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Electron transfer flavoprotein from pig liver mitochondria

A simple purification and re-evaluation of some of the molecular properties

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Electron transfer flavoprotein (ETF) from pig liver mitochondria has been purified to homogeneity by a three-step procedure with approx. 10-fold higher yields than previously reported. The purified ETF exhibits an absorption coefficient for the bound FAD of 13.5 $mM^{-1} \cdot cm^{-1}$ at 436 nm and an isoelectric point of 6.75. Gel filtration, sodium dodecyl sulphate gel electrophoresis and flavin analysis indicate that pig liver ETF is a dimer, composed of non-identical subunits (M_r 38000 and 32000) with only one FAD/dimer. Anaerobic reduction by dithionite produces anionic flavin semiquinone as a stable intermediate and establishes the flavin to be the only redox-active chromophore in ETF.

Electron transfer flavoprotein (ETF), a soluble protein located in the mitochondrial matrix, plays a crucial role in the β-oxidation of fatty acids and the oxidative demethylation reactions of the mitochondrial 'one-carbon cycle' by coupling several flavoprotein dehydrogenases to the electron transport system (Frisell & Mackenzie, 1962; Noda *et al.*, 1980). ETF was first purified by Crane & Beinert (1956) from pig liver mitochondria and characterized as a flavoprotein containing one mol of FAD per mol of protein. Hall & Kamin (1975) purified ETF to homogeneity by a new procedure and showed that some of the properties of their preparation were substantially different from those described in the original report. Thus, they found ETF to be a dimer (M_r 58000) consisting of identical subunits (M_r 27500), each containing one FAD. Neither of these two conflicting reports has yet been confirmed by an independent investigation.

Our interest in ETF derives from our recent study on the effect of tetrahydrofolate on the oxidative demethylations catalysed by the sarcosine and dimethylglycine dehydrogenases (Steenkamp & Husain, 1982). The need for large amounts of ETF, used as the physiological electron acceptor in these studies, prompted us to develop a simpler and more efficient procedure for preparing ETF.

The present report describes a three-step procedure for preparing homogeneous ETF with yields approx. 10-fold higher than those previously reported. Several properties of ETF were re-evaluated and found to be markedly different from those reported earlier. Evidence is presented to show that pig liver ETF is a dimer of two non-identical subunits, only one of which contains an FAD.

Abbreviations used: ETF, electron transfer flavoprotein; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

Experimental

All manipulations in the preparation of ETF were carried out at 4°C unless otherwise noted. Mitochondria from 2 kg of pig liver were prepared as described (Schnaitman & Greenawalt, 1968) using isolation medium H, and stored at −20°C until needed. The frozen mitochondria were thawed overnight on ice, diluted with an equal volume of 10 mM-Hepes (pH 7.8 at 25°C) and sonicated for a total of 5 min at 90 W. The sonicated mitochondria (approx. 400 ml) were centrifuged at 106000 ***g*** for 1 h and the supernatant was directly applied to a DEAE-cellulose column (5 cm × 30 cm) previously equilibrated with 25 mM-Hepes, pH 7.8. ETF was eluted from the column in the same buffer and could be detected in the effluent by its intense yellow-green fluorescence. Fractions containing ETF were pooled (approx. 500 ml, $A_{270}/A_{436} = 45$), concentrated to approx. 30 ml by ultrafiltration with an Amicon PM-30 membrane, and dialysed overnight against 50 vol. of 10 mM-Hepes/10% (v/v) ethylene glycol, pH 7.8. The dialysed sample was applied to a CM-Sephadex (C-25) column (3 cm × 60 cm) previously equilibrated with the dialysis buffer and the

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column was washed with the same buffer until the A_{280} was approx. 0.02. The column was then eluted with a linear KCl gradient prepared with 1 litre of the starting buffer and 1 litre of the starting buffer containing 0.15M-KCl. Fractions were collected until all the ETF was eluted. At this stage, the pooled ETF fractions (approx. 60ml) showed a 270/436nm absorbance ratio of approx. 10 with no contaminant evident in the visible region. The pooled ETF was concentrated to 5ml, diluted 4-fold with cold distilled water containing 10% (v/v) ethylene glycol to reduce the salt concentration, and then applied to a hydroxyapatite column (3cm × 20cm) equilibrated with 10mM-potassium phosphate/10% (v/v) ethylene glycol, pH7.1. After washing successively with approx. 300ml of the starting buffer and 400–600ml of 50mM-potassium phosphate/10% (v/v) ethylene glycol, pH7.1, ETF was eluted with 100mM-potassium phosphate/10% (v/v) ethylene glycol, pH7.1. Fractions with 270/436nm absorbance ratios $\leqslant$6.1 were pooled (approx. 70ml), concentrated, passed through a short column of Sephadex G-25 equilibrated with 50mM-Hepes/10% (v/v) ethylene glycol, pH7.8, and stored in the freezer. Under these conditions, ETF can be stored for a few months without any apparent loss of activity as judged by its characteristic absorbance and fluorescence properties as well as its ability to undergo rapid reduction in the sarcosine and dimethylglycine dehydrogenase-catalysed reactions (Steenkamp & Husain, 1982).

Isoelectric focusing was carried out on agarose (FMC Corporation, Rockland, ME, U.S.A.) and Sephadex G-200 superfine (Pharmacia) beds using Pharmacia flat bed apparatus FBE 3000. The gel beds were prepared according to the manufacturer's instructions. Analytical disc gel electrophoresis was performed as described by Davis (1964). SDS/polyacrylamide disc gel electrophoresis was carried out according to the method of Weber & Osborn (1969) using bovine serum albumin, ovalbumin, carbonic anhydrase and haemoglobin as protein standards. The absorption coefficient of the ETF at 436nm was determined by quantifying the FAD released from ETF upon treatment with either 5% (w/v) trichloroacetic acid (Mayhew & Massey, 1969) or 4.5M-guanidine hydrochloride (Thorpe *et al.*, 1979). Amino acid analyses were performed on a Durrum D 500 analyser. Anaerobic titrations were carried out in a Thunberg-type cuvette equipped with a gas-tight syringe (Williams *et al.*, 1979). The protein concentration was determined by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as standard.

Results

The three-step purification procedure detailed in the Experimental section is relatively simple and yields 30–50mg of purified ETF from 2kg of pig liver; this may be compared with a yield of approx. 4mg by previously described procedures (Crane & Beinert, 1956; Hall & Kamin, 1975). The improved recovery of ETF can most likely be attributed to the effect of ethylene glycol as a stabilizing agent and the absence of repeated $(NH_4)_2SO_4$ fractionation in the purification method described here. Purified ETF was homogeneous by the criteria of polyacrylamide-gel electrophoresis in the presence or absence of SDS (Figs. 1*a* and 3). Two contaminating bands of very minor intensity could, however, be detected on isoelectric focusing (Fig. 1*b*). Under non-denaturing conditions, FAD remained bound to ETF during electrophoresis as judged by the presence of a single fluorescent band which co-migrated with the protein. ETF focuses in the same region as the acidic band of horse myoglobin, indicating a pI of approx. 6.85 for ETF. This was confirmed by repeating the experiment using

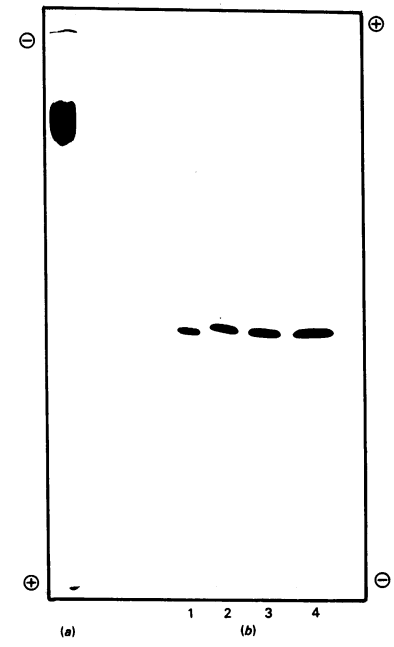

Fig. 1. *Homogeneity of the purified pig liver ETF by disc gel electrophoresis (a) and isoelectric focusing (b)*
Analytical disc gel electrophoresis was performed on 7.5% polyacrylamide gel using 10μg of ETF (A_{270}/A_{436} 5.8). Coomassie Blue was used as the staining agent. Isoelectric focusing was carried out in 1% agarose slabs (10cm × 15cm) containing 2.5% ampholine, pH range 3–10. Electrophoresis was started at 10mA (700V) and terminated after 3h. During the run, voltage rose to the limiting value of 1200V and current fell to 2mA. Lanes 1–4 contain varying amounts of ETF (A_{270}/A_{436} 5.8): 1, 1.7μg; 2, 3.4μg; 3, 5.1μg; 4, 6.8μg.

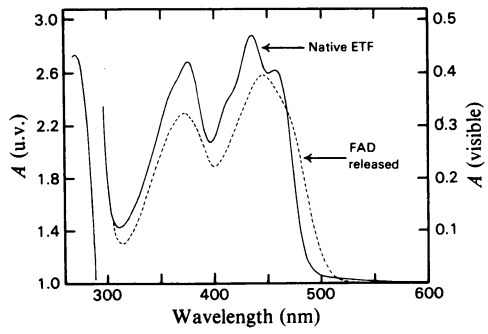

Fig. 2. *Spectral properties of ETF with and without guanidine hydrochloride*

—, peak fraction of ETF (2.45mg/ml) from hydroxyapatite column in 50mM-Hepes/10% (v/v) ethylene glycol, pH7.6; ---, after addition of guanidine hydrochloride (final concn. 4.5M, pH7.6) and appropriate volume correction.

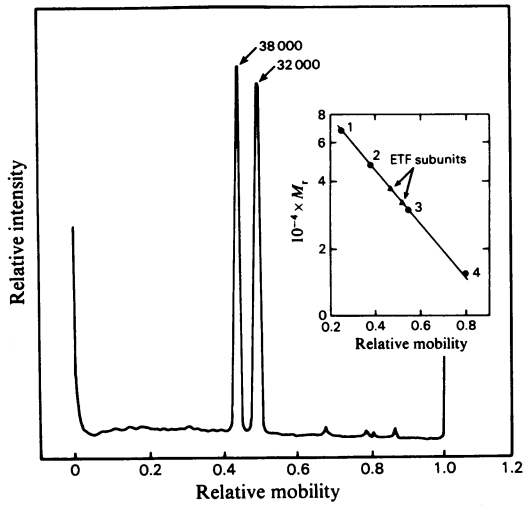

Fig. 3. *SDS/polyacrylamide disc gel electrophoresis of ETF*

Electrophoresis was performed on 10% polyacrylamide gels. ETF (A_{270}/A_{436} 5.8) was denatured with 1% SDS/1% mercaptoethanol for 3min in a boiling-water bath. The inset shows the calibration curve with bovine serum albumin (1), ovalbumin (2), human carbonic anhydrase (3) and haemoglobin (4) as the standards.

ampholine (pH 6–8 range) and directly measuring the pH of the ETF band. A value of 6.75 was determined, compared with 8.1 reported by Crane & Beinert (1956).

The visible spectrum of the purified ETF is similar to that reported by Crane & Beinert (1956) and Hall & Kamin (1975) with an absorbance maximum at

Table 1. *Amino acid composition of pig liver electron transfer flavoprotein*

Residue	Composition (residues/mol of FAD) Present study	Hall & Kamin (1975)
Aspartic acid	44.1	19.8 (39.5)*
Threonine	29·3	12.5 (25)
Serine	27.6	11.2 (22.4)
Glutamic acid	48.3	23.7 (47.4)
Proline	25	21 (42)
Glycine	46.9	19 (37.9)
Alanine	66.2	28.5 (56.9)
Half-cystine†	12	3.5–5 (7–10)
Valine	53.2	25.3 (50.6)
Methionine	6.7	4 (7.9)
Isoleucine	28.3	12.3 (24.6)
Leucine	53.7	25.3 (50.6)
Tyrosine	9.5	3.2 (6.3)
Phenylalanine	13.2	5.6 (11.1)
Lysine	43.8	25.8 (51.5)
Histidine	8.8	4.8 (9.5)
Arginine	22.8	11.1 (22.1)
Tryptophan‡	3	2.3 (4.5)

* The numbers in parentheses are residues per mol of ETF (residues/58000).

† Determined by performic acid oxidation (Hirs, 1967).

‡ Determined spectrophotometrically (Edelhoch, 1967) after removal of the bound FAD by precipitation of ETF with 5% (w/v) trichloroacetic acid.

436nm. The absorption coefficient of the bound FAD at 436nm is 13.5 $mM^{-1} \cdot cm^{-1}$ (Fig. 2). When excited at 450nm, purified ETF exhibited a fluorescence emission maximum at 493nm and, under identical conditions (50mM-Hepes, pH7.8, 25°C), a fluorescence yield 2.1-fold higher than that of free FAD.

The apparent M_r of the native ETF was 68000 as determined by means of a calibrated Sephadex G-150 column. When subjected to SDS/polyacrylamide-gel electrophoresis, ETF migrated as two bands of equal intensity (Fig. 3), corresponding to subunit M_r values of 38000 and 32000, the sum of which is in agreement with the value given above by gel filtration. A value of 70000 per mol of FAD was obtained from the experimentally determined ε_{436} of 13.5 $mM^{-1} \cdot cm^{-1}$ for the bound flavin and protein by the biuret method. The amino acid composition of the purified ETF is shown in Table 1, along with that reported by Hall & Kamin (1975). Except for the large differences in the proline and tyrosine contents, the data are comparable. From these analyses, the minimum M_r per mol of FAD, however, is 58000.

The flavin content of ETF did not vary significantly from preparation to preparation and addition of free FAD to ETF fractions during purification was found to have no effect either on the overall yield or

Table 2. *Properties of ETF*

Property	Present study	Hall & Kamin (1975)	Crane & Beinert (1956)
Absorbance characteristics			
Peaks and troughs (nm)	270, (310)*, 375, 436, 460	270, 375, 438, 460	270, (310)*, 375, 437.5, 460
Ratios	5.8:0.16:0.9:1.0:0.86	5.74:0.92:1.0:0.85	6.5:0.3:0.9:1.0:0.86
M_r			
Native	68000	58000	30000–70000
Subunits	Nonidentical; 38000, 32000	Identical; 27500	—
Minimum M_r/FAD†			
By amino acid composition	58000	—	—
By protein determination	70000	26000	—
Absorption coefficient (ε_{436}) ($mM^{-1} \cdot cm^{-1}$)	13.5–13.6	11.3	—
Isoelectric point (pI)	6.75	—	8.1

* The numbers in parentheses represent positions of troughs.

† The flavin content was determined using the experimentally determined absorption coefficient of 13.5 $mM^{-1} \cdot cm^{-1}$ at 436 nm.

the flavin content of the final ETF preparations. Further, addition of free FAD to the assay mixture failed to stimulate the rate of ETF-mediated bleaching of 2,6-dichloroindophenol in the presence of catalytic amounts of pig kidney general acyl-CoA dehydrogenase and an excess of octanoyl-CoA. Finally, the protein to flavin absorbance ratio (A_{270}/A_{436}) of our ETF preparation is comparable with that reported by Hall & Kamin (1975) (Table 2). From the above data, we conclude that flavin is not lost during the preparation and that pig liver ETF is composed of two nonidentical subunits, only one of which contains the flavin chromophore.

Fig. 4 illustrates the course of anaerobic reduction of ETF by dithionite. The reduction occurs in two phases. The first phase, which requires 0.55 mol of sodium dithionite/mol of ETF for completion, results in a spectrum characteristic of an anionic flavin semiquinone (Massey *et al.*, 1966). The second phase, which proceeds slowly upon each addition of dithionite, produces fully reduced ETF and requires an additional 0.55 mol of dithionite/mol of ETF. A total of 1.1 mol of sodium dithionite per mol of ETF was consumed in the overall titration. The results of the dithionite titration rule out the possibility of any other redox-active chromophore in the ETF.

Discussion

The availability of substantial quantities of highly purified ETF prepared by the simple and efficient procedure reported in this paper has allowed us to re-evaluate some of the basic characteristics of ETF. Table 2 summarizes our findings along with those of previous studies. The most notable differences between our data and those of Hall & Kamin (1975) are in the nature of the two subunits, and in the flavin content. We consistently find that ETF dissociates into two non-identical subunits under denaturing conditions, whereas Hall & Kamin (1975) found two identical subunits. In addition, we always find 1 mol of FAD per mol of ETF, in contrast with 2 FAD/mol as reported by Hall & Kamin (1975). Partial loss of flavin during our isolation of ETF appears an unlikely explanation for the discrepancy in flavin content, since the stoichiometry is the same from preparation to preparation and added FAD has no effect on activity. Further, such a loss would have been reflected in a difference in the A_{270}/A_{436} ratio, which was not the case. The observation of two non-identical, rather than identical, subunits (Hall & Kamin, 1975) cannot be readily accounted for in terms of limited proteolysis of one of the two subunits, since the two bands obtained on SDS/polyacrylamide-gel electrophoresis not only migrate as proteins larger than reported by Hall & Kamin (1975) but stain equally in all ETF preparations. Thus, from our data we conclude that pig liver ETF (M_r 68000) is composed of two nonidentical subunits (M_r 38000 and 32000) with only one FAD per dimer. Similar conclusions have been reached with ETF from other sources. For example, the ETF from rat liver mitochondria, which is identical with pig liver ETF in its absorption and kinetic properties, has been shown to consist of two heterogenous subunits (M_r 33500 and 25000) (Furuta *et al.*, 1981); the flavin content was not reported. Further, ETF from pig kidney has been shown to be a dimer of nonidentical subunits with one FAD per dimer (R. Gorelick, J. Mizzer & C. Thorpe, personal communication). It is of interest to

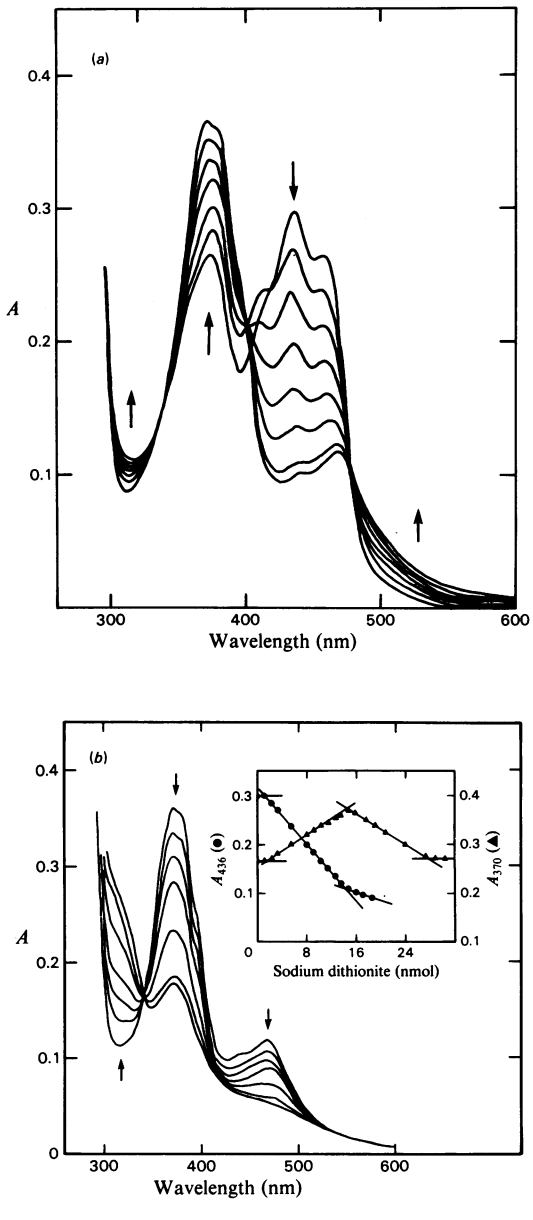

Fig. 4. *Dithionite titration of ETF*

ETF (22.9nmol) in 50mM-Hepes/10% (v/v) ethylene glycol, pH 7.6, was deoxygenated and titrated with anaerobic solution of dithionite. Spectra in (*a*) and (*b*) represent the two halves of the titration. The arrows indicate the direction of the spectral changes.

note that similar characteristics have been reported for ETF from a bacterial source. Thus, ETF from the methylotrophic bacterium W_3A_1 (M_r 77000), which exhibits absorbance characteristics similar to those of pig liver ETF, is a dimer of two non-identical subunits (M_r 42000 and 38000) with only one FAD per dimer (Steenkamp & Gallup, 1978). The non-identical nature of ETF subunits, a common feature, may suggest that the two subunits play a distinct role in the ETF-mediated electron transfer reactions.

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