The electron transfer flavoprotein from pig liver mitochondria is a 57,000-dalton electron transferase which links several primary flavoprotein dehydrogenases with the mitochondrial electron transport system. The protein was previously reported to be a dimer of apparently identical subunits. There are conflicting estimates in the literature regarding the FAD content of the protein. The results presented here clearly show that the protein contains nonidentical subunits based on polyacrylamide gel electrophoresis in the presence of 8 M urea and sodium dodecyl sulfate. The molecular weights of the subunits are 31,000 and 27,000. Analysis of peptides generated by cleavage of the subunits with cyanogen bromide show that the subunits have different primary structures. This result and amino acid analyses of the protein and the purified subunits show that the heterogeneity cannot be due to proteolysis. Using an experimentally determined molar extinction coefficient for the protein-bound flavin, a minimum of M₀ = 55,000 was calculated, indicating that the protein contains 1 mol of FAD/mol of protein.

The mitochondrial ETF is the specific electron acceptor for the fatty acyl-CoA dehydrogenases (1, 2), sarcosine and dimethyl glycline dehydrogenases (3), glutaryl-CoA dehydrogenase (4), and the branched chain acyl-CoA dehydrogenases (5). The protein acts as an electron transferase, linking the primary flavoprotein dehydrogenases to the main respiratory chain via ETF:ubiquinone oxidoreductase (6). During our investigations of the specific interaction of the general acyl-CoA dehydrogenases with ETF, it became necessary to investigate some aspects of the structure of ETF to evaluate our investigations of the specific interaction of the general acyl-CoA dehydrogenases with the mitochondrial electron transport system.

Crane and Beinert (7) initially reported that ETF from pig liver mitochondria contains 1 eq of FAD/80,000 daltons. More recent investigations by Hall and Kamin (8, 9) indicated that ETF is a dimer of apparently identical subunits containing 2 mol of FAD/58,000 daltons. The results of the present study are in agreement with the previously determined molecular weight for the ETF dimer (8); however, electrophoresis under denaturing conditions clearly shows that ETF is a heterodimer. Peptide mapping and amino acid analyses show that the subunit heterogeneity is not the result of limited proteolysis. In addition, our results from five different preparations show that ETF contains 1 FAD/54,000 daltons using an experimentally determined molar extinction coefficient for the protein-bound flavin.

**EXPERIMENTAL PROCEDURES**

Materials—Electron transfer flavoprotein was prepared from pig liver mitochondria as previously described (10). The purity of the protein was assessed by A₅₅₀/A₄₃₀ absorbance ratio (7), and by electrophoresis under nondenaturing conditions and in the presence of SDS as described below. Methanesulfonic acid (4 N) containing 0.2% tryptamine was obtained from Pierce Chemical Co. NBS, trimethobenzene sulfonic acid, DTNB, and iodoacetamide were purchased from Aldrich Chemical Co. NBS was recrystallized twice from acetone and iodoacetamide was recrystallized twice from water. Cyanogen bromide was obtained from Eastman. Ammonia solution, 25%, w/v, with a pH range of 3.5–10, was obtained from LKB. All other reagents were obtained from commercial sources and were the highest purity available.

Methods—The concentration of ETF flavin was determined spectrophotometrically using an ε₅₄₆ cm₃ = 13.4 × 10⁶ M⁻¹ cm⁻¹ (see below). FAD was released from the protein by three different methods to determine the molar absorbivity of the protein-bound flavin. Identical results were obtained in each case. Flavin was released by heat denaturation (90°C for 15 min), by treatment with 4 M guanidine hydrochloride in 0.1 M potassium phosphate, pH 7.1 (11), and by treatment with 5% dodecyl sulfate in 5 mM Na₂HPO₄, pH 7.6. Spectral comparison to authentic FAD under identical conditions permitted the calculation of the molar absorbivity for the protein-bound flavin.

All absorption spectra were measured on a Perkin-Elmer 559 spectrophotometer; samples were maintained at 25°C unless indicated otherwise.

Electrophoresis of ETF was carried out under non-denaturing conditions according to Davis (12); the concentration of acrylamide was varied from 7.0-11.5% to estimate the molecular weight of ETF (13). Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate was carried out according to the procedure of Weber and Osborn (14) at the concentration of acrylamide indicated in the individual experiments. The products obtained after cleavage of the protein with cyanogen bromide were subjected to electrophoresis in the dodecyl sulfate-urea system of Swank and Munkres (15). All acrylamide gels run in the presence of SDS were stained with 0.25% Coomassie Blue R-250 in 50% methanol, 10% acetic acid.

Isoelectric focusing was carried out in 5.6% polyacrylamide gels containing 0.2% Ampholine (pH 3.5–10) and 12% glycerol. The upper cathodic reservoir contained 0.4% ethanolate, pH 10, and the lower anodic reservoir contained 0.2% phosphoric acid (16). This orientation was employed since ETF is stable at least pH 10 but is labile under acidic conditions (17). Gels were polymerized with (NH₄)₂SO₄, and were prerun for 10 min prior to loading the protein on the gels. Isoelectric focusing was conducted under conditions of constant power at 5°C with an initial current of 1 mA/gel, until the current reached a minimum value and a mixture of acetylated cytochrome c charge isomers had reached their pl values. The bands of fluorescence due to ETF flavin had become stationary at this time. The pH gradient was determined in triplicate with a glass electrode after eluting 0.5-cm gel slices with 0.5 ml of glass-distilled water. Electrofocusing gels were stained with Amido black 10B after removal of ampholytes by soaking the gels in 5% trichloroacetic acid.

After staining and electrophoretic destaining, gels were analyzed using a Gilford gel scanning attachment with a 0.3-mm slit. This procedure was especially useful for estimating protein purity from 12.5% SDS gels (see Table I), which contained from 10–50 μg of protein. We observed approximate linearity of integrated absorbance...
Subunit Structure of ETF

with protein load, in agreement with previous investigations (14). This method also gave the correct per cent purity of a sample of 90% (w/w) ovalbumin with bovine serum albumin and soybean trypsin inhibitor as known impurities.

Amino acid analyses were carried out on ETF and ETF subunits which had been reduced and alkylated with iodoacetamide by the procedure of Noyes and Bradshaw (18). The protein was hydrolyzed at 110°C for 24 h in 6 M hydrochloric acid as described by Simpson et al. (19). The analyses were carried out by Dr. Daniel Omilianowski, University of Wisconsin-Madison. Exposed sulphydryl groups were quantitated with DTNB using an ε_{280nm} = 13.6 mM⁻¹cm⁻¹ (20). The native enzyme in 50 mM potassium phosphate, pH 8.0, containing 12.5% glycerol was reacted with a 70-fold molar excess of DTNB over ETF protein at 15°C using the appropriate reference. The increase in absorbance at 412 nm was recorded with time and the data were analyzed according to the procedure of Goldfarb (21). Total sulphydryl groups were determined with DTNB at 15°C in 50 mM sodium phosphate buffer, pH 8.0. After accessible sulphydryl groups had reacted with a 165-fold molar excess of DTNB over flavin, the reaction mixtures were made 1% in dodecyl sulfate by the addition of 10% dodecyl sulfate to the sample and reference cuvettes. The reaction was complete after 15 min.

Tryptophan in the native and alkylated ETF was quantitated independently by spectrophotometric titration with NBS (22, 23). Lyophilized protein (1-2 mg) was dissolved in 1.5 ml of 1% SDS made slightly alkaline, pH 8, with NaOH. After the protein had completely dissolved, an equal volume of 0.2 M formic acid-sodium acetate buffer, pH 4.15, containing 0.2% SDS was added. The final pH was 4.18. Aliquots of protein solution (0.70 ml) were then background corrected against an equal volume of buffer from 250-350 nm to obtain a zero base-line. Additions of NBS (7 m in glass-distilled water) were made at 280 nm. The maximal decrease in absorbance and the protein concentration as determined by the modified Lowry method allowed the calculation of moles of tryptophan/mol of ETF. The validity of this method was confirmed by quantitating 6.0 and 2.1 tryptophan residues in hen egg lysozyme and bovine serum albumin, respectively (24, 25).

Lysine was also quantitated by an independent method involving reaction of ETF containing 0.1% dodecyl sulfate with trinitrobenzene sulfonic acid according to the procedure of Habeob (26).

Protein concentrations were determined by the method of Lowry et al. (27), as modified by Miller (28), using bovine serum albumin as the standard. Since glycerol was routinely included in all buffers to stabilize ETF (10), blanks containing identical concentrations of glycerol and FAD were included in all protein determinations.

The chemical quantitations of lysine and tryptophan residues/mole of ETF were determined by the procedures described by Lowry and agree with the results obtained in the amino acid analyses. Therefore, the color yield in the protein assay, which can vary among proteins (27), accurately assays the concentration of ETF protein. The microbiuret method was found to yield essentially identical results (8); however, the Lowry procedure was routinely used.

**Subunit Preparation**—Subunits for amino acid analyses and peptide mapping were prepared electrophoretically on 5% polyacrylamide gels in the presence of 6 M urea at pH 4.5 (29). Approximately 200 µg of the reduced and alkylated ETF were electrophoresed on each gel column (0.5 × 11 cm) for 3 h at 8 mA/gel. The subunits were localized by fluorescence after soaking the gels in 0.03% anilinonaphthalene sulphonate for 30 min (30). Zones of the gels containing the subunits were excised, macerated, and eluted three times with 8 M guanidine hydrochloride in 0.1 M Tris, pH 9. The eluate was filtered through glass wool, exhaustively dialyzed against glass-distilled water and lyophilized. The purity of the polypeptides was assessed by electrophoresis in 13.4% polyacrylamide gels in the presence of dodecyl sulfate and 8 M urea (15). The yield of purified peptides was 8% of the weight of ETF initially alkylated (235 µg of α and 115 µg of β recovered from 4.0 mg of ETF).

**Peptide Mapping**—Peptide mapping was carried out after cleavage of reduced and alkylated ETF and of purified subunits with cyanogen bromide. Reduced and alkylated ETF (20-50 µg) and the purified subunits were reacted with 25 µm cyanogen bromide in 0.2 ml of 88% formic acid and 0.03% dodecyl sulfate in the dark at 25°C (31). After 24 h, the reaction mixture was diluted to 1 ml with H2O and lyophi- lized. Peptides were separated electrophoretically on 13.4% polyacrylamide gels by the procedure of Swank and Menkres (15). The system was capable of resolving peptides with molecular weights as low as 1800. The amount of protein applied to the gels was determined by the procedure of Lowry et al. No difference in the peptide maps was noted as the concentration of cyanogen bromide was varied from 10 to 200 mM.

**RESULTS**

**Molecular Weight, Flavin Content, and Subunit Composition**—Electrophoresis of ETF on polyacrylamide gels of varying porosities yields M, = 50,800 ± 3,000 (Fig. 1) (12,13). This value for the molecular weight is in good agreement with the minimum molecular weight based on flavin content. Using the experimentally determined molar absorptivity for the protein-bound flavin, ε_{450nm} = 13.4 ± 0.4 × 10^3 M⁻¹ cm⁻¹ (seven determinations), a minimum M, = 55,000 ± 4,000 was determined after correction for sample purity (Table 1). Subunit molecular weights were determined by electrophoresis on polyacrylamide gels in the presence of SDS and 2-mercaptoethanol (14); acrylamide concentrations were varied from 7.5-15%. From the slopes of primary plots of mobility as a function of acrylamide concentration, secondary plots (Fig. 2) were constructed (32). Using this procedure, the molecular weights

![Fig. 1. Estimation of the molecular weight of native ETF by gel electrophoresis.](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th>Protein/FAD</th>
<th>Purity</th>
<th>M, minimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td></td>
<td>g/mol</td>
</tr>
<tr>
<td>5.90</td>
<td>54,800</td>
<td>96.1</td>
</tr>
<tr>
<td>5.96</td>
<td>59,300</td>
<td>97.1</td>
</tr>
<tr>
<td>6.30</td>
<td>56,000</td>
<td>97.4</td>
</tr>
<tr>
<td>6.68</td>
<td>60,300</td>
<td>85.5</td>
</tr>
<tr>
<td>6.80</td>
<td>66,700</td>
<td>87.4</td>
</tr>
</tbody>
</table>

Average: 54,600 ± 3,100

*This ratio of absorption maxima is for the native enzyme in 2.5 mM K2HPO4 containing 3% glycerol.

Protein concentration was determined by the method of Lowry (see "Methods"), and flavin concentration of the same solution was determined spectrophotometrically using ε_{450} = 13.4 mM⁻¹ cm⁻¹.

Sample purity was assessed by densitometry of 12.5% SDS gels stained with Coomassie R-250. Each gel contained 10-50 µg of total protein.
of the α and β subunits were determined to be 31,100 ± 1,400 and 26,800 ± 1,400, respectively. The subunits were observed in the presence and absence of 2-mercaptoethanol. Flavin fluorescence has not been observed in either band (8). The sum of subunit molecular weights agrees with the molecular weight of the native protein within experimental error. These values agree well with the Mᵣ = 58,000 ± 3,000 determined by Hall and Kamin (8); however, our results differ significantly in that our data indicate that ETF is a heterodimer containing 1 FAD/dimer.

**Isoelectric Focusing**—Isoelectric focusing of ETF reveals one major band and several minor bands which exhibit characteristic of ETF flavin before staining (Fig. 3). The major band, which constitutes at least 75% of the total, has a pH of about 7.0. Other bands are observed with pH values of 7.50, 6.87, and 6.39-6.72. This pattern was observed with two different preparations. Electrophoresis in a second dimension in the presence of dodecyl sulfate indicated that each band has the heterodimeric structure of the bulk preparation.

**Amino Acid Analyses and Peptide Mapping**—The amino acid analysis of the native protein (Table II) compares well with the analysis previously reported (8). ETF contains 9.4 ± 0.3 sulphydryl groups as determined by reaction with DTNB after denaturation with 1% sodium dodecyl sulfate. In the native protein, two free sulphydryl groups were detected by reaction with DTNB (Table III). These two groups differ in reactivity with DTNB by about 10-fold. These results are consistent with the demonstration of nonequivalent subunits. Quantitation of lysine residues with trinitrobenzenesulfonate by the method of Habeeb (26) gives a value of 40.8 ± 3.5 lysines/54,000 daltons based on the protein concentration determined by the method of Lowry et al. (27). Since this chemical determination agrees with the amino acid analysis, the protein assay gives a reliable value for ETF protein concentration within 10%.

Previous results indicated that ETF could be a homodimer or at least a heterodimer with subunits of identical molecular weight (8). Fig. 4 shows densitometric scans of dodecyl sulfate-urea-polyacrylamide gels after electrophoresis of the ETF and the purified α and β subunits. To demonstrate that the results

---

**Table II**

<p>| Amino acid composition of ETF and isolated α and β subunits |
|---------------------------------|-------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>α subunit¹</th>
<th>β subunit²</th>
<th>αβ (calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>6.0</td>
<td>2.8</td>
<td>21.8</td>
</tr>
<tr>
<td>Thr</td>
<td>30.6</td>
<td>16.8</td>
<td>12.4</td>
</tr>
<tr>
<td>Ser</td>
<td>23.4</td>
<td>19.0</td>
<td>10.5</td>
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<tr>
<td>Glu</td>
<td>52.9</td>
<td>25.4</td>
<td>31.4</td>
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<tr>
<td>Pro</td>
<td>24.3</td>
<td>17.4</td>
<td>15.8</td>
</tr>
<tr>
<td>Gly</td>
<td>39.4</td>
<td>27.9</td>
<td>22.6</td>
</tr>
<tr>
<td>Ala</td>
<td>63.8</td>
<td>37.4</td>
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</tr>
<tr>
<td>Val</td>
<td>49.1</td>
<td>26.4</td>
<td>28.4</td>
</tr>
<tr>
<td>Met</td>
<td>6.2</td>
<td>2.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Ile</td>
<td>24.8</td>
<td>15.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Leu</td>
<td>50.3</td>
<td>27.8</td>
<td>24.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>7.4</td>
<td>6.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Phe</td>
<td>13.0</td>
<td>8.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Lys</td>
<td>44.4</td>
<td>23.7</td>
<td>19.1</td>
</tr>
<tr>
<td>His</td>
<td>8.7</td>
<td>5.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Arg</td>
<td>20.4</td>
<td>7.2</td>
<td>12.8</td>
</tr>
<tr>
<td>Trp</td>
<td>1.5</td>
<td>2.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Cys</td>
<td>9.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Average of analyses of preparations with A₁₁₀ = A₂₂₀ = 5.9 and 6.3.
² Average of two analyses of two different preparations.
³ Determined by reaction with trinitrobenzene sulfonate (26).
⁴ Average of four determinations by titration with NBS, 2.40 ± 0.25
⁵ Subunits.
⁶ Single determinations on the amino acid analyzer.
⁷ Determined by reaction with DTNB in the presence of 1% dodecyl sulfate. Carboxymethyl cysteine was not determined.
TABLE III

Kinetic parameters for the reaction of DTNB with the native ETF
ETF (\(A_{270\, \text{nm}}A_{438\, \text{nm}} = 5.8\)) was reacted with DTNB in 50 mM potassium phosphate, pH 8.0, at 10 °C; Experiment I was carried out in the presence of 20% glycerol and II was carried out in 12.5% glycerol.

<table>
<thead>
<tr>
<th>FAD [DTNB] [FAD]</th>
<th>(n_1^+)</th>
<th>(k_1)</th>
<th>(n_2^+)</th>
<th>(k_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu M)</td>
<td>(M^{-1}, \text{min}^{-1})</td>
<td>(M^{-1}, \text{min}^{-1})</td>
<td>(M^{-1}, \text{min}^{-1})</td>
<td>(M^{-1}, \text{min}^{-1})</td>
</tr>
<tr>
<td>I</td>
<td>6.26</td>
<td>143</td>
<td>1.45 ± 0.28</td>
<td>110 ± 11</td>
</tr>
<tr>
<td>II</td>
<td>8.53</td>
<td>70</td>
<td>1.23 ± 0.03</td>
<td>153 ± 9</td>
</tr>
</tbody>
</table>

* The data are expressed as moles of sulfhydryl groups reacted/mol of FAD. Values of \(n_1^+\) and \(n_2^+\), the number of sulfhydryl groups reacted, and the second order rate constants were calculated as described by Goldfarb (21).

![Polyacrylamide gel electrophoresis of peptides derived from cyanogen bromide cleavage of ETF](image)

**Fig. 5.** Polyacrylamide gel electrophoresis of peptides derived from cyanogen bromide cleavage of ETF. Cleavage with cyanogen bromide was carried out in 200 \(\mu l\) of 88% formic acid containing 25 mM cyanogen bromide, 0.05% dodecyl sulfate for 24 h at 25 °C. Peptides were separated on 13.4% polyacrylamide gels in the presence of dodecyl sulfate and 8 M urea under reducing conditions (15). The tracings show peptides derived from cleavage of (a) reduced and alkylated ETF, 25 \(\mu g\); (b) reduced and alkylated \(\alpha\) subunit, 25 \(\mu g\); (c) reduced and alkylated \(\beta\) subunit, 21 \(\mu g\). Subunits were isolated as described in Fig. 4.

DISCUSSION

We have reexamined the structure of ETF from pig liver mitochondria as part of our investigation of the interactions between ETF and flavoprotein dehydrogenases. Our principal finding is that ETF is a heterodimer containing 1 FAD/54,000 ± 4,000 daltons. The original characterization of liver ETF by Crane and Beinert (7) indicated an 80,000-dalton protein containing 1 FAD, with a \(A_{270\, \text{nm}}A_{438\, \text{nm}}\) ratio of 6.5. The purity of their preparation was not estimated. However, from their data in Fig. 4 of Ref. 7, we calculate a molar absorbivity at 438 nm of \(13.7 \times 10^3\) and a minimal \(M_r = 52,000\). Therefore, our data are in excellent agreement. Furuta et al. (33) have recently reported that rat liver ETF is composed of nonidentical subunits, \(M_r = 25,100\) and 33,500. The \(A_{270\, \text{nm}}A_{438\, \text{nm}}\) ratio of their preparation is 5.7. They did not rule out limited proteolysis and did not address the flavin content of the enzyme. Our peptide mapping data and amino acid analyses of the isolated subunits strongly support the conclusion that the \(\alpha\) and \(\beta\) subunits have different primary structures. Our
data regarding subunit structure and flavin content conflict with those of Hall and Kamin (8) who proposed that ETF is a dimer of apparently identical subunits, \( M_r = 26,000 \pm 1,500 \), containing 2 eq of FAD/dimer. They occasionally observed subunit heterogeneity on SDS-polyacrylamide gels. Despite their finding of 2 mol of FAD/58,000 g of protein, they report a \( A_{279\text{nm}}/A_{438\text{nm}} \) ratio of 5.5-5.9. We calculate that the hypothetical incorporation of a second mole of FAD into our ETF samples would lower the \( A_{279\text{nm}}/A_{438\text{nm}} \) ratio to less than 5.0. A molar absorptivity of \( 11.4 \times 10^3 \) at 438 nm was determined by Hall and Kamin based on comparison with salicylate hydroxylase (8). This would still not account for the difference between the two determinations of the minimum molecular weight based on flavin content. We are unable to resolve this discrepancy.

Heterodimeric electron transfer flavoproteins have been isolated from two bacterial species; however, the biological functions of the two proteins differ significantly. The ETF from the methylotrophic bacterium W3A1 is the electron acceptor from the primary dehydrogenase and an electron donor to ETF:ubiquinone oxidoreductase. Although their finding of 2 mol of FAD/58,000 g, they report a theoretical incorporation of a second mole of FAD into our ETF which contains only 1 flavin/dimer (7, 37) acts as an electron acceptor from the tetrameric acyl-CoA dehydrogenase to the ETF. It is well established that the kinetically significant reduced form of ETF flavin is the anionic semiquinone (38). The present work indicates a simple molecularity at the level of the electron transfer reaction in the sense that a single ETF molecule would react with a single dehydrogenase subunit. The finding that the liver ETF is a dimer of dissimilar subunits with a single redox center further suggests the possibility of separate electron acceptor and donor sites, or binding and electron transfer domains.

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Subunit structure of electron transfer flavoprotein.
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