Electron-Transferring Flavoprotein from Pig Kidney: Flavin Analogue Studies†

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ABSTRACT: Apo-electron-transferring flavoprotein from pig kidney (apo-ETF) has been prepared by an acid ammonium sulfate procedure and reconstituted with FAD analogues to probe the flavin binding site. The 8-position of the bound flavin is accessible to solvent as judged by the reaction of 8-Cl-FAD–ETF with sodium sulfide and thiophenol. A series of 8-alkylmercapto-FAD analogues containing increasingly bulky substituents bind tightly to apo-ETF and can be reduced to the dihydroflavin level by octanoyl-CoA in the presence of catalytic levels of the medium-chain acyl-CoA dehydrogenase. Bulky substituents severely slow the rate of these interflavin electron-transfer reactions. In the case of the 8-cyclohexylmercapto derivative, this decrease reflects a sizable increase in the K_m for ETF (approximately 14-fold) with only a 20% decrease in V_{max}. Reduction of all of these 8-substituted derivatives involves the accumulation of ETF anion radical intermediates. Dihydro-5-deaza-FAD dehydrogenase, unlike the corresponding 1-deazaflavin substitution, is unable to reduce native ETF despite a strongly favorable redox potential difference. These results, together with data from the native proteins, are consistent with obligatory 1-electron transfer between dehydrogenase and ETF possibly involving the exposed dimethylbenzene edge of ETF. Irradiation of apo-ETF reconstituted with the photoaffinity analogue 8-azidoflavin leads to approximately 10% covalent incorporation of the flavin. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of apo-ETF labeled with tritiated 8-azido-FAD shows preferential labeling of the smaller subunit (88%, M_r 30000 subunit; 12%, M_r 33000 subunit). These data suggest that the smaller subunit of ETF may comprise at least part of the flavin binding site in this heterodimeric electron acceptor.

Mammalian electron-transferring flavoprotein [ETF[1] (Crane & Beinert, 1956)] is a rather unusual electron carrier. First, it is a dimer of dissimilar subunits containing one FAD molecule per dimer (Furuta et al., 1981; Gorelick et al., 1982; McKean et al., 1983; Husain & Steenkamp, 1983). Second, it collects reducing equivalents from several mitochondrial dehydrogenases and delivers them to the electron-transport chain at the level of the membrane bound ETF-CoQ oxidoreductase (Ruzicka & Beinert, 1977; Beckman & Frerman, 1985):

substrates → flavoprotein dehydrogenases → ETF → ETF-CoQ oxidoreductase → CoQ → electron-transport chain

ETF was first recognized as the physiological electron carrier for the short, medium- and long-chain acyl-CoA dehydrogenases (Crane & Beinert, 1956; Beinert, 1963), then for sarcosine and dimethylglycine dehydrogenases (Beinert & Frisell, 1962; Frisell et al., 1966) and more recently for glutaryl-CoA dehydrogenase (Besrat, 1969, Noda et al., 1980), isovaleryl-CoA dehydrogenase (Noda et al., 1980) and 2-methylbutyryl-CoA dehydrogenase (Ikeda et al., 1983). Thus these interflavin redox reactions between primary dehydrogenases and ETF play a central role in several catabolic sequences.

The reoxidation of substrate-reduced dehydrogenases by ETF has been shown to involve the initial accumulation of ETF semiquinone, followed by the slower appearance of the dihydroflavin form (Hall & Lambeth, 1980; Reinsch et al., 1980; Beckmann et al., 1981; Steenkamp & Husain, 1982). Recent work with the medium-chain acyl-CoA dehydrogenase from pig kidney has clarified this reaction (Gorelick et al., 1985). The octanoyl-CoA-reduced dehydrogenase is rapidly oxidized in apparently 1-electron steps by two molecules of oxidized ETF:

$$dH_{2e}\text{-octenoyl-CoA} + ETF_{ox} \rightleftharpoons dH_{1e}\text{-octenoyl-CoA} + ETF_{1e}$$

$$dH_{1e}\text{-octenoyl-CoA} + ETF_{ox} \rightleftharpoons dH_{ox}\text{-octenoyl-CoA} + ETF_{1e}$$

Further reduction of the ETF semiquinone by the 1- and 2-electron-reduced forms of the dehydrogenase is much more sluggish, leading to the slower phases seen in rapid reaction experiments (Gorelick et al., 1985).

This paper discusses the use of FAD analogues to probe the flavin environment in pig kidney ETF. We show that interflavin redox reactions between the medium-chain acyl-CoA dehydrogenase and ETF apparently involve obligatory 1-electron steps. The 8-position of the bound flavin ring is exposed to solvent in ETF, and bulky substituents at this locus impede reduction by the dehydrogenase. Finally, a photoaffinity FAD analogue has been used to selectively label the

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; CoA, coenzyme A; CoQ, coenzyme Q; DCPIP, 2,6-dichlorophenolindophenol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ETF, electron-transferring flavoprotein; ETF_{ox}, oxidized ETF; ETF_{1e}, semiquinone ETF; FAD, flavin adenine dinucleotide; $FADH_2$, fully reduced FAD; FMN, riboflavin 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; dH, dehydrogenase; dH_{ox}, oxidized dH; dH_{1e}, semiquinone dH; dH_{2e}, fully reduced dH; NADH, reduced nicotinamide adenine dinucleotide; NaDodSO$_4$, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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smaller subunit of ETF, suggesting that this subunit may comprise at least part of the binding site for FAD.

Experimental Procedures

Materials. Ultrapure ammonium sulfate and guanidine hydrochloride were from Schwarz/Mann. PM-30 ultrafiltration membranes were from Amicon. Collodion bags (M_r 25 000 cutoff) and a vacuum concentrator were from Schleicher and Schuell. Protocatechuic acid, Folin and Ciocalteu's phenol reagent, DCPIP, DTNB, Coomassie Brilliant Blue R-250, FAD [further purified by the method of Massey & Swoboda (1963)], Norit A activated charcoal, and ATP were from Sigma. CoA thioesters were from P-L Biochemicals. Bradford protein assay concentrate, Bio-Lyte pH 4/6 ampholytes, acrylamide, and bisacrylamide were from Bio-Rad. Thiophenol, methyl disulfide, and cyclohexyl mercaptan were from Aldrich. Sodium sulfide was from Mallinckrodt. [2,8-$^3H_2$]ATP was purchased from ICN. *Brevibacterium ammoniagenes* ATCC 6872 was purchased from the American Type Culture Collection, Rockville, MD. The ACS scintillation cocktail was from Amersham. The following were generous gifts: 8-F-FAD, 8-F-riboflavin, 1-deazariboflavin, 5-deazariboflavin, and 6-OH-FAD (from Dr. Vincent Massey, University of Michigan); 8-Cl-riboflavin (from Dr. John Lambooy, University of Maryland); protocatechuate 1,3-dioxygenase (from Dr. David Ballou, University of Michigan). Pig kidney ETF and general acyl-CoA dehydrogenase were purified according to the modified methods described by Gorelick et al. (1985).

General Methods. Visible and ultraviolet spectra were obtained on a Cary 219 or a Perkin-Elmer 552A UV/vis spectrophotometer. NaDodSO$_4$–polyacrylamide gel electrophoresis was performed on an LKB Model 2117 Multiphor equipped with an LKB Model 2197 power supply. Gels were scanned on a gel scanner attachment for the Cary 219. All buffers were Tris/HCl, pH 8.5 at 4 °C, containing 0.3 mM EDTA and 5% v/v glycerol unless otherwise noted. Small-scale ultrafiltrations were performed with microconcentrators (Amicon Corp.: Centricon-10 and -30), following the manufacturer's instructions.

Preparation and Reconstitution of Apoprotein. ETF and general acyl-CoA dehydrogenase apoproteins were prepared essentially according to the method of Mayer and Thorpe (1981). ETF and the dehydrogenase apoproteins were precipitated with 10 volumes of resolving solution maintained at −10 °C in an ice–salt water bath. After centrifugation, the proteins were immediately redissolved in an equal volume of 150 mM Tris buffer and recentrifuged to remove the charcoal suspension. Apo-ETF was reconstituted immediately with either FAD or various analogues (see text; with approximately 30 min allowed for reconstitution at 4 °C). Reconstituted proteins were concentrated either by ultrafiltration (in a 10-mL stirred cell using a PM-30 ultrafiltration membrane followed by two 10-mL rinsings with buffer) or by vacuum concentration/dialysis via a collodion bag apparatus.

Assays. Enzyme assays were conducted as described in Gorelick et al. (1982). Protein determinations were performed either by the method of Lowry et al., (1951) or by the method of Bradford (1976) using the Bio-Rad protein assay concentrate. Titration of cysteine residues was as described in Gorelick et al. (1982). The covalent incorporation of FAD derivatives was assessed by precipitation and washing with TCA as described previously (Fitzpatrick et al., 1985).

Anaerobic Experiments. Anaerobic experiments were performed as described in Gorelick et al. (1982); however, the oxygen scrubber system used consisted of adding 10 μL of 30 μM protocatechuate 1,3-dioxygenase, and 200 μL of 15 mM Tris buffer to the side arm of an anaerobic cuvette containing a 0.4 × 5 cm piece of fluted filter paper. The cuvette was flushed with nitrogen for 1 min, and then 20 μL of 100 mM protocatechuate in 15 mM Tris buffer was added. The contents were then immediately deoxygenated by repeated evacuation as described earlier.

Preparation of Flavin Analogues. FAD analogues were prepared from the corresponding riboflavin derivatives and ATP with the FAD–synthetase from *Brevibacterium ammoniagenes* (Spencer et al., 1976).

Alkyl(aryl)mercapto-FAD analogues were prepared in the dark at 25 °C by mixing 8–10 μM 8-Cl-FAD with 5–10 mM thiol in deoxygenated 150 mM Tris buffer, pH 9.0, with continuous stirring under nitrogen. Thiols that were insufficiently soluble were dissolved by the addition of up to 70% tetrahydrofuran. After completion of the spectral changes (judged by the increase in absorbance at 480 nm, see later), 6 M HCl was added to give a pH of about 4.0–5.0, and tetrahydrofuran was evaporated with a stream of nitrogen. Except for CoASH, excess thiol was removed by ether extractions, and the residual ether was removed under a stream of nitrogen. 8-CH_3S-FAD was prepared in aqueous solution by first mixing 50 mM methyl disulfide with 25 mM dithiothreitol in 150 mM Tris buffer, pH 9.0 (10 mL total volume), under rigorously anaerobic conditions (Moore et al., 1979). A deoxygenated solution of concentrated 8-Cl-FAD was introduced into the incubation mixture after 10 min to a final concentration of 0.85 μM and flavin concentrated by lyophilization in the dark. The 8-alkylmercapto analogues were then mixed with equimolar apoprotein as described earlier. 8-N_3-FAD was prepared in the dark from 8-F-FAD and NaN_3 by the method of Fitzpatrick et al. (1985). Tritiated 8-F-FAD was prepared with the FAD–synthetase system using 1.0 mCi of [2,8-$^3H_2$]ATP diluted to give a specific activity of 0.265 Ci/mmol. The titriated 8-F-FAD was then converted to the azido derivative as described above.

Photolysis of Azidoflavin. Photolysis of ETF with bound 8-N_3-FAD was performed by using a Kodak 750H Carousel projector on the low lamp setting. The sample was photolyzed in an ice bath 10 cm from the front of the projector, which was equipped with a 102-mm f/2.8 Kodak Ektanar C projection lens.

Reduction of Deazaflavins. Preparation of reduced 5-deaza-FAD for reconstitution with general acyl-CoA dehydrogenase apoprotein was performed as described previously (Thorpe & Massey, 1983). Preparation of reduced 1-deaza-FAD-dehydrogenase was performed by reconstitution with dehydrogenase apoprotein and then photoreduction as described above without the use of the oxygen scrubber system.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. NaDodSO$_4$–polyacrylamide gel electrophoresis (using 10% slab gels in the presence or absence of 1% 2-mercaptoethanol) was performed by the method of Weber and Osborn (1969) on an LKB Multiphor and run according to the manufacturer's instructions. Slab gel electrophoresis was performed on photolyzed samples of tritiated 8-N_3-FAD bound to ETF according to the method of Laemmli (1970) employing the LKB Multiphor, without the use of a stacking gel. The protein samples were concentrated at 30 mA for 10 min and separated at 130 mA for 2 h. Gels were stained with Coomassie Brilliant Blue R-250, destained, photographed, and then sliced into sections (see Results and Discussion). Segments were placed in separate scintillation vials containing 1 mL of 30% H_2O_2 and digested overnight in a 60 °C water bath. The samples

were then allowed to cool before opening and adding 10 mL of ACS liquid scintillant. They were then kept in the dark for 1 h before scintillation counting in a Beckman LS-100C.

Results and Discussion

Preparation, Properties, and Reconstitution of ETF Apoprotein. At the outset of this work several methods for the preparation of ETF apoprotein were tried. The only successful procedure, a modification of the acid ammonium sulfate method (Mayer & Thorpe, 1981; see Experimental Procedures), gave 50% recovery of protein and a 4% residual flavin content determined spectrophotometrically. Attempts to lower this somewhat high residual FAD level by manipulation of a variety of experimental conditions were unsuccessful. ETF apoprotein solutions are labile and were therefore used immediately after the precipitated protein was redissolved from the charcoal pellet (see Experimental Procedures).

Reconstitution of apoprotein with a 5-fold molar excess of FAD at 4 °C for 30 min followed by gel filtration in 15 mM Tris buffer, pH 8.5, gave a visible spectrum indistinguishable from that of the native protein. Reconstituted ETF shows a slightly increased 272 nm/436 nm absorbance ratio (6.2 vs. 5.9) presumably reflecting a small level of soluble apoprotein unable to bind flavin under these conditions. Reconstituted and native enzymes have identical turnover numbers in the standard assays (see Experimental Procedures). Finally, like native ETF, the reconstituted material shows two equally staining bands (30 000 and 33 000) on $NaDodSO_4$–polyacrylamide gel electrophoresis (Gorelick et al., 1982). Thus, the resolution procedure does not result in the selective loss of one subunit type.

In general, the reconstitutions described in the remainder of this work were performed with 1 equiv of FAD analogues. At 10 μM levels, binding of all but the most bulky flavin analogues (see later) was complete in less than 30 s in 150 mM Tris buffer, pH 8.5, at 4 °C. The holoenzymes were then freed from the excess salts carried over from the resolution procedure by ultrafiltration or vacuum dialysis vs. 15 mM Tris buffer pH 8.5 at 4 °C (see Experimental Procedures).

It should be noted that the standard ETF assay (see Experimental Procedures) cannot be used to assess the amount of residual holoenzyme in ETF apoprotein preparations. This is because the terminal electron acceptor, DCPIP, reacts with ETF cysteine residues that are exposed upon removal of the FAD (data not shown). This reaction leads to an initial bleaching of the dye absorbance at 600 nm with subsequent appearance of a band at 660 nm as the reduced dye–cysteine species undergo reoxidation. Analogous reactions of DCPIP with proteins and model thiols have been described previously (Benitez & Allison, 1973; Hadler & Erwin, 1963; Rafter & Colowick, 1957; Coffey & Hellerman, 1964, 1965). Consistent with these observations, treatment of ETF apoprotein with 0.5 mM DTNB in 50 mM KPi, pH 7.6, at 4 °C results in the comparatively rapid reaction of five thiols ($t_{1/2}$ about 2 min; data not shown). In contrast, the holoprotein reacts sluggishly with DTNB with release of 0.3 mol of thionitrobenzoate after 10 min under identical reaction conditions (Gorelick et al., 1982).

8-Cl-FAD–ETF. The addition of 8-Cl-FAD to apoprotein yields a highly resolved visible spectrum (Figure 1) very similar to that seen with native pig kidney ETF (Gorelick et al., 1982). 8-Cl-FAD–ETF may be reduced with catalytic levels of general acyl-CoA dehydrogenase in the presence of octanoyl-CoA first to the red anionic semiquinone and then to the dihydroflavin form in analogy with the behavior of the native enzyme (Hall & Kamin, 1975; data not shown). The reactivity of

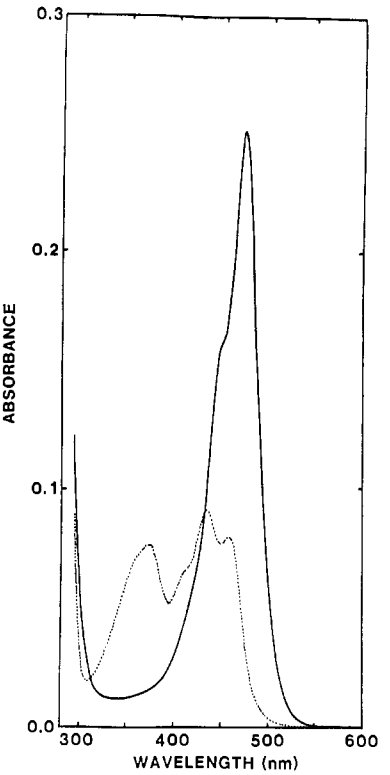

FIGURE 1: Effect of thiophenol on the visible spectrum of ETF reconstituted with 8-Cl-FAD. Apo-ETF (7.8 μM) was reconstituted with equimolar 8-Cl-FAD, and the protein was desalted by ultrafiltration (see Experimental Procedures) to give a concentration of 7.8 μM in 80 mM Tris buffer, pH 8.5, at 4 °C, containing 0.3 mM EDTA and 5% glycerol (···). Thiophenol was then added to a concentration of 1 mM and the final spectrum recorded after 1 h (—).

8-Cl-flavins toward thiols was used to examine the solvent accessibility around the 8-position of the bound isoalloxazine ring (Massey & Hemmerich, 1980). Figure 1 shows the spectral changes encountered on the addition of 1 mM thiophenol to 8-Cl-ETF. The typical two-banded spectrum is replaced ($t_{1/2}$ of 6 min) by an intense single maximum at 479 nm with a distinct shoulder at 452 nm. This spectrum is clearly different from that of free 8-phenylmercapto-FAD (Moore et al., 1978). Further, the analogue is readily reduced by catalytic levels of substrate reduced general acyl-CoA dehydrogenase suggesting that this derivative is bound in a nativelike state (see later). Thus it appears that the 8-position of the flavin is accessible to thiophenol and that the protein can accommodate this somewhat bulky substitutent without subsequent release of flavin. The use of 8-Cl-flavin as an active-site probe has identified a number of flavoproteins with apparently solvent-exposed 8-positions (Massey & Hemmerich, 1980). Of particular relevance to the present work is the small electron carrier, flavodoxin, whose crystal structure shows that the dimethylbenzene edge is exposed at the surface of the protein (Burnett et al., 1974; Watenpaugh et al., 1973).

Solvent accessibility of the 8-position of ETF has also been examined under the same conditions as in Figure 1 but with 5 mM sulfide used as a nucleophile. A new peak at 596 nm was obtained (data not shown), which corresponds closely to that expected for the benzoquinoid anion of 8-mercaptoflavin (Massey et al., 1979). In this form, the negative charge is carried over the N-1—C-2=O locus rather than on the benzene subnucleus (Massey et al., 1979). The stabilization of this species is in accord with the appearance of the red anionic radical upon reduction of ETF (Massey & Hemmerich, 1980). Complicating the spectral changes observed with

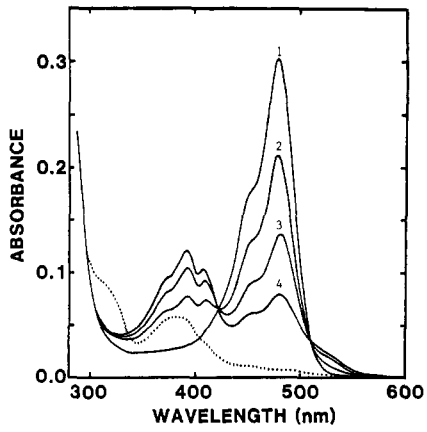

FIGURE 2: Catalytic reduction of 8-methylmercapto-FAD–ETF by the medium-chain acyl-CoA dehydrogenase from pig kidney. A solution of 9.4 μM 8-methylmercapto-ETF and 6.5 nM medium-chain acyl-CoA dehydrogenase in a total volume of 1 mL was deoxygenated, and spectrum 1 was recorded. Octanoyl-CoA was then added from a sidearm of the anaerobic cuvette to a concentration of 60 μM. Spectra were recorded at 2 nm/s intervals from 700 to 300 nm at 0.4, 6.4, and 24.3 min after mixing (curves 2–4, respectively). The dotted spectrum was recorded 18 h after tipping.

sulfide is a subsequent reduction of the bound 8-mercaptoflavin to yield a spectrum very similar to that of the corresponding 2-electron-reduced form (Massey et al., 1979). Thus full formation of the 8-mercaptoflavin species does not occur because of this competing reaction (maximal apparent ϵ_{596} = 11.9 mM^{-1} cm^{-1} at 40 min).

Preparation and Catalytic Reduction of Alkylmercapto-FAD–ETF Analogues. Since it appeared from the above that the 8-position of the bound isoalloxazine in mammalian ETF is exposed to solvent, a variety of 8-alkylmercapto-FAD derivatives were preformed (with methanethiol, 2-mercaptoethanol, octanethiol, cyclohexanethiol, and CoASH; see Experimental Procedures) and their binding to apo-ETF was examined. Except for the CoA analogue, where binding required 30 min for completion, the flavin derivatives were bound rapidly and were retained on ultrafiltration or dialysis. All gave spectra very similar to that of Figure 1 (see below). Thus even the bulky CoA moiety can be accomodated at the 8-position of the bound flavin.

Figure 2 shows the reduction of the 8-methylmercapto-FAD–ETF derivative by catalytic levels of the dehydrogenase in the presence of excess octanoyl-CoA. Reduction is comparatively rapid (see later) and proceeds to the dihydroflavin form via an apparently stoichiometric accumulation of the anionic semiquinone, judging by the sharp isosbestic points observed at 423 and 508 nm (Figure 2). Sizable accumulation of the red anionic radical is also observed with native ETF during catalytic reductions (Hall & Kamin 1975; Reinsch et al., 1980; Beckmann et al., 1981; Steenkamp & Husain, 1982; Gorelick et al., 1982). The methanethiol group used in Figure 2 is a small substituent, and so thiol groups of increasing size (see above) were tested under the same conditions. All gave spectral changes essentially identical with those shown in Figure 2 (data not shown). The rate of reduction of these 8-alkylmercapto-ETF derivatives is compared to that of native ETF in Figure 3. The rate of reduction is clearly sensitive to the bulk of the substituent at the 8-position. Thus the methanethiol substituent has a minimal effect on the rate compared to the native enzyme, followed by a grouping of 2-mercaptoethanol, cyclohexanethiol, and octanethiol with an intermediate rate. CoA-FAD–ETF is reduced extremely slowly (Figure 3; but again through the intermediacy of the flavin semiquinone).

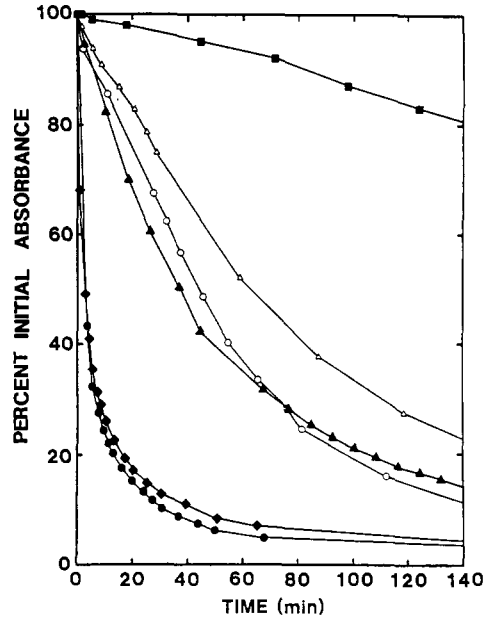

FIGURE 3: Comparison of the rate of reduction of ETF and 8-alkylmercapto-FAD–ETF derivatives by pig kidney medium-chain acyl-CoA dehydrogenase. Catalytic reductions were performed under the conditions described in Figure 2, plotting the changes as a percentage of the initial absorbance at 436 (native ETF, ●) and 482 nm for 8-CH_3S-(◆), 8-$HOCH_2CH_2S$- (▲), $CH_3(CH_2)_7S$- (○), 8-cyclohexylmercapto- (△), and 8-CoA-FAD-substituted ETF (■).

The decrease in apparent rate (Figure 3) for the reduction of the ETF analogues with native dehydrogenase could, in principle, be due to a combination of V_{max} and K_m effects. This was examined by analyzing the kinetic parameters for the reduction of 8-cyclohexylmercapto-FAD bound to ETF in the standard assay system (using 10 nM general acyl-CoA dehydrogenase; see Experimental Procedures). Substitution of the analogue reduced the V_{max} slightly, to 80% of the value shown by the native enzyme. In contrast, the K_m was drastically raised from 0.79 μM with ETF to 11.0 μM with the ETF analogue. It should be noted that the behavior seen in Figure 3 cannot be ascribed to differences in redox potentials between the various analogues, since 8-alkylmercaptoflavin derivatives have 2-electron-oxidation–reduction potentials very similar to those of normal flavins (Moore et al., 1979).

Interaction of ETF with Reduced 5-Deaza- and 1-Deaza-FAD General Acyl-CoA Dehydrogenase. 5-Deaza-FAD-substituted dehydrogenase is used in this section to test whether interflavin redox reactions between dehydrogenase and ETF involve obligatory 1-electron transfers or whether, for example, transfer of a hydride equivalent is possible between the two redox partners. The feasibility of this approach rests on the extreme thermodynamic destabilization of the 5-deazaflavin radical in comparison to normal flavin (Blankenhorn, 1976). As noted earlier, the ETF anionic radical is an intermediate in the reduction of this electron carrier by substrate-reduced general acyl-CoA dehydrogenase. Similarly, the reduction of ETF by photoreduced general acyl-CoA dehydrogenase also involves the transient appearance of ETF semiquinone together with concomitant formation of the blue neutral dehydrogenase radical (Gorelick et al., 1985).

Figure 4 represents the incubation of 1,5-dihydrodeaza-FAD dehydrogenase (see Experimental Procedures) with equimolar ETF under anaerobic conditions (see legend). The solid curve, recorded immediately after adding oxidized ETF from a side arm, represents the summation of the spectra of the reactants. After 5 h almost no reduction of ETF had occurred (see Figure 4). In contrast, the native dihydroflavin dehydrogenase reduces

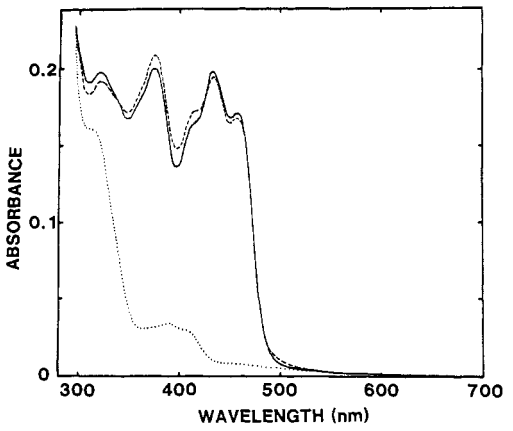

FIGURE 4: Interaction between 1,5-dihydro-5-deaza-FAD medium-chain-length acyl-CoA dehydrogenase and oxidized ETF. The reduced dehydrogenase derivative (13.3 μM in 1.0 mL of 50 mM phosphate buffer, pH 7.6, 25 °C) was deoxygenated in the main space of an anaerobic cuvette. Spectra were recorded before (···), immediately after (—), and 300 min after equimolar oxidized native ETF was added from a side arm (- -).

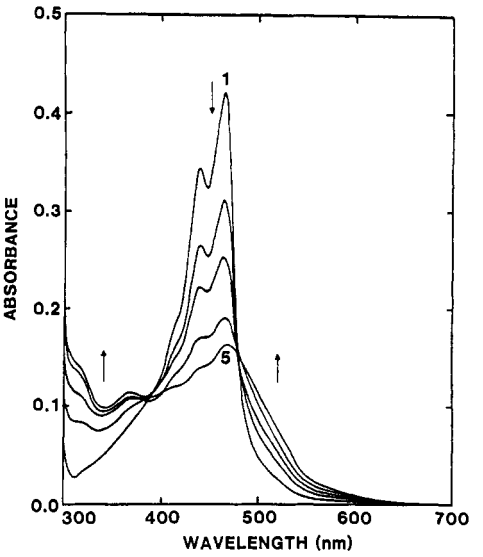

FIGURE 5: Spectral changes accompanying the photolysis of 8-azido-FAD–ETF. Apoprotein was reconstituted with 8-azido-FAD as described in Experimental Procedures and dialyzed vs. 15 mM Tris buffer, pH 8.5, at 4 °C, containing 0.3 mM EDTA and 5% glycerol. The holoprotein (17 μM, curve 1) was illuminated for a total of 2.5, 5, 14, and 1500 s (curves 2–5, respectively; see Experimental Procedures).

ETF comparatively rapidly to the semiquinone and hydroquinone states with half-times of 0.6 and 60 s under similar conditions (Gorelick et al., 1985). The failure of the 5-deaza-FADH$_2$-substituted dehydrogenase to reduce ETF cannot simply be ascribed to thermodynamic considerations, since the analogue is a thermodynamically stronger two-electron reductant than normal flavin [by about 100 mV; see Stankovich and Massey (1976) and Walsh et al. (1978)]. Finally it should be noted that, in contrast to the oxidative half-reaction, 1,5-dihydrodeaza dehydrogenase is competent in the reductive half-reaction (Ghisla et al., 1984). Thus, crotonyl-CoA is rapidly reduced with transfer of a hydride equivalent from the 5-position of the flavin to C-3 of the bound thio ester (Ghisla et al., 1984).

The experiment shown in Figure 4 was repeated with general acyl-CoA dehydrogenase reconstituted with 1-deaza-FAD (Thorpe & Massey, 1983) and photoreduced prior to the anaerobic addition of ETF (see Experimental Procedures). This flavin analogue has a redox potential similar to that of 5-deazaflavin (Walsh et al., 1978) but, like normal flavin, has an accessible semiquinone state. Reduction of ETF with dihydro-1-deaza dehydrogenase showed a half-time of 30 min (data not shown). This is much more rapid than in the 5-deazaflavin case (see Figure 4) consistent with the hypothesis of obligatory 1-electron transfers, but considerably slower than for the native reduced dehydrogenase (see above). The molecular basis of this difference is unknown.

Thus in agreement with rapid reaction studies (Hall & Lambeth, 1980; Reinsch et al., 1980; Gorelick et al., 1985), interflavin communication between general acyl-CoA dehydrogenase and ETF probably occurs via discrete 1-electron steps thermodynamically inaccessible to 5-deazaflavins. Clearly, reduction does not involve the direct transfer of a hydride equivalent between the N-5 positions of the reactants. In any case, such a geometry would appear unlikely in view of the hydrophobicity of the flavin binding site in ETF and the considerable molecular reorganization such an interflavin hydride reaction would entail. In view of the inhibition of electron transfer by bulky substituents at the 8-position, a favorable geometry for this reduction may require approach of dehydrogenase flavin to the exposed dimethylbenzene edge of ETF. As has been observed in a number of other redox proteins (Salemme, 1977; Poulos & Kraut, 1980; Adman, 1979), the orientation of dehydrogenase to ETF appears to involve the association of complementary charged surfaces (Beckman & Frerman, 1985). The present work shows that ETF, like the electron carrier flavodoxin (Mayhew & Ludwig, 1975), has at least part of the dimethylbenzene edge of the bound flavin prosthetic group exposed to solvent. Thus, in analogy with the studies of flavodoxin (Simondsen et al., 1982), this feature may be important in electron-transfer reactions of ETF. In contrast, the complementary region of the dehydrogenase flavin appears to be solvent inaccessible by the criteria used in this paper (Thorpe & Massey, 1983). However, electron transfer, e.g. via tunneling (Marcus & Sutin, 1985), does not necessarily require van der Waals contact of the prosthetic groups of donor and acceptor and may be less sensitive to the orientation of reactants than hydride transfer mechanisms.

Covalent Linkage of 8-Cl-FAD with ETF Apoprotein. In the experiments described earlier, reconstitution of apo-ETF with 8-Cl-FAD was followed immediately by dialysis or ultrafiltration to remove ammonium sulfate and KBr carried over from the resolution procedure (estimated to be about 0.1 M and 0.01 M respectively; see Experimental Procedures). However if the holoenzyme is incubated at these relatively high ionic strengths, slow formation of a covalent linkage between flavin and a protein cysteine residue is observed ($t_{1/2}$ = 500 min, 93% covalent by TCA precipitation, data not shown). A similar reaction has been reported for dihydrolipoamide dehydrogenase (Moore et al., 1978) and for electron-transferring flavoprotein from *Megasphaera elsdenii* (Massey & Hemmerich, 1980; O'Nuallain & Mayhew, 1986; see later). Thus this observation might enable the specific labeling of one of the two dissimilar subunits of ETF. However, analysis by NaDodSO$_4$–polyacrylamide gel electrophoresis, by HPLC using C_4 or C_{18} reverse-phase columns, or by chromatofocusing were all thwarted by the tendency of the labeled ETF derivative to polymerize to yellow aggregates, which contain both subunits in approximately equal amounts Gorelick, 1985; data not shown). Accordingly, an alternative approach to locating the flavin binding site was explored.

Photoaffinity Labeling of ETF with 8-Azido-FAD. The use of 8-azido-flavins as photoaffinity labels for a variety of

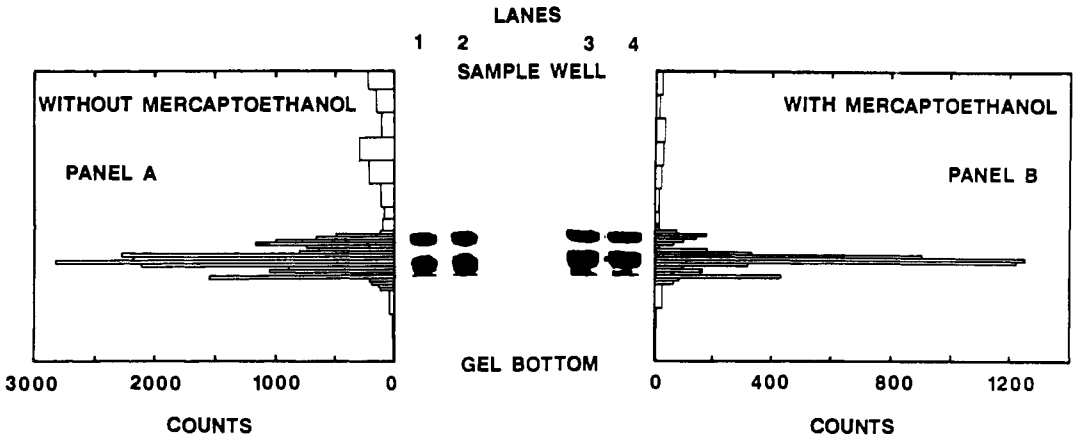

FIGURE 6: NaDodSO$_4$–PAGE of tritiated 8-N$_3$-FAD labeled ETF. [2,8-^{3}H$_2$]Adenine-labeled 8-N$_3$-FAD was reconstituted with ETF and photolyzed as described in Figure 5. A 10-μL aliquot of a 3.0 mg/mL solution of labeled ETF was applied to a horizontal NaDodSO$_4$ gel and separated according to the method of Laemmli (1970) as described in the Experimental Procedures. Lanes 1 and 2 contain enzyme incubated without 2-mercaptoethanol. Lanes 3 and 4 contain enzyme incubated with 1% 2-mercaptoethanol. Panels A and B represent the number of counts per millimeter of gel, plotted as a function of distance from the top of the gel. Counts are corrected to account for the differences in the widths of the slices. The distance from the sample well to the bottom of the gel prior to slicing was 7.6 cm. The counts are corrected for a background of 50 counts/min.

flavoproteins has recently been described with covalent incorporations of about 7–30% after photolysis (Fitzpatrick et al., 1985). Reconstitution of apo-ETF with 8-azido-FAD results in an increased resolution and a red shift of the absorbance maximum to 466 nm together with the appearance of a minor peak at 441 nm (Figure 5, curve 1). Photolysis effects rapid changes (Figure 5), which are similar to those reported for a number of other flavoproteins, and which presumably involve the generation of a number of products (Fitzpatrick et al., 1985). Analysis of the material from curve 5 of Figure 5 revealed 10% covalent incorporation (see Experimental Procedures). This somewhat low value is not unexpected for photoaffinity labels, particularly in view of the accessibility of the 8-position of the bound isoalloxazine to solvent in ETF (Fitzpatrick et al., 1985; see earlier). The experiment shown in Figure 5 was then repeated with 8-azido-FAD containing tritiated adenine, and the photolyzed protein was washed twice by ultrafiltration. Figure 6 depicts this material separated by NaDodSO$_4$–polyacrylamide gel electrophoresis. The gel was stained for protein and then sliced such that each band was divided into several sections. In the absence of 2-mercaptoethanol, the two bands were separated quite well (Gorelick et al., 1982) with 74% of the counts in the smaller subunit and 26% of the counts in the larger subunit. In the presence of 2-mercaptoethanol, the smaller subunit contained 88% of the counts with the larger subunit containing 12% of the counts. Note that the overall recovery of counts and the amount of radioactivity between the sample well and the M_r 33 000 subunit is considerably lower in the 2-mercaptoethanol-treated sample. The inclusion of 2-mercaptoethanol may result in the displacement of certain flavin-protein linkages notably 8-*S*-alkyl ones (see earlier). The photolysis experiment was then repeated under the same conditions as before but with 0.9 equiv of 8-N$_3$-FAD mixed with holo-ETF containing FAD. After 2 min, the mixture was photolyzed and analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. Approximately 3% incorporation of covalent label was noted, with 96% of this associated with the smaller subunit and about 4% with the larger subunit. It is at present uncertain why this control experiment yields such an asymmetric labeling of the two subunits.

The finding that 8-N$_3$-FAD preferentially labels the smaller subunit represents the first indication for the location of a flavin binding site in mammalian ETF. This considereation should be regarded as preliminary in view of the small percentage of incorporation obtained. Further, one could imagine an extreme situation in which the 8-position of the bound flavin was adjacent to the subunit interface. Photolysis might then result in the preferential labeling of the subunit that contained little if any of the FAD binding site. Obviously other covalent probes with reactive functions at different positions on the FAD molecule are needed for comparison. If all approaches lead to preferential labeling of the smaller subunit, then it would appear reasonable that this subunit contains the flavin binding site.

Recently, the interaction 8-Cl-FAD with apo-ETF from the rumen microorganism *Megasphaera elsdenii* has been reported (O'Nuallain & Mayhew, 1986). In this organism, ETF transfers reducing equivalents from D-lactate dehydrogenase to butyryl-CoA dehydrogenase in the excretion of butyrate (Baldwin & Milligan, 1964). Unlike ETF from mammalian sources (Beinert 1963), the *M. elsdenii* protein can also be reduced by NADH with transfer of reducing equivalents either to the short-chain acyl-CoA dehydrogenase or to redox dyes (Whitfield & Mayhew, 1974; Brockman & Wood, 1975). In common with other ETF proteins, the *M. elsdenii* protein has two dissimilar subunits (Whitfield & Mayhew, 1974), but unlike the mammalian and certain other bacterial ETFs [e.g. *Paracoccus denitrificans* and *Methylcoccus W3Al*; see Husain and Steenkamp (1983) and Steenkamp and Gallup (1978)], it contains two molecules of FAD per heterodimer (Whitfield & Mayhew, 1974). 8-Cl-FAD binds to both subunits of apo-ETF from *M. elsdenii* but reacts covalently only with the smaller subunit (O'Nullain & Mayhew, 1986). This covalent derivative retains diaphorase activity but is now no longer able to transfer reducing equivalents to the short-chain acyl-CoA dehydrogenase (O'Nuallain & Mayhew, 1986). It will be interesting to know whether the smaller subunit of *M. elsdenii* and mammalian ETF proteins are functionally and perhaps evolutionarily related.

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Registry No. FAD, 146-14-5; 8-N$_3$-FAD, 96099-56-8; 8-Cl-FAD, 68385-36-4; CoASH, 85-61-0; 8-CH$_3$-S-FAD, 76510-49-1; 8-

CH_3CH_2-S-FAD, 104487-54-9; 8-$CH_3(CH_2)_7$-S-FAD, 104505-50-2; 8-cyclohexyl-S-FAD, 104465-99-8; 8-CoA-FAD, 104487-55-0; MeSH, 74-93-1; $HO(CH_2)_2SH$, 60-24-2; $Me(CH_2)_7SH$, 111-88-6; MeS_2Me, 624-92-0; dithiothreitol, 3483-12-3; cyclohexanethiol, 1569-69-3; acyl-CoA dehydrogenase, 9027-65-0.

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