A New Iron-Sulfur Flavoprotein of the Respiratory Chain

A COMPONENT OF THE FATTY ACID $\beta$ 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 ($g_1 = 1.886$; $g_2 = 1.939$; $g_3 = 2.086$, 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 $-55$ mV higher than that of the flavin. The Fe-S group was reduced with a $t_{1/2}$ of $<10$ ms by the reduced form of the electron-transferring flavoprotein (ETF) of the $\beta$ oxidation pathway of fatty acids and when reduced was reoxidized with a $t_{1/2}$ of $\sim20$ 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 Flitmark 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 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 an 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 with dithionite, other anaerobic procedures, rapid freeze-quenching, EPR spectroscopy, and evaluation of spectra were carried out as described previously (18).

Elecrophoresis of the native protein was performed essentially according to the method of Davis (19) in a slab electrophoresis cell. The system contained 2.5% acrylamide stacking gel (0.6 x 15.5 cm
Iron-Sulfur Flavoprotein from Mitochondria

(1.5 mm thickness) (0.6 M Tris/HCl pH 6.8) on 10% acrylamide separating gel (11 x 15.5 cm) (0.38 M Tris/HCl pH 8), and electrode buffer (0.005 M Tris, 0.003 M glycine, pH 9.0). Protein samples (0.3 to 12 mg) 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°C 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 pg) were applied in 0.5-cm wells after incubation at 50°C for 30 min in 0.0025 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°C.

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) were collected and dissolved in 0.01 M Tris/HCl, pH 7.4, containing 1 mM dithiothreitol and 5 mM succinate, and suspended in the same buffer to a protein concentration of 30 mg/ml. Potassium chloride (4% w/v, pH 7.4) was added to a concentration of 0.18 mg/mg of protein. The mixture was stirred on ice for 20 min and centrifuged for 3 h at 78,000 x g. The pellet was resuspended to a protein concentration of 30 mg/ml with the same buffer. Potassium chloride (20% w/v, pH 7.4) was added to 0.25 mg/mg of protein followed by the addition of 1 M potassium phosphate (pH 7.4) to 0.25 M/ml of protein solution. The solution was stirred on ice for 20 min followed by centrifugation at 78,000 x g for 1 h. The supernatant which contained the Fe-S flavoprotein was fractionated at pH 7.4 by the addition of solid ammonium sulfate. The fraction precipitating between 50 and 80% saturation was collected and dissolved in approximately 40 ml of 0.01 M Tris/HCl (pH 7.4) containing 1 M dithiothreitol and frozen overnight in liquid nitrogen (Fraction II, Table I). After thawing, it was dialyzed for 3 h in 1-cm (collapsed diameter) dialysis tubing in the same buffer as suspended and centrifuged for 1 h at 144,000 x g.

The supernatant (Fraction III, Table I) was applied to a column consisting of DEAE-Bio-Gel layered over Bio-Beads SM-2 and the protein eluted as shown in Fig. 1. Bio-Beads SM-2 were used to remove most of the nonionic detergent. The presence of Triton X-100 was necessary for successful elution of the Fe-S flavoprotein which appeared as a yellow fraction. The fractions were combined, treated with solid ammonium sulfate to 60% saturation, and centrifuged at 48,000 x 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>
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<tbody>
<tr>
<td>1 I. (mg)</td>
<td>(mg)</td>
<td>(mg)</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>1300</td>
<td>52,000</td>
<td></td>
</tr>
<tr>
<td>0.025</td>
<td>1250</td>
<td>49,500</td>
<td></td>
</tr>
<tr>
<td>0.025</td>
<td>1290</td>
<td>51,600</td>
<td></td>
</tr>
<tr>
<td>1 II.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.21</td>
<td>250</td>
<td>1,230</td>
<td></td>
</tr>
<tr>
<td>0.18</td>
<td>260</td>
<td>1,490</td>
<td></td>
</tr>
<tr>
<td>0.18</td>
<td>240</td>
<td>1,380</td>
<td></td>
</tr>
<tr>
<td>0.24</td>
<td>210</td>
<td>660</td>
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</tr>
<tr>
<td>0.22</td>
<td>245</td>
<td>1100</td>
<td></td>
</tr>
<tr>
<td>0.23</td>
<td>230</td>
<td>1030</td>
<td></td>
</tr>
<tr>
<td>1 IV.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.12</td>
<td>120</td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>11.5</td>
<td>140</td>
<td>10.9</td>
<td></td>
</tr>
</tbody>
</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%.

Properties of Fe-S Flavoprotein

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 stages 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 as the purification procedure was worked out. 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
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

![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."](image)

![Fig. 3. Anaerobic reductive titration of Fe-S flavoprotein with dithionite. Of protein solution, 0.450 ml in 10 mM Tris/HCl of pH 7.4 was used, containing 30 nmol of flavin and 112 nmol of iron. The values are plotted in terms of reducing equivalents recovered in the EPR signals, flavin semiquinone, and Fe-S signal (ordinate), versus reducing equivalents added in the form of dithionite, both with respect to the iron concentration in the protein. The symbol (O) indicates radical signal and the symbol (●) indicates Fe-S signal intensity. The dashed line shows the maximal total recovery of equivalents possible with the number of equivalents added.](image)

### Table II

<table>
<thead>
<tr>
<th>Flavin, iron, and labile sulfur content and EPR signal intensities of Fe-S flavoprotein at different states of purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavin I</td>
</tr>
<tr>
<td>Flavin IX₃</td>
</tr>
<tr>
<td>ng/mol protein</td>
</tr>
<tr>
<td>1a</td>
</tr>
<tr>
<td>1b</td>
</tr>
<tr>
<td>1c</td>
</tr>
<tr>
<td>2a</td>
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<tr>
<td>2b</td>
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<tr>
<td>3a</td>
</tr>
<tr>
<td>3b</td>
</tr>
<tr>
<td>3c</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.*
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 I (1-butanol/acetic acid) and 8 (t-butyl alcohol/ammonia) of Kilgour et al. (12), the flavin migrated with FAD and not FMN. The observed R_f 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 (flavoprotein), 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, dihydrocorotate, and choline did not show rates of cytochrome c reduction significantly exceeding those of the control.

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_1 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
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 absorption 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 be easily 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 O2, 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 of 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 final 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°C in a microcell attachment of a recording Beckman DU Spectrophotometer (UPate 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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