1 μM 8-Cl-FAD–enzyme is shown in 50 mM phosphate buffer, pH 7.6, 4 °C (—), with 12.1 μM octanoyl-CoA (···) or with 40 μM acetoacetyl-CoA (– –). The inset plots intermediate points in the octanoyl-CoA titration. A double-reciprocal plot of A_{570} vs. acetoacetyl-CoA indicates that the spectral changes shown are 80% complete at 40 μM ligand concentration (apparent K_d = 8 μM). The spectral changes shown were complete before measurement could be made. (Panel B) The spectrum of 5.1 μM native enzyme under the same conditions (—) with 12 μM octanoyl-CoA (···) or with 40 μM acetoacetyl-CoA (– –). No significant reoxidation of the reduced enzyme occurred over the duration of these aerobic titrations.

reductive titration of the enzyme with octanoyl-CoA, the extinction coefficient ϵ_{440} of the oxidized enzyme can be calculated as 14 000 M^{-1} cm^{-1}.

Very similar spectral changes are obtained on the addition of butyryl- or palmitoyl-CoA to the oxidized 8-Cl enzyme (data not shown). This is in marked contrast to the behavior of the native enzyme in which the internal equilibrium K_2 is

$$E{\cdot}SH_2 \rightleftharpoons EH_2{\cdot\cdot}S$$

strongly dependent on the chain length of the acyl thioester substrate (Beinert & Page, 1957; Thorpe et al., 1979; Hall et al., 1979). Thus with octanoyl-CoA, K_2 lies to the right; whereas with palmitoyl-CoA, the spectrum is dominated by the oxidized flavin component, and only a very weak long-wavelength band is seen. In addition, little or no long-wavelength band is observed on the addition of butyryl-CoA to the

native kidney enzyme (Thorpe et al., 1979). Thus the discrimination in K_2 observed in the native enzyme is suppressed by using the higher redox potential analogue such that the reduced flavin species is favored in each case.

Figure 1 also shows the spectrum of the acetoacetyl-CoA complex of the 8-Cl enzyme. In native acyl-CoA dehydrogenases the corresponding spectra (Figure 1B) are believed to represent charge-transfer complexes between the enolate form of acetoacetyl-CoA and oxidized flavin as acceptor (Engel & Massey, 1971; Massey & Ghisla, 1974; Benecky et al., 1979). The red shift in the position of this band (from 550 to 570 nm, Table I) is consistent with this assignment. The acetoacetyl-CoA complex (Figure 1A) is transformed immediately to the reduced flavin–octenoyl-CoA complex on the addition of 11 μM octanoyl-CoA.

Recent work has shown the usefulness of 8-chloro flavins as active site probes of flavoproteins (Massey & Hemmerich, 1980). For example, reconstitution of apo-lipoamide dehydrogenase with 8-Cl-FAD produces marked spectral changes due to the formation of an 8-*S*-cysteinyl adduct (Moore et al., 1978). An analogous reaction is not encountered in this work even after prolonged (1 year) storage of the enzyme at −20 °C, indicating that none of the six cysteine residues per subunit are similarly reactive. Incubation of the 8-Cl enzyme with thiophenol (0.1 mM) or sodium sulfide (3.7 mM) showed a similar lack of reactivity to externally added nucleophiles. Thus the 8-position of the isoalloxazine ring may be shielded from solvent, or alternatively, nucleophilic attack may be unfavorable for steric or electrostatic reasons (Moore et al., 1979; Massey & Hemmerich, 1980). Further evidence supporting the inaccessibility of the 8-position to solvent is presented later.

Interestingly, incubation of the 8-Cl enzyme with 3.7 mM sodium sulfide at 25 °C leads to reduction of the flavin after a lag period during which dissolved oxygen is removed from the solution. Reduction to the dihydroflavin form proceeds via appreciable levels of the blue 8-Cl-FAD semiquinone (λ_{max} = 570 nm, apparent ϵ = 2.8 mM^{-1} cm^{-1}; attained about 5 min after the end of the lag phase). This phenomenon is not restricted to the analogue, since an anaerobic incubation of the native enzyme with 3 mM sodium sulfide at 4 °C yields a high concentration of blue radical (ϵ_{560} = 3.94 mM^{-1} cm^{-1}) followed by the slower accumulation of fully reduced enzyme (results not shown). Sulfide solutions may contain varying amounts of polysulfide species and the actual reducing agent, and oxidized products in this reaction have not been identified.

Although kidney general acyl-CoA dehydrogenase forms the expected (Massey & Palmer, 1966; Massey & Hemmerich, 1980) blue neutral flavosemiquinone on reduction by a variety of methods (Thorpe et al., 1979; Mizzer & Thorpe, 1981), saturated or enoyl-CoA derivatives preferentially bind to the red anion radical markedly depressing the apparent pK (Mizzer & Thorpe, 1981). The addition of a few grains of solid sodium dithionite to an aerobic solution of the 8-Cl enzyme in phosphate buffer, pH 7.6, containing 160 μM crotonyl-CoA gave a typical anion radical spectrum with peaks at 476, 408, and 384 nm (the latter partially obscured by dithionite end absorbance). Despite the continued presence of excess dithionite, further reduction to the dihydroflavin form was extremely slow and was only partially complete after 6 h at 4 °C (results not shown).

7-Bromo-FAD–Enzyme. The spectrum of the 7-Br-FAD–enzyme shows a λ_{max} at 446 nm. Octanoyl-CoA (1.4 equiv) effects a rapid approximately 50% bleaching of the flavin chromophore with the appearance of a long-wavelength band (ϵ_{536} = 2.1 mM^{-1} cm^{-1}). Further additions of substrate do not increase the extent of reduction. However, the residual oxidized 7-bromoflavin component can be rapidly reduced by dithionite without bleaching the long-wave length band, as was originally observed with the native enzyme (Beinert & Page, 1957). Palmitoyl-CoA produces very similar spectral changes to those observed with octanoyl-CoA with about 50% bleaching of the 446-nm absorbance and a long-wavelength band at 536 nm. The explanation for this partial reduction of the flavin is not yet clear. Butyryl-CoA effects similar partial bleaching of the main absorbance peak, but the long-wavelength band is only about half the intensity as that obtained with octanoyl-CoA. The acetoacetyl-CoA complex of the 7-Br-FAD–enzyme shows a long-wavelength band at 580 nm (ϵ = 1.9 mM^{-1} cm^{-1}).

2-Thio-FAD–Enzyme. 2-Thio-FAD binds tightly to apoacyl-CoA dehydrogenase. On the addition of an excess of apoprotein, there is an increase in absorbance of the flavin by about 20%, to give an ϵ_{495} for the enzyme-bound flavin of 24 600 M^{-1} cm^{-1}. Unlike the two previous examples, only about half of the enzyme flavin is reduced rapidly by octanoyl-CoA; the remainder requires several hours for complete reduction. Both phases involve the same spectral changes. A weak long-wavelength band extends into the main oxidized-flavin absorbance peak. Dithionite reduction of the mixture did not allow the position of this band to be estimated with confidence. Both butyryl- and palmitoyl-CoA effect similar biphasic spectral changes to those observed with octanoyl-CoA but with little or no long-wavelength band. Acetoacetyl-CoA effects small spectral changes with a very weak long-wavelength tail overlapping the 497-nm peak.

All three enzyme derivatives described thus far show appreciable activity in the standard assay system (Table I), as might be expected on the basis of their reduction by substrates. Conclusions concerning the significance of these lowered rates cannot be drawn at present, since the rate-limiting step in the turnover of octanoyl-CoA in this assay system is not yet known. Reconstitution with the remaining analogues (Table I) does not yield active holoenzymes, and reduction of the bound flavin is not observed on the addition of thioester substrates.

5-Deaza-FAD–Enzyme. Again, binding of the flavin is accompanied by a marked increase in extinction coefficient, ϵ_{393} = 16 700 M^{-1} cm^{-1}. Addition of octanoyl-CoA to the 5-deazaflavin-substituted enzyme (Figure 2) perturbs the oxidized flavin chromophore, with no significant reduction of the bound analogue and no long-wavelength band formation. This is consistent with the considerably more negative oxidation–reduction potential of 5-deazaflavin compared to FAD (Table I). Thus the internal equilibrium, K_2, lies far to the left (see later). Figure 2 also shows that binding of acetoacetyl-CoA is accompanied by a slight decrease in absorbance at 400 nm and a decreased resolution of the shoulder at 420 nm. No long-wavelength band is observed.

Since octanoyl-CoA was an ineffective reductant of the 5-deaza enzyme, it was of interest to examine the reverse reaction: whether the reduced enzyme could be reoxidized by enoyl-CoA derivatives. Accordingly, the reduced enzyme was prepared by reconstituting apoprotein with 5-deaza-$FADH_2$ (see Methods). Only about 10% reoxidation of the bound reduced analogue occurs on aerobic dialysis, in keeping with the very slow reaction of 5-deaza-$FADH_2$ with molecular oxygen (Edmondson et al., 1972; Spencer et al., 1976). The addition of 24 μM crotonyl-CoA to the reduced enzyme causes rapid reoxidation of the flavin, which was complete before measurement could be made. The extreme thermodynamic

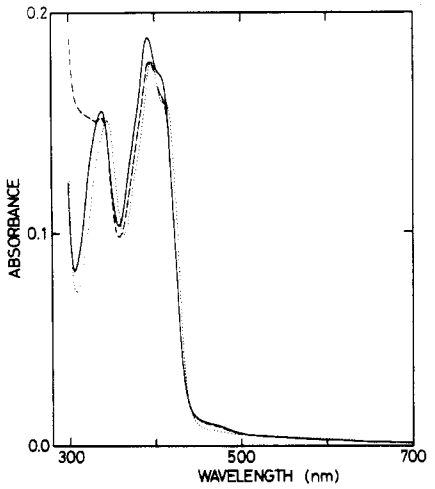


FIGURE 2: Perturbation of spectrum of 5-deaza-FAD general acyl-CoA dehydrogenase. A solution of the enzyme (11 μM) in 0.8 mL of 50 mM phosphate buffer, pH 7.6, 4 °C (—), was treated with 39 μM octanoyl-CoA (···) or 64 μM acetoacetyl-CoA (– –). No further spectral changes were observed on the addition of more ligand.

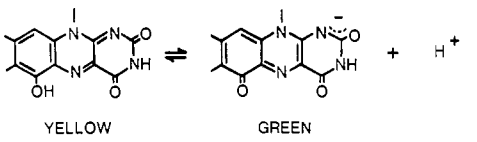
destabilization of the 5-deazaflavin radical in comparison to normal flavins (Blankenhorn, 1976) suggests that reoxidation of the reduced enzyme derivative is unlikely to proceed via discrete obligatory one-electron steps in this particular instance.

The reduced dialyzed enzyme is approximately 10-fold more fluorescent than free 5-deaza-$FADH_2$ at pH 7. Furthermore, the enzyme appears to bind preferentially the neutral dihydrodeazaflavin species. The emission maximum at pH 7 of the enzyme-bound form is at 420 nm (excitation maximum 320 nm). The emission maximum shifts to 370 nm, and the excitation maximum to ~310 nm on raising the pH to 10.8, with an apparent pK ~ 9.0 at 4 °C. This change, which is accompanied by only a small increase in fluorescence intensity, is perfectly reversible on lowering the pH again. The pK of reduced 5-deazariboflavin has been reported to be in the range 7.0–7.2 (Stankovich & Massey, 1976; Fisher et al., 1976). The strong fluorescence of the reduced enzyme disappears on reoxidation by crotonyl-CoA; uncomplexed oxidized 5-deaza-FAD–enzyme is also nonfluorescent. Although 15 μM octanoyl-CoA has no significant effect on the visible spectrum of the 5-deaza-$FADH_2$–enzyme, the fluorescence of the reduced enzyme is quenched by about 20-fold upon complex formation. This concentration of octanoyl-CoA also markedly slows the reoxidation of the reduced enzyme by crotonyl-CoA (the increase in 400-nm absorbance is now easily observed with a $t_{1/2}$ of about 30 s at 4 °C). Acetoacetyl-CoA (15 μM) effects a 1.15-fold enhancement of the fluorescence of the reduced enzyme at pH 7 without noticeable perturbation of the visible spectrum. When added to the reduced enzyme at higher pH values, it causes a shift of the emission spectrum toward that of the neutral dihydroflavin species.

1-Deaza-FAD–Enzyme. On binding to the apoenzyme, the absorption maximum shifts from 540 to 546 nm, and the extinction coefficient increases from 6800 (Spencer et al., 1977) to about 9000 M^{-1} cm^{-1}. Like 5-deazaflavin, apoenzyme reconstituted with 1-deazaflavin is neither active in the standard assay nor reduced by octanoyl-CoA. The long-wavelength edge of the main absorbance peak (λ_{max} = 546 nm) is red shifted by about 10 nm on binding octanoyl-CoA, with only very minor perturbations at other regions of the spectrum (data not shown).

6-Hydroxy-FAD–Enzyme. 6-Hydroxyflavins have been suggested as active site probes for flavoproteins (Mayhew et al., 1974; Massey & Hemmerich, 1980). In free solution,

Scheme I



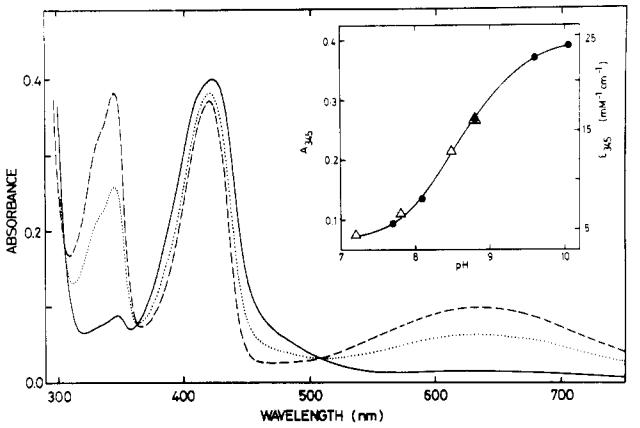


FIGURE 3: pH dependence of the spectrum of 6-OH-FAD–enzyme. The pH of the enzyme solution (16 μM in 0.9 mL of 50 mM phosphate buffer, 4 °C) was adjusted by the addition of small increments of solid sodium carbonate to an upper limit of 10.05 [see inset (●)] and then lowered to pH 7.2 with solid KH_2PO_4 [inset (△)]. The spectra shown correspond to pH 7.7 (—), 8.8 (···), and 10.05 (– –).

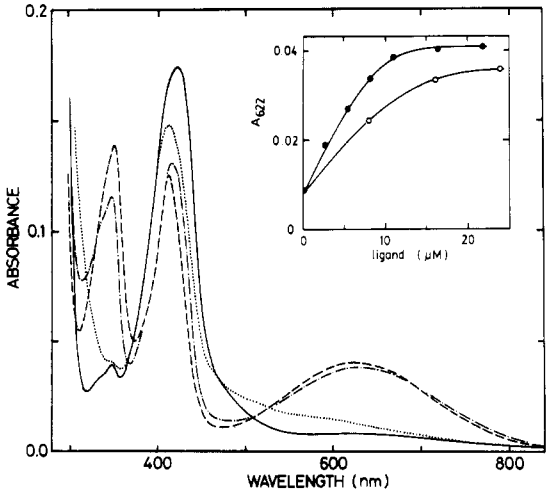
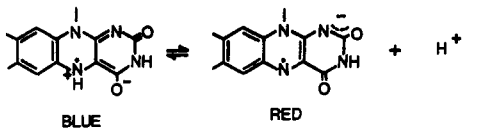


FIGURE 4: Effect of octanoyl-, crotonyl-, and acetoacetyl-CoA on the spectrum of 6-OH-FAD–enzyme: oxidized enzyme, 6.7 μM in 0.8 mL of 50 mM phosphate buffer, pH 7.6, 4 °C (—), and in the presence of 16 μM octanoyl-CoA (– –), 40 μM crotonyl-CoA (–·–), and 67 μM acetoacetyl-CoA (···). The inset is a plot of the absorbance at 622 nm vs. octanoyl-CoA (●) and crotonyl-CoA (○) concentration.

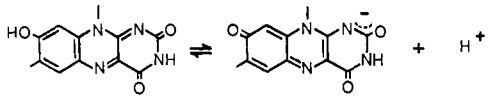
ionization of the yellow neutral species (pK = 7.1; Mayhew et al., 1974) may be represented as shown in Scheme I. For simplicity, only the dominant tautomeric and mesomeric forms of each ionization state is depicted. A pH titration of the 6-OH enzyme (Figure 3) clearly shows that the apoprotein exhibits a preferential binding (by about 40-fold) of the neutral yellow form, elevating the pK of the bound chromophore to 8.7.

Octanoyl-CoA does not reduce the 6-OH-FAD–enzyme; rather, it induces ionization of the bound flavin to give a green enzyme–substrate complex (Figure 4). The spectrum of this species, while clearly similar to that shown by the uncomplexed enzyme at high pH (Figure 3), is blue shifted in the long-wavelength region (from 632 to 618 nm on complex formation). The spectral changes in Figure 4 were complete before measurements could be made, and no further absorbance changes occurred. In accord with this failure to reduce enzyme

Scheme II



Scheme III



flavin, the derivative is not catalytically active in the standard assay system (Table I). Figure 4 also shows that crotonyl-CoA promotes formation of the green anion, with a spectrum very similar to that observed using octanoyl-CoA. This similarity also argues against significant reduction of the 6-hydroxyflavin chromophore on the addition of octanoyl-CoA, since crotonyl-CoA is an enoyl derivative: the product of substrate dehydrogenation. In contrast, acetoacetyl-CoA does not effect these characteristic spectral changes. Rather, the spectrum of the neutral flavin is perturbed with the formation of a weak long-wavelength band and without the appearance of the 343-nm peak characteristic of the green anion (Figure 4).

These results are of considerable interest to another line of investigation with native general acyl-CoA dehydrogenase. Recently, both saturated and enoyl-CoA derivatives, but not acetoacetyl-CoA, have been found to promote the conversion of the native blue radical form to the red anionic semiquinone by lowering the pK of this ionization by at least 2.5 pH units (Mizzer & Thorpe, 1981). The red anion radical is also believed to carry a negative charge at the N-1—C-2=O locus (Massey et al., 1979; Massey & Hemmerich, 1980; Scheme II). Thus the effect of acyl-CoA derivatives on the ionization of the oxidized 6-OH analogue mirrors that on the native flavosemiquinone, underscoring the usefulness of 6-OH-FAD as an active site probe in the acyl-CoA dehydrogenases. Additional evidence that acyl-CoA substrates and their enoyl-CoA products profoundly stabilize anionic flavin species comes from experiments with 8-OH- and 8-SH-FAD derivatives, and further discussion of this general effect is deferred until later.

8-Hydroxy-FAD–Enzyme. The spectrum of freshly prepared 8-OH-FAD–enzyme at pH 7.6 (Figure 5) suggests that the benzoquinoid anion (Ghisla & Mayhew, 1976, 1980; Massey & Hemmerich, 1980) is the predominant form at this pH. The corresponding ionization in free solution has a pK of 4.8 (Ghisla & Mayhew, 1976; Scheme III). Octanoyl-CoA perturbs the spectrum of the enzyme, leading to an intensification and red shift of the main absorbance peak (from 470 to 483 nm) without significant reduction of the enzyme and without formation of a long-wavelength band. Crotonyl-CoA generates similar spectral changes (λ_{max} = 486 nm) as does acetoacetyl-CoA, but the latter shows a less marked increase in absorbance at 487 nm upon complex formation (Figure 5). Experiments with the 8-hydroxyflavin–enzyme (and with the analogous 8-mercapto derivative, see later) are complicated by a marked tendency of the holoenzyme to lose flavin. Under the conditions of Figure 5, this reaction is half complete in about 120 min at 4°C (i.e., $k_{off} \sim 6 \times 10^{-3}$ min^{-1}). Interestingly, 24 μM octanoyl- or crotonyl-CoA strongly protects against this loss, whereas 25 μM acetoacetyl-CoA is much less effective. After 1 day, the samples from Figure 5 were dialyzed for a further day vs. 50 mM phosphate buffer, pH 7.6, 4 °C. The control sample, in the absence of thioester ligands, lost 90% of its flavin; the acetoacetyl-CoA-treated enzyme,

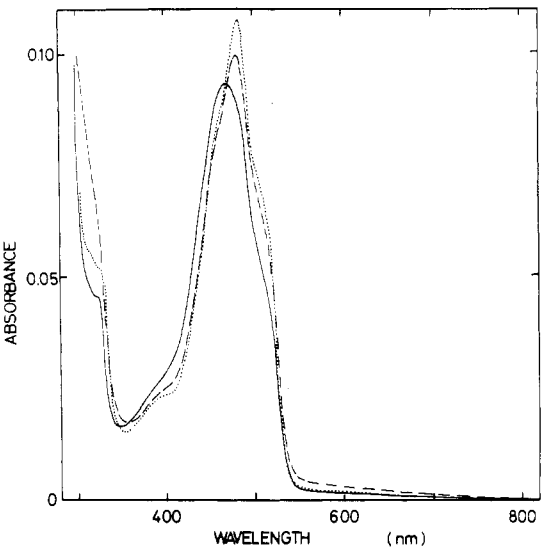


FIGURE 5: Effect of octanoyl- and acetoacetyl-CoA on the spectrum of 8-OH-FAD–acyl-CoA dehydrogenase. The spectrum of the derivative (1.8 μM in 0.8 mL of 50 mM phosphate, pH 7.6, 4 °C) was recorded immediately after gel filtration alone (—) or in the presence of 16 μM octanoyl-CoA (···) or 27 μM acetoacetyl-CoA (– –).

about 50%, whereas the octanoyl- and crotonyl-CoA-treated samples retained almost all of their flavin. This release of flavin is clearly reversible, since the addition of 24 μM crotonyl-CoA to enzyme allowed to dissociate in the absence of ligands for 1 day leads to the slow appearance of the spectrum of the crotonyl-CoA holoenzyme complex ($k_{on} \sim 8 \times 10^2$ M^{-1} min^{-1}, data not shown). If the binding of 8-OH-FAD is so weak, it might well be asked how a holoenzyme is formed in the first place. This presumably occurs because typically the apoprotein was allowed to incubate with an excess of the flavin at 25 °C for several hours; the mixture was then chilled in ice and promptly passed through a column of Sephadex G-25. With the slow dissociation rate at 4 °C, the holoenzyme slowly readjusts to equilibrium at 4 °C. From the kinetic behavior described above the K_d at pH 7.6, 4 °C, may be calculated

$$\text{E} + \text{8-OH-FAD} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{E·8-OH-FAD} \xrightarrow{\text{crotonyl-CoA}} \text{E·8-OH-FAD·crotonyl-CoA}$$

as 7×10^{-6} M from the estimated values of k_{on} and k_{off}.

8-Mercapto-FAD–Enzyme. The spectrum of the 8-mercapto analogue immediately after gel filtration (Figure 6) shows a resolved main absorbance peak at 592 nm together with peaks at 424 and 365 nm, suggestive of the benzoquinoid form of the 8-mercaptoflavin anion (Massey et al., 1979). Octanoyl-CoA and acetoacetyl-CoA perturb the spectrum with no detectable long-wavelength bands. As observed with the 8-OH enzyme, the 8-mercaptoflavin dehydrogenase is unstable, releasing flavin in a reaction half complete in about 280 min under the conditions of Figure 6. Dissociation of flavin is accompanied by a marked change in color of the solution from blue to red. Again, a dramatic stabilization is afforded by octanoyl- and crotonyl-CoA (Figure 6) with no release of flavin apparent in 60 h at 4 °C.

The reactivity of the 8-mercapto position can be probed by using the relatively small, highly reactive and uncharged reagent methyl methanethiosulfonate (Kenyon & Bruice, 1978). Incubation of freshly gel-filtered enzyme with 0.56 mM methyl methanethiosulfonate leads to the very slow ($t_{1/2}$ about 240 min at 4 °C) conversion of the spectrum to that expected for 8-(methylmercapto)-FAD (Moore et al., 1979). Probably, flavin is initially released from the holoenzyme to be attacked

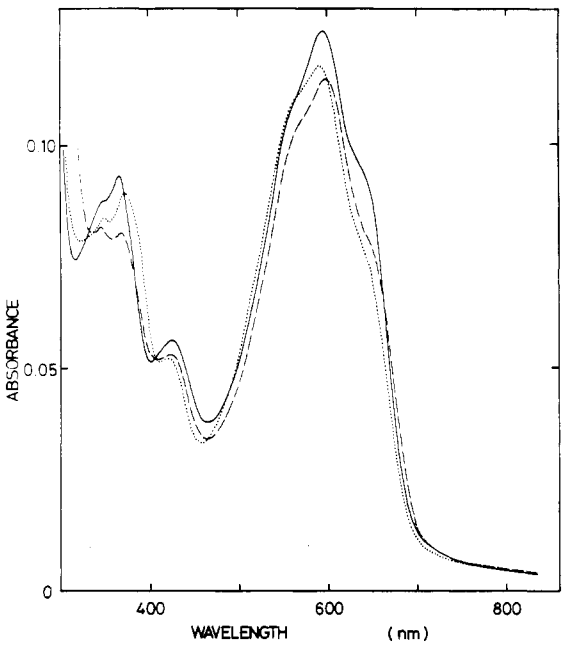


FIGURE 6: Perturbation of visible spectrum of 8-mercapto-FAD–acyl-CoA dehydrogenase using crotonyl- and octanoyl-CoA. The 8-mercaptoflavin enzyme was subjected to gel filtration, and the spectrum of 0.8 mL of 3.3 μM bound flavin was recorded immediately in 50 mM phosphate buffer, pH 7.6, 4 °C (—) and in the presence of 54 μM octanoyl-CoA (···) or 200 μM crotonyl-CoA (– –). See the text.

by the sulfhydryl reagent (see above). The complete protection against these spectral changes that octanoyl- and acetoacetyl-CoA afford is consistent with this explanation. These results corroborate the lack of reactivity found with the 8-Cl enzyme and suggest that the 8-position is shielded from solvent.

Stabilization of Anionic Flavin Species. Several lines of evidence suggest that acyl-CoA dehydrogenase apoprotein binds flavins that carry a negatively charged isoalloxazine ring more weakly than the corresponding neutral forms. Thus, chemical or photochemical reduction of the native dehydrogenase generates the blue neutral semiquinone up to pH values of at least 9.6 (Mizzer & Thorpe, 1981), indicating a considerable preference for binding the neutral as opposed to the anionic semiquinone (pK of ionization of free coenzyme = 8.5; Draper & Ingraham, 1968). Similarly, the pK for the conversion of the oxidized 6-OH-FAD to the green anion is raised from 7.1 to 8.7 on binding to the apoprotein. Finally, the benzoquinoid anions of both 8-OH- and 8-SH-FAD are bound noticeably more weakly than any of the other oxidized analogues used in this study. The fact that these two analogues are bound in their anionic forms at pH 7.6 is not inconsistent with the hypothesis that apoenzyme preferentially binds neutral isoalloxazine species. In this case, the pK values of the free flavins [4.8 for 8-OH-FAD (Ghisla & Mayhew, 1976, 1980) and 3.8 for 8-SH-FAD (Moore et al., 1979)] may be simply too low to be elevated into the neutral pH range upon binding to the dehydrogenase. Discrimination against these anions by the general acyl-CoA dehydrogenase may reflect an unfavorable accomodation of negatively charged flavin within a hydrophobic or already negatively charged protein environment. However, once bound, as in the case of 8-OH- and 8-mercapto-FAD anions, the flavin adopts the benzoquinoid form, carrying a negative charge at the N-1—C-2=O locus, rather than the corresponding phenolate or thiolate forms in which the negative charge is carried on the benzene subnucleus (Massey & Hemmerich, 1980). The finding that the spectrum of the unstable 8-SH- or 8-OH-FAD enzymes is typical of the benzoquinoid anion may not represent a particular stabilization of this form by a complementary positively charged residue in the vicinity of the N-1—C-2=O locus, as is observed with the flavoproteins of the dehydrogenase–oxidase class (Massey et al., 1979; Massey & Hemmerich, 1980), but rather an inability to stabilize the alternative thiolate or phenolate forms. This conclusion is supported by the results obtained with the 6-OH-FAD–enzyme, where the pK of the bound flavin is raised instead of lowered. The latter result would be expected if there were stabilization from a positively charged residue around the N-1—C-2=O locus (Massey & Hemmerich, 1980). In this connection, several observations can be made concerning the environment of the flavin in the acyl-CoA dehydrogenases. Electron transfer between general acyl-CoA dehydrogenase and electron-transferring flavoprotein may involve electrostatic interactions between a negatively charged surface adjacent to the dehydrogenase flavin and a complementary positively charged region on the acceptor (Frerman et al., 1980; Beckman & Frerman, 1982). A negatively charged flavin environment in the dehydrogenase is also consistent with the fact that anionic oxidants such as ferricyanide and 2,6-dichlorophenolindophenol are much more sluggish acceptors than the cationic phenazine methosulfate (Beinert, 1963; Thorpe et al., 1979). Finally, the work described in this paper suggests that the 8-position of the flavin is protected from solvent and is probably in a hydrophobic environment.

The marked stabilization of anionic flavin species on binding acyl-CoA substrates or products is evident by a decrease in the pK of the native blue semiquinone by at least 2.5 pH units (Mizzer & Thorpe, 1981), a sizable lowering of the pK of the oxidized bound 6-OH flavin chromophore, and the strong protection afforded the 8-OH and 8-SH enzymes against loss of flavin. Stabilization of a negative charge at the N-1—C-2=O locus has recently been suggested to involve a favorable electrostatic or hydrogen-bonding interaction with an adjacent amino acid side chain (Massey & Hemmerich, 1980). Possibly, binding of acyl- and enoyl-CoA derivatives elicits a conformational change or an adjustment in the polarity of this region of the active site favoring such a stabilization. Whatever the mechanism of this stabilization, the ineffectiveness of CoA derivatives that are neither substrates nor products suggests that this effect may be important catalytically. These data also predict that the two-electron-reduced native enzyme is a neutral dihydroflavin species and that formation of the characteristic enoyl-CoA–reduced flavin charge-transfer complex involves loss of a proton.

Registry No. FAD, 146-14-5; 8-Cl-FAD, 68385-36-4; 7-Br-FAD, 73358-81-3; 2-thio-FAD, 43203-75-4; 5-deaza-FAD, 57818-88-9; 1-deaza-FAD, 64183-67-1; 6-OH-FAD, 52301-43-6; 8-OH-FAD, 50909-80-3; 8-SH-FAD, 71800-97-0; octanoyl-CoA, 1264-52-4; acetoacetyl-CoA, 1420-36-6; crotonyl-CoA, 992-67-6; acyl-CoA dehydrogenase, 9027-65-0.

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S-Adenosyl-L-methionine Synthetase from Human Erythrocytes: Role in the Regulation of Cellular *S*-Adenosylmethionine Levels†

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ABSTRACT: The properties of human erythrocyte *S*-adenosyl-L-methionine synthetase (ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) were studied with respect to the role of *S*-adenosylmethionine in transmethylation reactions. Kinetic values obtained with both a cytosolic and a 350-fold purified preparation of enzyme were compared with measured intracellular concentrations of substrates and products. This analysis revealed that effective regulation of enzyme activity and product concentration can occur through feedback inhibition by *S*-adenosylmethionine (K_i = 2.0–2.9 μM; the endogenous concentration is 3.5 μM). This enzyme can be distinguished from *S*-adenosylmethionine synthetases found in other tissues and appears to be specialized for its role in erythrocyte methyl group metabolism, especially with regard to protein carboxyl methyl-transfer reactions.

S-Adenosyl-L-methionine is the source of methyl groups for a wide variety of biological methyl transfer reactions (Cantoni, 1975). In the red blood cell, this compound has been shown to be the substrate for protein carboxyl methylation reactions (Kim et al., 1980; Freitag & Clarke, 1981) and has been implicated in possible methylation reactions of histamine (Axelrod & Cohn, 1971), catechols (Axelrod & Cohn, 1971; Quiram & Weinshilboum, 1976), and phospholipids (Hirata & Axelrod, 1978). In the human erythrocyte, specific cytoskeletal and membrane proteins are reversibly methylated at D-aspartyl residues (Freitag & Clarke, 1981; McFadden & Clarke, 1982), and it has been proposed that the physiological role of this *S*-adenosylmethionine-dependent reaction is involved in the metabolism of aging proteins (McFadden & Clarke, 1982; Barber & Clarke, 1983).

Red cell *S*-adenosyl-L-methionine is formed in a cytosolic enzymatic reaction from L-methionine and ATP (Cohn et al., 1972; Tallan, 1979). Plasma L-methionine is rapidly equili-

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