Purification and Properties of Electron-Transferring Flavoprotein from Pig Kidney†

Robert J. Gorelick, John P. Mizzer, and Colin Thorpe*

ABSTRACT: Electron-transferring flavoprotein has been isolated from pig kidney by a simple procedure with a 7-fold higher yield over a previous method using pig liver. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, gel filtration, amino acid analysis, peptide mapping, and measurement of flavin content indicate that pig kidney electron-transferring flavoprotein contains nonidentical subunits (M_r 30000 and 33000) with one flavin adenine dinucleotide per dimer. These data contrast with reports that the liver protein is a dimer of identical subunits containing two flavin molecules. Dithionite and ferricyanide titrations indicate that flavin is the only redox-active moiety in pig kidney electron-transferring flavoprotein. Disproportionation of the anionic semiquinone is very slow, requiring about 10 h for half-completion. In contrast to results obtained with the liver protein, pig kidney electron-transferring flavoprotein does not bind crotonyl coenzyme A (crotonyl-CoA) significantly, and the semiquinone form is not reoxidized by crotonyl-CoA directly. These data do not support recent suggestions for a broader role of electron-transferring flavoprotein in β oxidation.

Three distinct flavoproteins participate in the transfer of reducing equivalents from acyl coenzyme A (acyl-CoA) derivatives to the electron-transport chain during mitochondrial fatty acid oxidation. The initial flavin-catalyzed dehydrogenation involves the acyl-CoA dehydrogenases which are classified according to their chain length specificity (Beinert, 1963a) for short- (green et al., 1954), medium- (Crane et al., 1956; Hall & Kamin, 1975; Thorpe et al., 1979), or long-chain acyl-CoA thioesters (Hauge et al., 1956; Hall et al., 1976). The second flavoprotein, electron-transferring flavoprotein (ETF;[1] Crane & Beinert, 1956; Beinert, 1963b; Hall & Kamin, 1975), mediates the transfer of reducing equivalents to the respiratory chain at the level of a membrane-bound iron–sulfur flavoprotein, ETF dehydrogenase (Ruzicka & Beinert, 1977). In addition to its important role in β oxidation, ETF also serves as an electron carrier in other mitochondrial oxidation reactions, for example, during the dehydrogenation of isovaleryl-CoA (Noda et al., 1980), 2-methylbutyryl-CoA (Ikeda & Tanaka, 1982), and glutaryl-CoA (Besrat et al., 1969; Noda et al., 1980) and in the oxidative N-demethylation of sarcosine and dimethylglycine (Beinert & Frisell, 1962; Frisell et al., 1966).

Pig liver ETF has been the subject of several papers in recent years. ETF from this source has been reported to be a dimer of M_r 28000 subunits each containing one FAD (Hall & Kamin, 1975; Hall, 1981). Our studies on flavoenzymes in fatty acid oxidation have used pig kidney as the source tissue, and during the purification of ETF, it became apparent that the properties of pig kidney ETF markedly differed from those reported for the liver preparation. In particular, pig kidney ETF is a heterodimer containing only one FAD per dimer. While this work was in progress, Hashimoto and colleagues (Furuta et al., 1981) reported that rat liver ETF is also composed of two subunits of unequal size, although the flavin content was not specified. In view of these marked differences, we report below the properties of pig kidney ETF in some detail. In particular, the suggestion that ETF plays an additional role in β oxidation by binding acyl-CoA derivatives has been addressed.

Experimental Procedures

Materials

CoA derivatives were purchased from P-L Biochemicals. Phenylmethanesulfonyl fluoride, phenazine methosulfate, 2,6-dichlorophenolindophenol, 5,5′-dithiobis(2-nitrobenzoic acid), Trizma base, Coomassie Brilliant Blue R-250, superoxide dismutase, cytochrome *c*, glucose oxidase, catalase, diphenylcarbamyl chloride treated trypsin, iodoacetic acid, dithioerythritol, fluorescamine, $NaDodSO_4$ gel electrophoresis molecular weight standards, $NaDodSO_4$, and FAD [further purified by the method of Massey & Swoboda (1963)] were obtained from Sigma. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman. Matrex gel blue A and PM-30 ultrafiltration membranes were from Amicon. Acrylamide was from Bio-Rad. Guanidine hydrochloride and ammonium sulfate, both ultrapure, were purchased from Schwarz/Mann. Sephacryl S-200 superfine and a gel filtration molecular weight calibration kit were purchased from Pharmacia. Cibacron Blue F3GA was obtained from Polysciences Inc. Amino acid standards were purchased from Beckman. Oxygen and ultra high purity grade nitrogen were obtained from Linde. *N*,*N*′-Methylenebis(acrylamide), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and 20 × 20 cm cellulose-coated plates were obtained from Eastman. Calcium phosphate gel was prepared by the method of Swingle & Tiselius (1951). 3,10-Dimethyl-5-deazaisoalloxazine and lumiflavin 3-acetate were gifts from Dr. Vincent Massey. All other chemicals were reagent grade.

Methods

Visible and ultraviolet spectra were obtained by using a Cary 219 spectrophotometer. Fluorescence spectra were recorded by using a Perkin-Elmer 650-10S fluorescence spectrophotometer. Assays were performed on either a Cary 219 or a Beckman DB spectrophotometer. $NaDodSO_4$ slab gel densitometry was performed on a Biomed Instruments Model SL504 soft laser scanning densitometer. $NaDodSO_4$ tube gel

† From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received July 26, 1982.* This work was supported by a grant from the U.S. Public Health Service (GM 26643).

[1] Abbreviations: ETF, electron-transferring flavoprotein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Purification of Electron-Transferring Flavoprotein and Acyl-CoA Dehydrogenase from Pig Kidney

step	total A_{280}	total[a] A_{450}	A_{280}/A_{450}
(1) supernatant from homogenate of 1004 g of pig kidney cortex	121000	8600	14.0
(A) ETF Purification			
(2A) filtrate from DE-52 batch step	83300	4750	17.5
(3A) 50–85% ammonium sulfate precipitation	16100	1900	8.5
(4A) first DE-52 column	3040	68	45
(5A) calcium phosphate gel–cellulose column	584	15.3	38
(6A) second DE-52 column	48	7.6	6.3
(7A) CM-52 column	29	5.3	5.5
(B) Dehydrogenase Purification			
(2B) DE-52 batch adsorption of supernatant; column elution of yellow fractions	3060	96	31.9
(3B) 35–75% ammonium sulfate precipitation	1530	66.5	23.0
(4B) calcium phosphate gel–cellulose column	332	31.6	10.5
(5B) Matrex gel blue A column			
(a) primary	79.5	13.8	5.8
(b) secondary	38.3	5.1	7.5

[a] Includes contributions from other chromophores, e.g., hemoproteins and iron–sulfur centers.

densitometer scans were done by using a Varian gel scanner accessory for the Cary 219. Thin-layer peptide mapping was performed on a Desaga thin-layer electrophoresis apparatus by using a Gelman regulated power supply.

Concentrations of pig kidney general acyl-CoA dehydrogenase and pig kidney electron-transferring flavoprotein are expressed with respect to bound flavin by using the extinction coefficients of 15.4 mM^{-1} cm^{-1} (Thorpe et al., 1979) and 13.3 mM^{-1} cm^{-1}, respectively. The extinction coefficient of ETF-bound flavin was measured by using 12.5 μM enzyme in 4 M guanidine hydrochloride in 100 mM phosphate buffer, pH 7.6, and 0.3 mM EDTA, by the method described previously (Thorpe et al., 1979).

Enzyme Purification. All procedures were performed at 0–4 °C with buffers containing 0.3 mM EDTA. Ammonium sulfate saturation levels refer to 25 °C. A summary of the purification scheme is shown in Table I. Cortex from 14 partially thawed pig kidneys (stored at −70 °C) was cut into small pieces, and 1004 g was combined with 2.5 L of 50 mM phosphate buffer, pH 7.2, containing 250 mg of phenylmethanesulfonyl fluoride. The mixture was homogenized for 1 min at low speed and then 2 min at top speed in a Waring blender and centrifuged at 21000*g* in a Sorvall GSA rotor for 30 min. The supernatant was mixed with 400 mL of packed wet DE-52, previously equilibrated in 50 mM phosphate buffer, pH 7.2. The slurry was gently stirred for 1.5 h before being filtered through Whatman 4 filter paper supported on a large Büchner funnel. The DE-52 containing adsorbed general acyl-CoA dehydrogenase was washed with 2 L of 50 mM phosphate buffer, pH 7.2, and treated as described later (Table I, steps 2B–5B).

(*A*) *ETF Purification.* Buffers for the ETF purification are Tris, pH 8.5 (at 4 °C), with 5% v/v glycerol unless otherwise noted. The filtrate (step 2A, 2.72 L) was adjusted to pH 7.8 with 1 M NH_4OH, and 5% v/v glycerol and 0.115 g of FAD were added. The filtrate was then fractionated with ammonium sulfate. The 50–85% red precipitate was redissolved in 150 mL of 300 mM Tris buffer and dialyzed for 9.5 h against 7 L of 5 mM Tris buffer containing 30 mg of FAD and then for a further 7 h vs. the same buffer without added flavin. The dialyzed protein material was then loaded onto a DE-52 (5 × 41 cm) column previously equilibrated with 15 mM Tris buffer and run at 120 mL/h. Red hemoproteins bound tightly at the top. A band of flavin fluorescence moved relatively rapidly down the column following a broader fainter yellow band containing ETF. ETF could be clearly discriminated from the more slowly moving band by the distinctly greener fluorescence of ETF under a long-wavelength UV lamp, and by its clearly different fluorescence emission spectrum (see text). The fluorescent ETF fractions were combined, and the yellow-brown solution was applied to a 2.5 × 20 cm calcium phosphate gel–cellulose column (Massey, 1960) equilibrated with 15 mM Tris buffer. The column was washed at 80 mL/h with 500 mL of 15 mM Tris buffer, removing some contaminating proteins, and ETF was then eluted by using a linear gradient formed by mixing 200 mL of 15 mM Tris buffer and 100 mM phosphate buffer, pH 8.0. The fluorescent ETF fractions were pooled and concentrated to 4.7 mL with 30 μM added FAD via ultrafiltration using a PM-30 membrane. The sample was then dialyzed against 4 L of 15 mM Tris buffer for 10 h and applied to a DE-52 column (2.5 × 100 cm) equilibrated at 35 mL/h with the same buffer. The fluorescent ETF band moved fairly rapidly down the column, separating from a closely running faint brown band. Fluorescent fractions with an A_{280}/A_{436} ratio of less than 7.5 were pooled and concentrated by ultrafiltration to a volume of 2.5 mL. The resulting concentrate is free of heme contaminants and acyl-CoA dehydrogenase activity. It is usable at this point for many spectrophotometric experiments but showed the presence of minor protein contaminants (a total of about 5%) on $NaDodSO_4$–polyacrylamide gels. Homogeneous ETF was prepared by dialyzing the sample for 12 h against 4 L of 5 mM Tris buffer, pH 7.8, and applying it to a CM-52 column (2.1 × 41 cm) equilibrated with the same buffer at 17 mL/h. Fractions with an A_{272}/A_{436} ratio of less than 6 were pooled and concentrated to 1.1 mL, yielding 462 nmol of ETF with a UV/visible ratio of 5.88. Concentrated samples were stored at −20 °C.

(*B*) *General Acyl-CoA Dehydrogenase Purification.* Phosphate buffers are used for the dehydrogenase purification unless otherwise stated. General acyl-CoA dehydrogenase, adsorbed to DE-52 (step 2B), was washed with 2 L of 50 mM buffer, pH 7.2, suspended in 300 mL of the same buffer, and poured into a 3-cm diameter column. The column was washed with the same buffer until no further protein emerged. The dehydrogenase eluted from the brown DE-52 column by using 0.3 M phosphate buffer, pH 7.2, as a sharp yellow-brown band moving with the buffer front. Fractions containing acyl-CoA dehydrogenase were combined, taken to 35% ammonium sulfate, and centrifuged at 21000*g* in a GSA rotor for 40 min. The beige precipitate was discarded, and the yellow supernatant was taken to 75% ammonium sulfate and centrifuged. The yellow precipitate was redissolved in 20 mM buffer, pH 7.6, and dialyzed overnight against 4 L of the same buffer. The dialyzed solution (73 mL) was applied to a 5.5 × 17 cm calcium phosphate gel–cellulose column (Massey, 1960) equilibrated with 20 mM buffer, pH 7.6. The enzyme was washed at 20 mL/h with an additional 500 mL of buffer and then eluted by using 0.15 M buffer, pH 7.6. Yellow fractions were combined (360 mL) and dialyzed against 4 L of 20 mM buffer, pH 7.6. The dehydrogenase was applied at 20 mL/h

to a Matrex gel blue A column (1.5 × 18 cm) equilibrated with 20 mM buffer, pH 7.6, and washed with the equilibration buffer until no further protein was eluted. Cibacron Blue–Sepharose synthesized from free Cibacron Blue F3GA dye and Sepharose CL-4B according to the procedure of Atkinson et al. (1981) gave similar results to those obtained with the commercial material. The binding affinity of the dehydrogenase is proportional to the amount of dye attached to the Sepharose CL-4B (J. P. Mizzer, unpublished results). The dehydrogenase was then eluted by using a linear gradient formed from 100 mL of 20 mM buffer, pH 7.6, and 50 mM buffer, pH 7.6, containing 1.5 M KCl. The salt gradient must be run at a low flow rate (20 mL/h) to ensure proper equilibration and to prevent smearing of the enzyme on the column. Fractions were combined, yielding 896 nmol of dehydrogenase with an A_{280}/A_{450} ratio of 5.8, and secondary fractions yielded 332 nmol with a ratio of 7.5. If desired, the dehydrogenase may be purified to a ratio of 5.3 on Sephacryl S-200 (Thorpe et al., 1979). Fractions were dialyzed against 50 mM buffer, pH 7.6, and stored frozen.

Assays. General acyl-CoA dehydrogenase assays were performed at 25 °C as described previously (Thorpe, 1981). ETF-mediated enzyme assays were performed by the addition of 1–30 μL of ETF to a total volume of 0.7 mL of 20 mM phosphate buffer, pH 7.6, and 0.3 mM EDTA containing 6.0 nM general acyl-CoA dehydrogenase, 37.5 μM 2,6-dichlorophenolindophenol, and 60 μM octanoyl-CoA at 25 °C.

ETF Stability Experiments. ETF was stored in 5% v/v glycerol for 4 weeks at 4 and −20 °C in 50 mM buffers at pH values of 7.0 and 7.5 (phosphate), and 8.0 and 8.5 (Tris, pH adjusted at 4 °C).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Assistance with slab gel experiments was kindly given by J. Keller, Frederick Cancer Research Facility, Frederick, MD, by using the procedure described by Weber & Osborn (1969). Approximately 10 μg each of the standard proteins (bovine serum albumin, ovalbumin, pepsin, trypsinogen, β-lactoglobulin, and lysozyme) and 20 μg of ETF were applied to 10% gels. Tube gels were run under the same conditions. The proteins were stained with Coomassie Brilliant Blue R-250 (Weber & Osborn, 1969).

Discontinuous Polyacrylamide Gel Electrophoresis. Anodic discontinuous polyacrylamide gel electrophoresis was performed according to the method of Davis (1964) by using 10- and 20-μg samples of purified ETF.

Isoelectric Focusing. LKB Ampholine polyacrylamide gel electrophoresis plates (pH 3–10) were run with 20 μg of ETF according to the manufacturer's instructions. Flavin fluorescence was detected by using a long-wavelength UV lamp, and protein was stained with Coomassie Brilliant Blue R-250.

Gel Filtration. Estimation of native molecular weight was performed on a 1.3 × 74 cm Sephacryl S-200 column equilibrated with 100 mM phosphate buffer, pH 7.6, 4 °C, and run at a flow rate of 19 mL/h. Standard proteins were ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin, and aldolase, and results were calculated as described by Andrews (1964).

Amino Acid Analysis. Amino acid analyses were kindly performed by L. Parente, University of Delaware, by using a Beckman 119C analyzer. Samples were hydrolyzed at 110 °C in constant-boiling HCl (Moore & Stein, 1960).

Peptide Mapping. A 100-nmol sample of lyophilized ETF was dissolved in 1.5 mL of anaerobic buffer containing 0.5 M Tris, 0.25 M EDTA, a 2-fold molar excess of dithioerythritol over total cysteines, and 6 M guanidine hydrochloride adjusted to pH 8.5, and the mixture was stirred in a capped vial under an atmosphere of nitrogen. After 1 h, a 2-fold excess of iodoacetic acid over total thiols was added under nitrogen, and the mixture was incubated at 25 °C for 20 min with stirring. The carboxymethylated protein was desalted by dialysis vs. two changes of distilled water followed by two changes of 0.1 M ammonium bicarbonate, pH 8.0. The protein was recovered by lyophilization and resuspended in 1.5 mL of 0.2 M ammonium bicarbonate, pH 8.0. Digestion was performed by adding 2% w/w aliquots of diphenylcarbamyl chloride treated trypsin (dissolved in 1.0 mM HCl) at 0 and 3 h to the stirred sample at 37 °C. The reaction was stopped after 6 h by freezing, and the peptides were lyophilized twice against water.

Thin-layer peptide mapping was performed by applying 5–12 nmol of the digested sample dissolved in water, 8 cm from the edge (positive terminal side) and 2 cm from the bottom of a thin-layer cellulose plate. Electrophoresis at pH 5.6 was run in pyridine/glacial acetic acid/water buffer (10:3:300) for 90 min at 300 V and 20 mA. Electrophoresis at pH 3.7 used the same reagents in a 2:10:89 proportion. The plate was air-dried for 1 h and chromatographed in the second dimension by using a 1-butanol/pyridine/glacial acetic acid/water (50:30:1:40) solvent system. The plate was again air-dried and sprayed with fluorescamine (Gracy, 1977).

Redox Titrations. Anaerobic dithionite and ferricyanide titrations were performed as described previously (Mizzer & Thorpe, 1981). Photoreduction experiments used a 100-W tungsten bulb positioned 10 cm from the sample which was maintained at 20 °C in a water bath. An oxygen scrubber was used during anaerobic titrations by adding 200 μL of a 1.3 μM glucose oxidase solution, 40 μL of an 18 μM catalase solution, and 10 μL of a 1 M glucose solution to a side arm containing a fluted piece of filter paper (0.4 × 5 cm) immediately before anaerobiosis. Crotonyl-CoA solutions were deoxygenated by stirring for at least 2 h in a small capped vial under nitrogen.

Equilibrium Dialysis. Equilibrium dialysis was performed with a Kontron-Diapack Model 4000 apparatus using semimicro cells and Spectrapor dialysis membranes (M_r 12 000–14 000 cutoff). ETF (8 μM) was placed on one side of the cell and 16 μM crotonyl-CoA on the other in either 2.5 or 50 mM phosphate buffer, pH 7.6, with 0.3 mM EDTA. The cells were rotated for 20 h at 4 °C. The concentration of crotonyl-CoA had decreased to 8.0 μM in the buffer compartment, indicating that no significant binding to ETF had occurred. A control experiment performed in the absence of ETF gave the same result.

Results and Discussion

A purification method for pig kidney general acyl-CoA dehydrogenase has been described previously (Thorpe et al., 1979) which yields comparatively large amounts of pure dehydrogenase in a procedure which avoids the isolation of mitochondria. This scheme has been substantially modified so as to allow the simultaneous purification of the dehydrogenase and its complementary electron-transferring flavoprotein in good yield (Table I). An important change is that the initial homogenization is performed at pH 7.2, not 5.8, as the lower pH value results in a complete loss of ETF flavin (R. J. Gorelick, unpublished results). ETF is most stable at pH values greater than 7.5 (see Methods), and, as observed by McKean et al. (1979), addition of glycerol was found to significantly stabilize the flavoprotein. The initial DEAE-cellulose batch step (Table I, step 2A) adsorbs most of the general acyl-CoA dehydrogenase which is further purified in

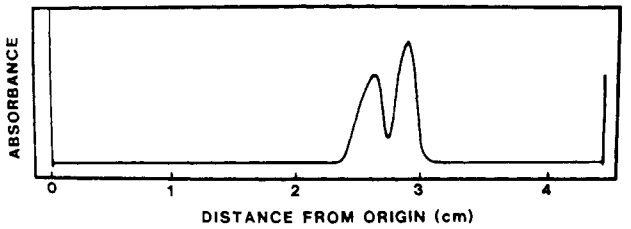

FIGURE 1: Densitometer scan of $NaDodSO_4$–polyacrylamide gel electrophoresis of pig kidney ETF. Electrophoresis of 20 μg of ETF was performed in 10% gels by the method of Weber & Osborn (1969). The tracking dye moved 4.4 cm from the origin.

stages 2B–5B. The use of Cibacron Blue–Sepharose (step 5B) is a significant improvement over the previous procedure, enabling an approximately 2-fold increase in yield of the dehydrogenase to be realized. Although the dehydrogenase can be eluted from the dye column with octanoyl-CoA, the use of a salt gradient is more practical for large-scale preparations. While this work was in progress, Furuta et al. (1981) reported the purification of three acyl-CoA dehydrogenases from rat liver by using a Blue Dextran–Sepharose column.

The filtrate from the batch step (Table I, step 2A) serves as the starting point for the isolation of ETF. After step 6A, ETF was approximately 95% pure as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis and was suitable for many spectrophotometric experiments, having no detectable heme or acyl-CoA dehydrogenase contaminants. The final step yields 28 mg of essentially homogeneous ETF per kg of kidney cortex, approximately 7-fold higher than that from the liver preparation (Hall & Kamin, 1975). This material elutes from a Sephacryl S-200 column as a single peak (see Methods) and shows a single band on discontinuous polyacrylamide gel electrophoresis after being stained for protein. Isoelectric focusing reveals a single major band accounting for 95% of the total protein (pI = 6.9; showing ETF fluorescence before staining) and a minor band (5% of the total; pI = 6.4) with no detectable fluorescence (see Methods).

Figure 1 shows a densitometer trace of pig kidney ETF after $NaDodSO_4$–polyacrylamide gel electrophoresis. The scan shows two well-resolved bands of equal intensity corresponding to molecular weights of 30 000 and 33 000 (see Methods), with no detectable contaminants. Partial proteolysis of ETF during isolation seems to be an unlikely explanation for these results, since the two bands stain equally in all preparations and the protease inhibitor phenylmethanesulfonyl fluoride is added immediately prior to homogenization of the tissue (see Methods). Further, peptide maps of a tryptic digest of carboxymethylated ETF yield approximately 55 spots on fluorescamine staining (see Methods). Amino acid analysis of kidney ETF (Table II) indicates a total of 62 lysine and arginine residues in the dimer, and thus 32 spots would be anticipated for a preparation containing identical subunits. These results, together measurement of flavin content (see later), suggest that kidney ETF is composed of nonidentical subunits. It is of interest that ETFs from rat liver (Furuta et al., 1981), *Megasphaera elsdenii* (Whitfield & Mayhew, 1974), and a methylotrophic bacterium (W_3A_1) (Steenkamp & Gallup, 1978) are all composed of dissimilar subunits (with molecular weights of 25 000 and 33 500, 33 000 and 41 000, and 38 000 and 42 000, respectively). Recently, a new procedure has been developed for isolating ETF from pig liver which similarly yields two bands on $NaDodSO_4$–polyacrylamide gel electrophoresis (M. Husain, unpublished results). The reasons for the discrepancy between these results and those described earlier for the liver protein (Hall & Kamin, 1975) are not clear, although two bands were reported to be present in aged preparations.

Table II: Amino Acid Composition of Pig Kidney Electron-Transferring Flavoprotein[a]

amino acid	residues/mol of FAD
aspartic acid	42.8
threonine	27.8
serine	20.4
glutamic acid	51.2
proline	22.5
glycine	38.4
alanine	63.8
half-cystine	14.7
valine	53.4
methionine	7.9
isoleucine	27.9
leucine	50.3
tyrosine	6.9
phenylalanine	12.4
lysine	41.2
histidine	8.9
arginine	21.2
tryptophan	4.0[b]

[a] Samples were hydrolyzed for 24, 48, and 72 h. Half-cystine was estimated as cysteic acid after hydrolysis in the presence of dimethyl sulfoxide for 24 h (Spencer & Wold, 1969). [b] From Hall & Kamin (1975).

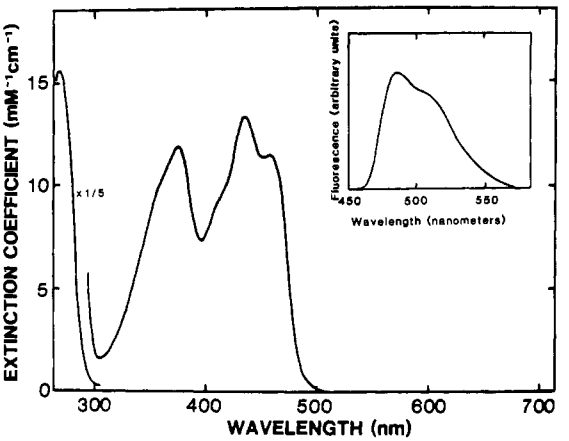

FIGURE 2: Visible and ultraviolet spectra of pig kidney ETF. Spectra were recorded in 15 mM Tris buffer, pH 7.6 at 25 °C. The inset shows the fluorescence emission spectrum of ETF recorded under the same conditions with an excitation wavelength of 450 nm.

The highly resolved visible spectrum of ETF (Figure 2) is similar to that reported for the pig liver protein (Crane & Beinert, 1956; Hall & Kamin, 1975) with an absorbance maximum at 436 nm; however, the kidney preparation exhibits a somewhat lower trough at 305 nm. The extinction coefficient at 436 nm was determined as 13.3 mM^{-1} cm^{-1} by release of ETF flavin in 4 M guanidine hydrochloride (see Methods). There is no evidence in these spectra for the presence of any 6-OH or 8-OH modified FAD derivatives, as is observed with ETF isolated from *Megasphaera elsdenii* (Whitfield & Mayhew, 1974). The fluorescence emission spectrum (maximum at 491 nm) is shown in the inset of Figure 2. The fluorescence of 2.5 μM ETF at 491 nm was enhanced 1.9-fold over the same concentration of FAD when both samples were excited at 450 nm at 25 °C in 15 mM Tris buffer, pH 7.9, containing 5% v/v glycerol.

The amino acid analysis of kidney ETF is presented in Table II and is similar to that reported for the liver enzyme (Hall & Kamin, 1975) with the exception of proline. The pig liver enzyme is reported to contain 42 prolines per dimer whereas

Table III: Properties of Mammalian Electron-Transferring Flavoprotein

properties	pig liver		pig kidney, this work
	Hall & Kamin (1975)	Crane & Beinert (1956)	
absorbance max (nm)	438	438	436
extinction coefficient at wavelength max (mM^{-1} cm^{-1})	11.1		13.3
protein/flavin absorbance ratio	5.5 (270 nm/438 nm)	6.5 (270 nm/438 nm)	5.88 (272 nm/436 nm)
subunit M_r	26 000		30 000, 33 000
native M_r	58 000	80 000	59 000
min M_r/FAD	26 000		55 000

23 are found in this work. A total of 11 cysteine residues react very rapidly with 0.5 mM DTNB in 50 mM phosphate buffer, pH 7.6, 25 °C, containing 4 M guanidine hydrochloride. However, cysteine residues in the native enzyme react sluggishly with DTNB; in the absence of perturbant, 0.3 thiol/FAD had reacted after 10 min.

Table III summarizes serveral properties of pig kidney ETF in comparison with the pig liver preparations. The amino acid composition of the kidney ETF (Table II) corresponds to a minimum molecular weight of 55 000 per FAD. Gel filtration of ETF on Sephacryl S-200 gives an apparent molecular weight of 59 000, and $NaDodSO_4$–polyacrylamide gel electrophoresis yields apparently equal amounts of subunits with molecular weights of 30 000 and 33 000 (see Methods; Table III). These results are consistent with a flavin content of one per dimer compared to two flavins per dimer reported for the liver enzyme (Hall & Kamin, 1975). It should be noted that both preparations actually contain comparable levels of flavin as judged by their similar protein to flavin absorbance ratios (Table III). The value of two flavins per dimer (Hall & Kamin, 1975) was obtained by combining microbiuret protein estimation with an assumed extinction coefficient of 11.3 mM^{-1} cm^{-1} for ETF-bound flavin. Use of the extinction coefficient determined in this work (Table III) would result in a 20% decrease in the apparent flavin content per dimer, which is clearly inadequate to resolve this discrepancy. Further, a recent purification table for liver ETF (Hall, 1981) shows material of highest purity having a flavin to protein ratio of 16–20 nmol of FAD/mg of ETF protein. These values correspond to a minimum molecular weight of 50 000–62 500 and indicate a flavin content of about one per dimer, although the significance of these data was not addressed. Kidney ETF undergoes no significant increase in flavin content on preincubation with 30 μM FAD for 3 h at 4 °C. Although FAD is added as a precautionary measure at several stages during the isolation of ETF (see Methods), further experiments have shown that the flavin content is unchanged when these additions are omitted. In summary, these results suggest that pig kidney ETF is a heterodimer containing one flavin molecule.

Figure 3 shows a dithionite titration of pig kidney electron-transferring flavoprotein at pH 7.6. The red semiquinone form (Massey & Palmer, 1966) is formed essentially quantitatively during the first phase of the titration as judged by the preservation of isosbestic points at 479, 402, and 343 nm, and by the linearity of the absorbance changes shown in the inset. Full reduction requires a total of 1.0 mol of dithionite/mol of FAD, consistent with the absence of additional redox-active groups in this flavoprotein. In contrast to the rapid generation of the red radical, further reduction to the dihydroflavin form is sluggish, requiring about 1 h between each addition of dithionite. Similar spectral changes are observed during photoreduction using 3,10-dimethyl-5-deazaisoalloxazine (see later). As expected, a ferricyanide back-

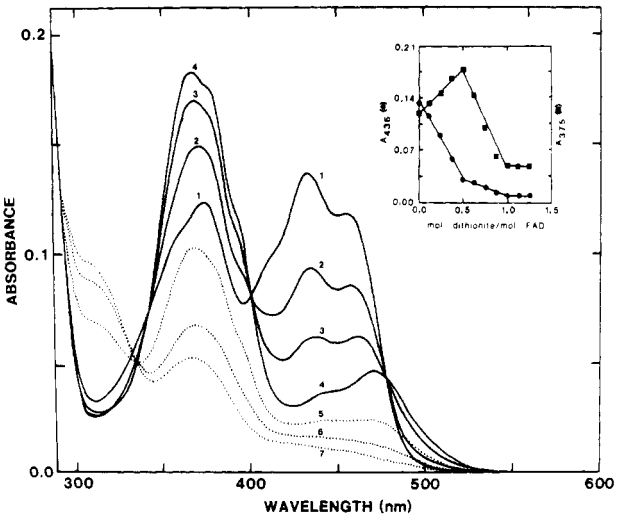

FIGURE 3: Dithionite titration of pig kidney ETF. The protein was deoxygenated as described under Methods, and 0.66 mL of 10 μM bound flavin in 50 mM phosphate buffer, pH 7.6, containing 0.3 mM EDTA, was titrated with 0.0, 1.5, 2.8, 3.7, 5.5, 6.4, and 7.3 nmol of dithionite (curves 1–7, respectively). Intermediate spectra are omitted for clarity. The inset plots the absorbance at 436 and 375 nm vs. moles of dithionite per mole of flavin.

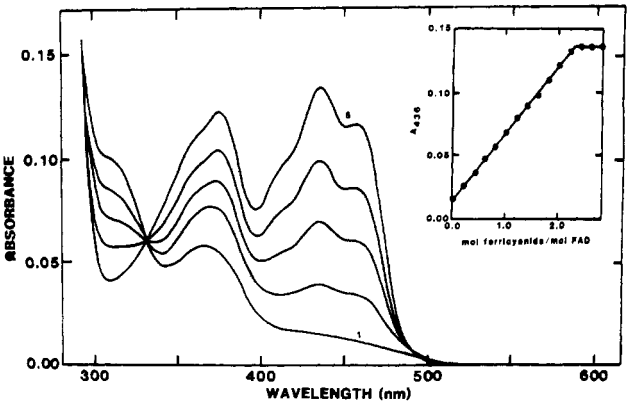

FIGURE 4: Ferricyanide back-titration of dithionite-reduced pig kidney ETF. Two-electron reduced ETF (0.67 mL; Figure 3) was titrated with 0.0, 4.0, 8.0, 11.9, and 17.2 nmol of ferricyanide (curves 1–5, respectively). Intermediate spectra are omitted for clarity. The inset plots the absorbance at 436 nm vs. moles of ferricyanide per mole of flavin.

titration of the fully reduced protein requires a total of 2.0 mol of ferricyanide/mol of flavin for completion (Figure 4); however, reoxidation proceeds without the detectable accumulation of semiquinone. Similarly, reoxidation of the 2-electron reduced protein by molecular oxygen (see later) does not generate detectable red radical. These data indicate that the disproportionation equilibrium

$$2ETFH\cdot \rightleftharpoons ETF + ETFH_2$$

is attained very slowly with ETF and that the yields of radical

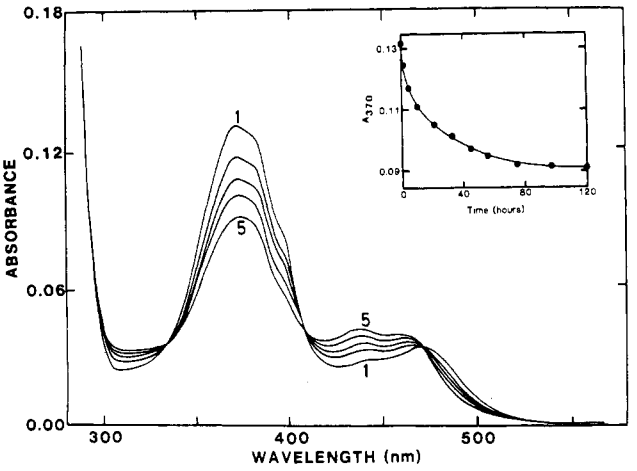

FIGURE 5: Disproportionation of pig kidney ETF semiquinone. ETF (8.1 μM flavin), containing 0.61 μM 3,10-dimethyl-5-deazaisoalloxazine and 6.1 mM EDTA in 50 mM anaerobic phosphate buffer, pH 7.6, was photoreduced as described under Methods. Spectra 1–5 were recorded after 0, 5.1, 15.9, 32.2, and 97.8 h, respectively. The value of the disproportionation equilibrium constant shown in the text was calculated by using extinction coefficients at 370 nm of 17.9, 11.3, and 5.1 mM^{-1} cm^{-1} for semiquinone, oxidized, and fully reduced ETF, respectively.

are determined kinetically, a situation observed with many other flavoproteins (Mayhew & Massey, 1973). The equilibrium constant was evaluated by carefully photoreducing 0.66 mL of 8.1 μM ETF to the semiquinone state under rigorously anaerobic conditions (see Methods). The spectral changes accompanying disproportionation were half-complete in 10 h (Figure 5), underscoring the slowness of this process. When the extinction coefficients for oxidized (ox), semiquinone (sq), and dihydroflavin (red.) forms (see legend of Figure 5) were used, a value of 0.49 could be calculated for the equilibrium constant by using the spectrum observed after 4 days.

$$K = \frac{[ox][red.]}{[sq]^2}$$

These data reinforce the conclusion that disproportionation of ETF semiquinone is not catalytically significant (Reinsch et al., 1980; Hall & Lambeth, 1980).

Beinert and colleagues first noted the sluggish reoxidation of reduced ETF by molecular oxygen (Beinert & Page, 1957; Beinert, 1963). This reoxidation rate was measured by mixing the 2-electron reduced protein, prepared by deazaflavin/light reduction (Massey & Hemmerich, 1978; see Methods), with 0.2 volume of oxygen-saturated buffer to give a final concentration of 11.6 μM ETF in 0.77 mL of phosphate buffer, pH 7.6 at 25 °C. Reoxidation did not generate significant levels of semiquinone (see above) and was pseudo first order over about 4 half-lives with a $t_{1/2}$ of 85 min. Under the same conditions, the red semiquinone form, prepared photochemically as above, was reoxidized much more rapidly in a pseudo-first-order reaction with a $t_{1/2}$ of 56 s. Thus, should the semiquinone state be formed during aerial reoxidation of dihydro-ETF, it would not accumulate significantly.

In addition to its role as an electron carrier (Crane & Beinert, 1956), liver ETF has been reported to bind enoyl-CoA derivatives tightly, based on dialysis and spectrophotometric experiments (Hall, 1976; Hall et al., 1979). Thus, dialysis of a solution of liver ETF containing 1–2 mol of crotonyl-CoA resulted in apparent binding which was stable to dialysis for 18 h vs. 2.5 mM phosphate buffer, under conditions where 95% crotonyl-CoA was removed in the absence of ETF (Hall, 1976). Equilibrium dialysis of pig kidney ETF using crotonyl-CoA in 2.5 or 50 mM phosphate buffer, pH 7.6, showed no significant binding (see Methods). Second, a 6% decrease in flavin fluorescence was observed when crotonyl-CoA was added to the liver protein (Hall, 1976). In contrast, no significant fluorescence quenching is observed with 1.6 μM kidney ETF using a range of phosphate buffer concentrations from 2.5 to 50 mM, pH 7.6, and concentrations of crotonyl-CoA up to 8.0 μM. A titration of the semiquinone form of liver ETF with 1 equiv of crotonyl-CoA has been reported to induce the reappearance of an oxidized flavin spectrum together with a featureless increase in absorbance to longer wavelengths (Hall et al., 1979), suggesting some form of direct communication between the flavin of ETF and crotonyl-CoA. Indeed, it has been proposed that acyl-CoA derivatives are transferred from the dehydrogenase to ETF before the completion of substrate dehydrogenation (Hall, 1980). This important experiment has been repeated with the kidney protein. The red semiquinone form was generated photochemically, and crotonyl-CoA was added under rigorously anaerobic conditions (see Methods). No reoxidation of the semiquinone was encountered even after the addition of 8.0 equiv of crotonyl-CoA. Rather, the spectral changes observed reflect the slow disproportionation seen in Figure 5.

The present paper emphasizes that despite the central role of ETF in several different mitochondrial oxidation pathways, many aspects of its structure and mode of action require clarification. The ready availability of kidney ETF should facilitate work on this important flavoenzyme and permit isolation and characterization of the individual subunits.

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Further Purification and Characterization of Scrapie Prions†

Stanley B. Prusiner,* David C. Bolton, Darlene F. Groth, Karen A. Bowman, S. Patricia Cochran, and Michael P. McKinley

ABSTRACT: The scrapie agent is prototypic of a novel class of small infectious pathogens called prions. Prolonged bioassays as well as the hydrophobicity of the agent have complicated its purification. A purification protocol for the agent in hamster brain has been developed, which results in preparations enriched for the agent between 100- and 1000-fold with respect to protein. The protocol includes Triton X-100/sodium deoxycholate extraction, poly(ethylene glycol) precipitation, nuclease and protease digestion, cholate/sodium lauryl sarcosinate extraction, $(NH_4)_2SO_4$ precipitation, Triton X-100/sodium dodecyl sulfate extraction, and sedimentation through a discontinuous sucrose gradient. The highest degree of purification was found in a fraction from the 25% and 60% sucrose interface near the bottom of the gradient. Examination by electron microscopy of rotary-shadowed samples from the gradient interface which contained $>10^{9.5}$ ID_{50} units/mL of the scrapie agent revealed aggregates composed of amorphous material and numerous flattened rods measuring 25 nm in diameter by 100-200 nm in length. Radioiodination of the interface fraction has identified a protein that is unique to preparations from scrapie-infected brains. The apparent molecular weight of the protein is between 27 000 and 30 000. The unique protein was also radiolabeled with [^{14}C]diethyl pyrocarbonate. Although the properties of the scrapie prion and this unique protein must be sufficiently similar to allow copurification, further experimental data will be needed to establish whether or not this protein is required for infectivity. In contrast to the protein radiolabeling studies, 5′-end labeling with [γ-^{32}P]ATP of nucleic acid molecules in the purified fraction failed to identify a unique nucleic acid. The results of these radiolabeling studies are consistent with earlier observations showing that prions are inactivated by procedures that modify proteins but are resistant to inactivation by procedures that modify nucleic acids.

The scrapie agent causes a degenerative disorder of the nervous system of sheep and goats many months or even years after inoculation or exposure. Its novel properties have prompted introduction of the term "prions" (Prusiner, 1982). The scrapie prion is prototypic of a unique class of small infectious pathogens. Similar pathogens probably also cause two human diseases, kuru and Creutzfeldt-Jakob disease (Gajdusek, 1977; Hadlow et al., 1980).

Recent studies on the scrapie agent have shown that it contains a protein that is required for infectivity (Prusiner et al., 1981b). These studies were made possible by the development of purification procedures that substantially reduced the amount of cellular protein and lipid contaminating scrapie agent preparations. To date, all attempts to inactivate the agent by hydrolyzing or modifying its hypothetical nucleic acid have been unsuccessful (Prusiner, 1982; Alper et al., 1967, 1978). Studies on the molecular size of the scrapie agent suggest that any nucleic acid contained within the agent would be too small to function as a gene coding for the protein(s)

† From the Departments of Neurology and of Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received August 2, 1982.* This work was supported by a grant from the National Institutes of Health (NS14069) and a gift from R. J. Reynolds Industries, Inc.

* Address correspondence to this author at the Department of Neurology, University of California.

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