A New Iron-Sulfur Flavoprotein of the Respiratory Chain

A COMPONENT OF THE FATTY ACID β OXIDATION PATHWAY*

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The iron-sulfur (Fe-S) flavoprotein from beef heart mitochondria previously reported (Ruzicka, F. J., and Beinert, H. (1975) Biochem. Biophys. Res. Commun. 66, 622-631) has been purified to apparent homogeneity by the criteria of polyacrylamide electrophoresis. The purity has been increased 3- to 4-fold over that achieved previously with no major change in the observed properties. The protein contains one flavin with chromatographic properties of FAD and a single [4Fe-4S] cluster (g Euler = 1.886; g = 1.939; g = 2.086, after reduction) per subunit (sodium dodecyl sulfate polyacrylamide electrophoresis). It is capable of taking up 3 electrons per flavin. The oxidation reduction midpoint potential of the Fe-S cluster is −85 mV higher than that of the flavin. The Fe-S group was reduced with a t1/2 of <10 ms by the reduced form of the electron-transferring flavoprotein (ETF) of the β oxidation pathway of fatty acids and when reduced was reoxidized with a t1/2 of ~20 ms by Q-1. It is shown that the light absorption at 438 nm of reduced ETF is readily restored by Q-1 only when the Fe-S flavoprotein is present. From these experiments, it is concluded that in vitro the Fe-S flavoprotein is an electron acceptor for ETF and a Q-1 reductase. Furthermore, from the observation of Flathmark et al. (1976) FEBS Lett. 63, 51-55), on the enhancement of the EPR signal of the Fe-S flavoprotein in brown adipose tissue of cold-acclimated guinea pigs, it is suggested that this Fe-S flavoprotein also functions in vivo as an electron carrier in fatty acid oxidation.

In 1975 we reported on the purification and partial characterization of an iron-sulfur (Fe-S) flavoprotein from beef heart mitochondria (1) which, to our knowledge, had not been recognized previously. The protein was purified following an EPR signal at g = 2.08 which had been observed in reduced mitochondria and submitochondrial particles in this and other laboratories (2-4).

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1 The abbreviations used are: Fe-S, iron-sulfur, with [4Fe-4S] indicating a cluster involving 4 iron and 4 labile sulfur atoms; ETF, electron-transferring flavoprotein (cf. Ref. 5); PMS, phenazine methosulfate; EPR, electron paramagnetic resonance.

This signal could not be attributed to any known component of the mitochondrial electron transfer system. The protein has a [4Fe-4S] center per flavin. The flavin is readily released by acid and has properties of FAD. No substrate of low molecular weight was found that was able to reduce this Fe-S flavoprotein or to transfer electrons to PMS in the presence of this protein. It was thought, therefore, that the protein may function as an electron carrier between other proteins. In experiments testing this idea, it was found (1) that the Fe-S group of the protein is reduced rapidly by the reduced form of the electron-transferring flavoprotein (ETF) (5) of the fatty acyl-CoA dehydrogenation system. In this paper we report on an improved purification procedure for the Fe-S flavoprotein that yields an enzyme of a purity 4-fold higher than previously achieved (1). Properties of this preparation and its ability to interact with ubiquinone as electron acceptor were also investigated.

EXPERIMENTAL PROCEDURES

Materials—NADH, NADPH, and butyryl-CoA were purchased from PL Laboratories; sodium succinate and sn-glycerol-3-phosphate from the Boehringer Mannheim Corp.; dihydroorotic acid, choline chloride, FMN, FAD, and Triton X-100 from Sigma Chemical Co.; and cholic acid from Matheson, Coleman and Bell. Cholic acid was recrystallized twice from 50% ethanol. Ubiquinone-1-(Q-1) was kindly given to us by Dr. O. Isler of Hoffmann-La Roche, Basel, Switzerland. DEAE-Bio-Gel A, Bio-Beads SM-2, and reagents for electrophoresis were obtained from Bio-Rad Laboratories. The yellow (general) fatty acyl-CoA dehydrogenase and ETF were purified from beef heart mitochondria according to the method of Beinert and Lee (6). Complexes I to III were prepared as described (7-9).

Methods—Acid-extractable flavin was determined according to the method of Beinert and Page (10), and protein was estimated by the biuret method applied to the trichloroacetic acid precipitate obtained in the flavin determination. For chromatography, the flavin of the Fe-S flavoprotein was released by heating to 80° for 20 min (11). Descending paper chromatography was carried out essentially according to the method of Kilgour et al. (12) and spots were located with a UV lamp. Iron was determined by a modification (13) of the method of Ref. 14, in which no ashing is required. Labile sulfur was determined according to the method of Brumby et al. (15).

Spectrophotometric assays for dehydrogenase activity with various substrates were carried out by the general PMS method of Singer (16) using cytochrome c as the terminal acceptor. Transhydrogenase activity from NADPH to acetylpyridine NAD+ was assayed according to Ref. 17. Anaerobic titrations were performed using dithionite, other anaerobic procedures, rapid freeze-quenching, EPR spectroscopy, and evaluation of spectra were carried out as described previously (18).

Electrophoresis of the native protein was performed essentially according to the method of Davis (19) in a slab electrophoresis cell.
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(1.5 mm thickness) (0.6 M Tris/HCl pH 6.8) on 10% acrylamide separating gel (11 × 15.5 cm) (0.38 M Tris/HCl pH 8), and electrode buffer (0.005 M Tris, 0.009 M glycerine, pH 9.0). Protein samples (0.3 to 12 µg) were applied in 0.5-cm wells in 0.05 M Tris/HCl, pH 8, and 0.25 M sucrose. All solutions contained 0.5% Triton X-100. Constant current electrophoresis was conducted at 40 mA at 5° for 14 h.

Sodium dodecyl sulfate-polyacrylamide electrophoresis was carried out according to the method of Laemmli (20) with slab gels (10% acrylamide in separating gels). Protein samples (0.3 to 15 µg) were applied in 0.5-cm wells after incubation at 50° for 30 min in 0.005 M Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 0.01 M dithiothreitol. Bromphenol blue (0.001%) was added to each sample as a tracking dye. Electrophoresis was conducted at 35 mA constant current at 20°.

RESULTS AND DISCUSSION

Purification of Fe-S Flavoprotein

The method for isolating the Fe-S flavoprotein is based on the previously published procedure (1). However, modifications have been made at all stages to warrant a complete description here. Beef heart mitochondria (Fraction I, Table I; approximately 50 g of protein according to the biuret reaction) were applied in 0.5-ml fractions after incubation at 50° for 30 min with the above buffer. The solution was stirred on ice for 20 min followed by centrifugation at 48,000 × g for 20 min. The yellow pellets were suspended in a total volume of approximately 1.5 ml of 0.01 M Tris/HCl, pH 7.4, dialyzed for 3 h in the same buffer, and frozen in liquid nitrogen (Fraction IV, Table I). The yield is 9 to 11 mg. Table I shows the recovery of Fe-S flavoprotein at several stages during the isolation procedure.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Fraction</th>
<th>Fe-S flavoprotein*</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/mg protein</td>
<td>mg total</td>
</tr>
<tr>
<td>1</td>
<td>I. Beef heart mitochondria</td>
<td>0.025</td>
<td>1300</td>
</tr>
<tr>
<td>2</td>
<td>0.025</td>
<td>1250</td>
<td>49,500</td>
</tr>
<tr>
<td>3</td>
<td>0.025</td>
<td>1290</td>
<td>51,600</td>
</tr>
<tr>
<td>1</td>
<td>II. Following cholate extraction and salt precipitation</td>
<td>0.21</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>0.18</td>
<td>260</td>
<td>1,400</td>
</tr>
<tr>
<td>3</td>
<td>0.18</td>
<td>240</td>
<td>1,380</td>
</tr>
<tr>
<td>1</td>
<td>III. Applied to DEAE-Bio-Gel column</td>
<td>0.24</td>
<td>210</td>
</tr>
<tr>
<td>2</td>
<td>0.22</td>
<td>245</td>
<td>1100</td>
</tr>
<tr>
<td>3</td>
<td>0.23</td>
<td>230</td>
<td>1090</td>
</tr>
<tr>
<td>1</td>
<td>IV. Purified Fe-S flavoprotein</td>
<td>11.2</td>
<td>120</td>
</tr>
<tr>
<td>2</td>
<td>13.0</td>
<td>140</td>
<td>10.9</td>
</tr>
<tr>
<td>3</td>
<td>11.5</td>
<td>140</td>
<td>10.4</td>
</tr>
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</table>

* The content of Fe-S flavoprotein in Fractions I to III was determined by comparison of the signal height of the low field resonance of the EPR signal (g = 2.08) to that of the purified Fe-S flavoprotein.

Because of signal to noise limitations, the content of Fe-S flavoprotein in heart mitochondria may be in error by ±20%.

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Composition and Capacity for Electron Uptake—Table II shows the flavin, iron, and labile sulfur content, and the number of unpaired spins represented in the Fe-S signal after reduction of the Fe-S flavoprotein at different states of purity. These values were not collected on individual preparations during the course of the purification procedure. Instead, they were obtained on various preparations in the course of months during the course of the purification procedure. Instead, they were obtained on various preparations in the course of months. The association of iron, labile sulfur, and flavin in the same proportion at various states of purity indicates that these substances are components of the same protein that is being purified. According to the data of Table II the protein has a single [4Fe-4S] center per flavin.

Fig. 3 presents results of a reductive titration of the Fe-S flavoprotein by dithionite. The ordinate shows electron equivalents recovered in the Fe-S signal (g = 2.08, 1.94, 1.89) or
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**Fig. 1.** Separation of Fe-S flavoprotein by column chromatography. Approximately 800 mg of dialyzed protein were applied to a column consisting of DEAE-Bio-Gel A (11 × 4 cm) layered over Bio-Beads SM-2 (2.5 × 4 cm) which had been previously equilibrated with 0.01 M Tris/HCl (pH 7.4). The flow rate of the column was set at 1.5 ml/min and 6-ml fractions were collected. The column was washed with equilibrating buffer until no protein was detected in the effluent. This was followed by a second wash with 500 ml of 0.1 M Tris/HCl (pH 7.4). Then a linear gradient was produced by mixing 750 ml each of 0.1 and 0.22 M Tris/HCl (pH 7.4) in 0.1% (w/v) Triton X-100 and the resulting mixture was used to elute the Fe-S flavoprotein.

**Fig. 2.** Polyacrylamide electrophoresis of Fe-S flavoprotein. Optical scans at 550 nm of polyacrylamide gels stained with Coomassie blue after electrophoresis of Fe-S flavoprotein. A, protein run under native conditions in 0.5% Triton X-100 at pH 8; B, protein run in the presence of 0.1% SDS. Cathode is to the left. For conditions, see “Methods.”

**Table II**

<table>
<thead>
<tr>
<th>Flavin</th>
<th>Iron</th>
<th>Labile sulfur</th>
<th>Fe²⁺</th>
<th>Fe³⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/mg protein</td>
<td>ng atoms/mg protein</td>
<td>ng atoms/mg protein</td>
<td>equiv/mg protein</td>
<td>equiv/mg protein</td>
</tr>
<tr>
<td>1a</td>
<td>3.7</td>
<td>12.9</td>
<td>13.4</td>
<td>2.7</td>
</tr>
<tr>
<td>1b</td>
<td>3.5</td>
<td>14.5</td>
<td>12.7</td>
<td>3.1</td>
</tr>
<tr>
<td>1c</td>
<td>2.9</td>
<td>14.2</td>
<td>12.7</td>
<td>2.7</td>
</tr>
<tr>
<td>2a</td>
<td>6.7</td>
<td>26.8</td>
<td>27.3</td>
<td>6.5</td>
</tr>
<tr>
<td>2b</td>
<td>7.3</td>
<td>27.4</td>
<td>22.2</td>
<td>6.0</td>
</tr>
<tr>
<td>3a</td>
<td>13</td>
<td>45.0</td>
<td>46.1</td>
<td>11.4</td>
</tr>
<tr>
<td>3b</td>
<td>13</td>
<td>48.0</td>
<td>46.0</td>
<td>13.0</td>
</tr>
<tr>
<td>3c</td>
<td>13</td>
<td>50.0</td>
<td>48.7</td>
<td>11.5</td>
</tr>
</tbody>
</table>

*a State 1 corresponds to the state of purification reached in our previous published work (1) and State 3 to that of the present work.

free radical signal ($g = 2.00$) and the abscissa electron equivalents added as dithionite, both with reference to atoms of iron present in the Fe-S flavoprotein. It can be seen that approximately 0.75 eq/iron or 3 eq/flavin are needed for complete reduction of the flavin and Fe-S groups. It is also apparent that the Fe-S group is titrated largely before the flavin.

From the data of Fig. 3, we calculate that the midpoint oxidation-reduction potential of the Fe-S cluster is about 55 mV higher than the average potential of the flavin group. If the value of 40 mV, found for the Fe-S group of the protein in mitochondria (4) is assumed to hold also for the purified
protein the midpoint potential values for the Fe-S and flavin groups would be +40 and −15 mV, respectively.

The flavin was readily released from the protein by heating or by acid. In chromatographic Systems 1 (1-butanol/acetate acid) and 8 (t-butyl alcohol/ammonia) of Kilgour et al. (12), the flavin migrated with FAD and not FMN. The observed 

\[ R_p \] values for the released flavin were 0.04 (FAD 0.04, FMN 0.09) and 0.23 (FAD 0.24; FMN 0.16) in the two systems, respectively.

**Light Absorption and EPR Spectra** —The absorption spectrum of the released flavin has been shown previously (1). Fig. 4 shows the spectrum of the Fe-S flavoprotein in the oxidized (Curve 1) and reduced (Curve 4) state and after addition of 0.48 (Curve 2) and 2.22 (Curve 3) reducing equivalents, respectively, per mol of flavin. The spectra have features similar to those seen with other Fe-S (flavo-) proteins, such as maxima in the 400 nm region (from Fe-S), a distinct shoulder between 450 and 470 nm (from flavin), and a tailing absorption toward longer wavelengths (from Fe-S). There is only a slight indication of heme contamination in the spectrum of the reduced protein between 410 and 420 nm. At states of lesser purity strong heme absorption is observed in this region.

The EPR spectrum was shown in Fig. 1 of Ref. 1. It is typical of Fe-S proteins, with \( g_1 = 1.886; g_2 = 1.939; \) and \( g_3 = 2.086. \) At the state of purity achieved at that time, we were not able to eliminate the radical signal by a large excess of dithionite. EPR spectra of the reduced protein at the present stage of purity no longer show a radical signal.

**Function of Fe-S Flavoprotein**

Neither with NADH nor with NADPH could any reduction of the flavin or the Fe-S center of the protein be detected, nor any significant oxidation of the nucleotide. Transhydrogenase activity from NADPH to acetylpyridine NAD+ could not be detected. Dehydrogenase assays (see "Methods") with sn-glycerol 3-phosphate, succinate, dihydroorotate, and choline did not show rates of cytochrome c reduction significantly exceeding those of the controls.

We considered the possibility, therefore, that the protein may be an electron carrier of the type of ETF of the \( \beta \) oxidation pathway of fatty acyl derivatives of CoA. The experiments to be described in the following have provided evidence that the flavoprotein is involved in electron transfer between ETF and the Q-bc<sub>1</sub> region of the mitochondrial electron transfer system. First, as reported previously in Fig. 2 of Ref. 1, the purified Fe-S flavoprotein is reduced by a mixture of yellow acyl-CoA dehydrogenase and ETF, pre-reduced by butyryl-CoA, with a \( t_{1/2} \) of ~10 ms at 16°. The appearance of the EPR signal of the reduced Fe-S center of the protein was monitored in this experiment. Control experiments showed that under analogous conditions, samples of purified Complexes I and II were not reduced by ETF, whereas Complex III was reduced in the time range of seconds.

Second, the reduced Fe-S flavoprotein is reoxidized with a \( t_{1/2} \) of ~20 ms by Q-1 as shown in Fig. 5. In the foregoing experiment on the reduction of the enzyme it was necessary to keep a high level of reduced ETF to effect rapid reduction. However, in the experiment of the reoxidation of the Fe-S flavoprotein, a minimal amount of the reducing systems was used, just sufficient to pre-reduce the protein anaerobically within minutes. Thus, in the reoxidation experiment with Q-1, very little reduction should take place during the sampling times of the experiment. It should be noted that under the conditions described 70 to 80% of ETF-ubiquinone oxidoreductase was reduced. This is, however, not an important consideration for the evaluation of the data. The experiment was carried out at 14° and a control shot against buffer containing ethanol but no Q-1 was made after every shot against Q-1. We have also tried to do an analogous experiment in which the protein was pre-reduced by a stoichiometric amount of

![Fig. 4. Light absorption spectra of the Fe-S flavoprotein at different oxidation states. The protein, 15 \( \mu \)M was dissolved in 0.1 M Tris/chloride of pH 7.5 (Curve 1). Complete reduction was carried out with a trace of solid dithionite (Curve 4). The partly reduced samples were derived from the experiment of Fig. 3 and they were prepared by the titration method of Orme-Johnson and Beinert (21) and their spectra were obtained as described by Hansen et al. (22). The absorbance values are plotted as \( a_\mu \).](http://www.jbc.org/content/843/1/8843/F5.large.jpg)

![Fig. 5. Reoxidation of reduced Fe-S flavoprotein by Q-1. 1.65 ml of a mixture of 0.25 \( \mu \)M ETF, 3 \( \mu \)M yellow acyl-CoA dehydrogenase, and 44 \( \mu \)M Fe-S flavoprotein (ETF-dehydrogenase) in 10 mM Tris/chloride of pH 7.4 were made anaerobic in a tonometer (23) by repeated evacuation and filling with nitrogen that had been passed over hot copper and BASF catalyst. While nitrogen was passed through the tonometer, 40 \( \mu \)l of 32 mM butyryl-CoA (C<sub>4</sub>CoA) were quickly added and evacuation and filling were repeated. This solution was then drawn anaerobically into a 1-ml syringe and left standing for 10 min at 18° before mixing the Q-1 solution. Q-1, 0.8 mM, dissolved in 10 mM Tris buffer (pH 7.4) containing 10% ethanol was made anaerobic in a tonometer and filled into a second 1-ml syringe. A third syringe for control shots contained the same anaerobic solution as the second one, except that Q-1 was omitted. Because of the possibility that the EPR signal of the Fe-S flavoprotein might increase further on standing (continued reduction) during the experiment, a control shot was made after every shot against the Q-1 solution. The changes in the control samples, which were minor, were corrected. The experiment was carried out at 14°. EPR spectroscopy was carried out as described previously (1, 18).](http://www.jbc.org/content/843/1/8843/F6.large.jpg)
dithionite. Unfortunately, the protein does not appear to be sufficiently stable under these conditions so that satisfactory results were not obtained.

Third, it had to be demonstrated that the rate of reoxidation of the Fe-S flavoprotein by Q-1 was significantly higher than that of ETF itself. In these experiments (Fig. 6), the reappearance of the absorbance of ETF at 438 nm was measured spectrophotometrically after reduction by acyl-CoA dehydrogenase and butyryl-CoA. A minimal quantity of the reducing ingredients was added so that continuing reduction of ETF would not significantly compete with oxidation by Q-1. The molar absorptivity of Q-1 at 438 nm is only about one-twentieth of that of ETF. Thus, oxidation of ETF simultaneous with reduction of Q-1 can easily be measured. In the experiment of Fig. 6, ETF is reoxidized in the course of minutes, with a 2- to 3-fold excess of Q-1. As shown in the figure, reoxidation occurs instantaneously when the Fe-S flavoprotein is added at a concentration ratio (flavin basis) of 1:500 to ETF. In view of the large difference in reaction rates in the absence and in the presence of the Fe-S flavoprotein, we are not certain that the rate observed without added Fe-S flavoprotein is the true reaction rate of ETF with Q-1, since traces of this protein or other contaminants in our ETF preparation might make a contribution. The rate observed in the absence of any added Fe-S flavoprotein can, therefore, only be considered as the maximal one. Reoxidation of reduced ETF by O₂ occurs only within minutes (10) slower yet than that by Q-1. In order to rule out the possibility that the Fe-S flavoprotein might merely accelerate the reoxidation of ETF by Q₈, experiments of the type shown in Fig. 6, lower curve, were performed, in which the Fe-S flavoprotein was added before Q-1. In this case an immediate increase in absorption occurs on addition of Q-1 which exceeds the absorption of the added Q-1 severalfold. The contributions to the absorbance at 438 nm of Q-1 or the Fe-S flavoprotein by themselves can be seen in the figure from the response to additions made following the reoxidation of ETF.

The fourth argument supporting the proposed function of the new Fe-S flavoprotein comes from experiments on brown adipose tissue of cold-adapted guinea pigs. In collaboration with Dr. T. Flatmark (24), we could show that the low field EPR signal typical of this Fe-S flavoprotein in brown adipose tissue is about 4 times that in heart relative to the signals of NADH dehydrogenase. The main oxidizable substrates of brown adipose tissue of guinea pigs are fatty acids and sn-glycerol 3-phosphate. We could detect only very little or no glycerol-3-phosphate dehydrogenase (not NADH-linked) activity in preparations of our Fe-S flavoprotein. Furthermore, in flight muscle mitochondria from flies, we did not find EPR signals at g = 2.08 (Fe-S flavoprotein) larger than those found in heart mitochondria despite a >100-fold difference in activity of glycerol phosphate dehydrogenase in these tissues (25). We feel, therefore, that the demonstration of a significantly enhanced level of this Fe-S flavoprotein in brown adipose tissue relative to other mitochondrial Fe-S proteins supports our contention that this Fe-S flavoprotein is involved in fatty acid oxidation.

We conclude from the evidence presented above, that at least in vitro, the Fe-S flavoprotein described is an efficient electron acceptor for the electron-transferring flavoprotein, ETF, and a reductase of ubiquinone. The last mentioned set of experiments on brown adipose tissue (24) supports the view that this also holds in vivo. We propose, therefore the trivial name ETF-ubiquinone oxidoreductase for this protein.

Acknowledgments—We acknowledge the expert collaboration of Mr. R. E. Hansen in rapid reaction experiments and EPR spectroscopy and the assistance of Dr. Robert Ake in the determination of the molecular weight of the subunit of the protein in the presence of guanidine HCl. A number of the labile sulfur analyses were kindly done for us by Ms. K. Vielhuber.

REFERENCES

![Fig. 6. Time course of reoxidation of ETF by Q-1 in the presence and absence of Fe-S flavoprotein. ETF, 32 μM, was reduced with 0.4 μM yellow acyl-CoA dehydrogenase and 300 μM butyryl-CoA. Then, 80 μM Q-1 and 60 nM Fe-S flavoprotein were added in different sequence (compare upper to lower level). The increase in absorbance due to the added absorbance of Q-1 and Fe-S flavoprotein by themselves can be seen from the additions of these ingredients made after oxidation of ETF was completed. The decrease in absorbance due to reduction of Q-1 should have been ≤0.017. The experiment was carried out aerobically at 18° in a microcell attachment of a recording Beckman DU Spectrophotometer (Uitlote Instrument, Inc.).]
Iron-Sulfur Flavoprotein from Mitochondria

A new iron-sulfur flavoprotein of the respiratory chain. A component of the fatty acid beta oxidation pathway.

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