A NEW MEMBRANE IRON-SULFUR FLAVOPROTEIN OF THE MITOCHONDRIAL ELECTRON TRANSFER SYSTEM

THE ENTRANCE POINT OF THE FATTY ACYL DEHYDROGENATION PATHWAY?*

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SUMMARY: An iron-sulfur (Fe-S) protein has been purified from beef heart mitochondria by following its EPR signal after reduction, which is characteristic of a ferredoxin-type Fe-S protein ($g_x = 1.886$; $g_y = 1.939$; $g_z = 2.086$). The signal intensity corresponds to one unpaired spin for 4 to 5 Fe atoms. The light absorption spectrum indicates the presence of flavin. Fe, labile S, and FAD are released by acid at a ratio of approximately 4:4:1. Neither prosthetic group of the protein is reduced by NADH, NADPH, succinate, glycerol-3-phosphate or dihydro-orotate. The Fe-S group is, however, reduced with a half-time of ∿5 msec, when the protein is mixed with an equivalent amount of electron transferring flavoprotein (ETF) of the β-oxidation cycle, prereduced with an acyl CoA dehydrogenase and a saturated fatty acyl CoA. In the presence of the two added flavoproteins the behavior of the flavin of the Fe-S flavoprotein could not be determined. Complexes I-III are not reduced by reduced ETF under analogous conditions. The low field EPR resonance ["center 5", Ohnishi et al. (1972), Biochem. Biophys. Res. Commun. 46, 1631-1638] of the protein is readily observed in whole tissue, mitochondria and sonic fragments from all species we have examined. Therefore, the protein appears to be a universal constituent of mitochondrial electron transfer systems.

Most of the resonances observed in heart mitochondria by EPR spectroscopy at presently attainable sensitivities have been identified. Among those that are yet unidentified is a resonance present in the reduced state at $g = 2.086$ (width at half height ∿20G) which has properties of a low field component (g_z) of a ferredoxin-type signal. Changes in the intensity of this resonance go parallel with changes at $g = 1.89$. These resonances have been attributed to a "center 5" by Ohnishi et al. (1). The signal is not present in Complex I-III or in purified soluble preparations of NADH or succinate dehydrogenase. According to the observed signal intensity, the concentration of the corresponding component is of the order of that of the individual Fe-S centers of NADH

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dehydrogenase. We have now been able to purify a protein from mitochondria by cholate solubilization, ammonium sulfate fractionation and DEAE-sepharose chromatography, in which these resonances can be observed after reduction with dithionite. The complete EPR spectrum of this protein is typical of that of a Fe-S protein containing a component which can exist in a free radical state, such as, e.g., flavin or ubiquinone (Q). The protein was found to contain FAD but no detectable Q.

MATERIALS AND METHODS.

Beef heart mitochondria were prepared according to Crane *et al.* (2). All purification procedures were carried out at 0-4°. The yellow (general) fatty acyl CoA dehydrogenase and the electron transferring flavoprotein (ETF) were purified from beef heart by a modification of described procedures (3,4). n-Butyryl CoA was purchased from P-L Laboratories, Milwaukee, Wisconsin. Glycerol-3-phosphate, manufactured by Eastern Chemical Company, was a gift of Dr. T. P. Singer. L-Dihydroorotic acid was a product of Sigma Chemical Company.

EPR spectroscopy was carried out as described (5) and Fe (6), labile S (7) and flavin (8) were determined as in previous work. Fe was also determined without ashing after denaturation by sulfuric acid. Chromatography of flavins after deproteinization at 80° for 20 min was carried out according to Kilgour *et al.* (9). Q was determined spectrophotometrically after extraction (10). Disc electrophoresis was carried out according to Davis (11) utilizing 5% acrylamide separating gels with 0.5% Triton X100. SDS-polyacrylamide electrophoresis was according to Fairbanks *et al.* (12). Assays for glycerol-3-phosphate and choline dehydrogenases were performed according to Ref. (13).

RESULTS

Purification of Fe-S flavoprotein. Mitochondria were washed once in 0.25 *M* sucrose, 0.01 *M* Tris-HCl (pH 7.4) containing 1 m*M* dithiothreitol (DTT) and 1 m*M* succinate, and suspended in the same buffer to a protein concentration of 30 mg per ml. Potassium cholate (20% w/v, pH 7.6) was added slowly to a concentration of 0.25 mg per mg of protein. The mixture was stirred on ice for 20 min and centrifuged for 3 hrs at 78,000 x g. The pellet was resuspended to a protein concentration of 30 mg per ml with the above buffer. Solid potassium succinate was added to a concentration of 20 mM, the pH of the solution adjusted to 7.4, and the solution stirred on ice for 30 min. Then potassium cholate was added to 0.25 mg per mg protein followed by the addition of 1 M potassium phosphate (pH 7.4) to 0.25 ml per ml of protein solution. The solution was stirred on ice for 20 min followed by centrifugation at 78,000 x g for 2 hrs. The supernatant which contained the Fe-S protein was fractionated at pH 7.4 by the addition of solid ammonium sulfate. The fraction precipitated between 50 and 60% was collected, dialyzed for 3 hrs in 0.01 M Tris-HCl (pH 7.4) containing 1 mM DTT, and applied to a short (5 cm) DEAE-sepharose column equilibrated with the same buffer as used for dialysis. Following column application, the protein was washed with the above buffer and a stepwise Tris-HCl (pH 7.4) gradient was prepared. The fraction eluted between 0.1 M and 0.25 M Tris-HCl (pH 7.4), 1 mM DTT was collected and centrifuged at 40,000 x g for 30 min. The supernatant was fractionated with solid ammonium sulfate at pH 7.4. The fraction precipitated between 45 and 55% ammonium sulfate contained the Fe-S protein. Approximately 15 mg protein are obtained from 200 ml mitochondrial paste.

Polyacrylamide gel electrophoresis in the presence of SDS or Triton X100 showed one major band and several minor bands. A preparation obtained from sonicated mitochondrial fragments by a somewhat different procedure (preparation I, Table I) showed the same main component but different minor bands. Since analyses for flavin and Fe-S gave values practically identical to those obtained from the other preparations, it is likely that the major band is derived from the Fe-S flavoprotein. The molecular weight of this subunit was estimated to be approximately 70,000. If it is assumed that the Fe-S flavoprotein consists only of this subunit and the results of the chemical analyses are taken into account, the protein at the present stage is 25-30% pure.

TABLE I

ANALYTICAL DATA ON Fe-S FLAVOPROTEIN

Prep #	A	B	C	D	E			
	Fe	labile S	flavin	e^- in Fe-S signal	e^- in radical signal	$\frac{\text{Fe}}{\text{labile S}}$	$\frac{\text{Fe}}{\text{Flavin}}$	$\frac{\text{Fe}}{e^-_{(\text{Fe-S})}}$
	$\frac{\text{ngat}}{\text{mg protein}}$	$\frac{\text{ngat}}{\text{mg protein}}$	$\frac{\text{nmoles}}{\text{mg protein}}$	$\frac{\text{neq}}{\text{mg protein}}$	$\frac{\text{neq}}{\text{mg protein}}$	$\frac{A}{B}$	$\frac{A}{C}$	$\frac{A}{D}$
1	12.5	13.1	3.7	2.3	1.4	0.96	3.4	5.4
2	14.5	12.7	3.5	3.1	1.15	1.14	4.1	4.7
3	14.2	12.6	2.9	2.7	1.0	1.13	4.9	5.3
4	11.0	-	3.7	2.7	1.0	-	3.0	4.1

EPR spectrum. Fig. 1 shows the EPR spectrum, after reduction with dithionite, of the purified protein (g_x = 1.886; g_y = 1.939; g_z = 2.086. Double integration of the signal, after correction for the superimposed radical signal, indicates the presence of one unpaired spin for 4-5 Fe atoms (Table I). In the standard Varian rectangular cavity and at 13°K, saturation of the signal sets in at ∿ 25 μwatt. It is interesting that in the presence of an excess of dithionite the component represented in the free radical signal is not further reduced. The radical signal, integrated at 117°K and 90 μW of power, accounts for the presence of approximately one unpaired spin for every 3 molecules of flavin.

Light absorption spectrum. The absorption spectrum with a maximum at 380 nm and a shoulder at 440-480, which disappears on reduction, is suggestive of a Fe-S flavoprotein. Contamination by heme to a variable extent was evident in all preparations from sharp absorption lines at 417 nm (oxidized) and 425 nm (reduced). However, in terms of iron, this contamination amounts to <10%.

Figure 1. EPR spectrum (first derivative) of Fe-S-flavoprotein from mitochondria. The protein, 5 mg per ml, was dissolved in 10 mM Tris-HCl (pH 8), upper trace: protein as isolated; lower trace, reduced with solid dithionite. The conditions of EPR spectroscopy were: Microwave power, 270 μwatt; modulation amplitude and frequency, 8G and 9.2 GHz, respectively; temperature 13°K; and scanning rate 400 gauss per minute. The radical signal at g=2 is strongly saturated under these conditions.

Flavin component. On heating or acidification a flavin compound was released which migrated with authentic FAD and not FMN in systems 1 and 8 of Ref. (9). The material released by heat has the absorption spectrum shown in Fig. 2. We conclude from this that, in addition to a Fe-S center, FAD is bound to the protein. The stoichiometric relationships observed with four independent preparations are presented in Table I. Q was present at a level <0.05 nmoles per mg protein.

Tests for activity. Under anaerobic conditions the flavin or Fe-S groups of the purified protein are not significantly reduced* by NADH, NADPH, succinate, glycerol-3-phosphate or dihydroorotate. We did not detect any choline dehydrogenase activity. Glycerol-3-phosphate dehydrogenase activity was absent in 2 and marginal in a third preparation.

When the Fe-S flavoprotein was mixed anaerobically with ETF, acyl CoA dehydrogenase and butyryl CoA, the EPR signal of the reduced Fe-S group appeared to its maximal extent within 30 sec. When butyryl CoA or ETF and acyl CoA de-

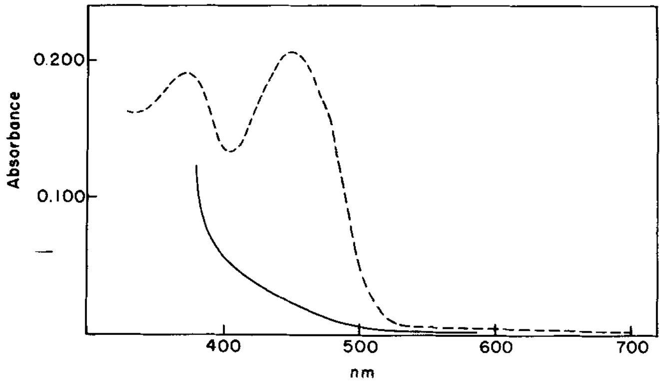

Figure 2. Absorption spectrum of pigment released from Fe-S-flavoprotein. Protein, dissolved as for Fig. 1, was heated to 80° for 20 min and then centrifuged. The spectrum was recorded directly on the supernatant (upper curve) and after addition of dithionite.

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* Under anaerobic conditions the Fe-S flavoprotein has a tendency toward autoreduction. This must be considered, when one wants to determine, whether the protein is reduced by other agents.

hydrogenase were omitted, no significant reduction occurred. With butyryl CoA and acyl CoA dehydrogenase, in the absence of ETF, 20% reduction occurred in 30 sec. This partial reduction is probably due to some contamination of the dehydrogenase by ETF, since in this experiment the dehydrogenase was not used in catalytic amounts (cf. legend to Fig. 3) but at a 1.5-fold excess over the Fe-S-flavoprotein, with respect to flavin. It was then demonstrated by rapid freeze-quenching that reduction of the Fe-S flavoprotein by the complete system, including ETF, is in fact much faster and occurs within milliseconds, as shown in Fig. 3. Neither Complex I nor Complex II was reduced by ETF, acyl CoA dehydrogenase and butyryl CoA under analogous conditions and Complex III was not reduced in 1 sec, but its Fe-S center and cytochrome c_1 were ∿ 70% reduced in 30 sec.

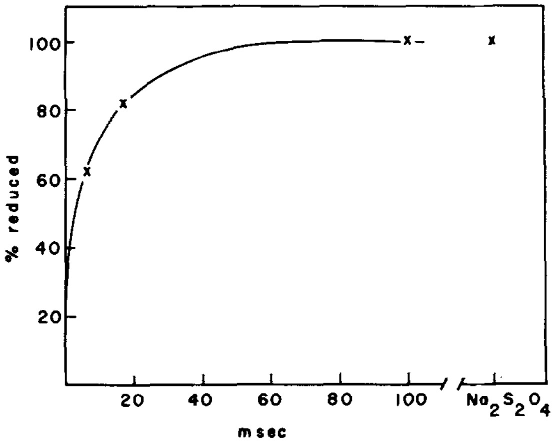

Figure 3. Time course of reduction of EPR signals of Fe-S-flavoprotein after mixing with ETF, acyl CoA dehydrogenase and butyryl CoA. ETF equivalent to 30 nmoles of flavin per ml was mixed with yellow fatty acyl CoA dehydrogenase equivalent to 6.7 nmoles of flavin per ml and made anaerobic in a tonometer. Butyryl CoA, 715 nmoles per ml were then added from a sidearm. The solution was drawn into a syringe. The concentrations given refer to the final volume, which was 1.8 ml. The buffer was 0.1 M Tris, pH 7.4. This solution was left at room temperature for 10 minutes and was then mixed with one fifth its volume of a solution of the Fe-S-flavoprotein containing 100 nmoles of flavin per ml. The ordinate shows % reduction of the Fe-S signal of the Fe-S-flavoprotein; 100% reduced refers to a sample of the protein reduced by dithionite and 0% reduced to a control sample of the protein mixed from the syringe with anaerobic Tris buffer instead of ETF etc. The behavior of the free radical signal was not followed as radical signals from all flavoproteins present may be superimposed.

DISCUSSION

The results presented above indicate that the protein purified from mitochondria is a hitherto unknown, membrane-bound Fe-S flavoprotein with one flavin (FAD) per Fe-S cluster. Although the analytical data suggest that the protein has 4 Fe and 4 labile S per flavin, the Fe/flavin ratios on two of the 4 preparations analyzed so far are sufficiently low that we would like to consider such a conclusion with reservation at the present state of purity of our preparations. We nevertheless, would like to present our data now, since at this stage the protein is free of EPR detectable contaminating Fe-S groups and since final purification of membrane-bound Fe-S proteins under preservation of activity is notoriously tedious.

In view of the presently achieved purity the question may be raised whether the flavin and Fe-S group are components of the same protein. The assumption, that this is indeed so, is supported by the simultaneous enrichment of Fe and flavin and the relatively consistent ratio of these 2 constituents in four separate preparations. Most convincing to us, however, appears the comparison of results of SDS polyacrylamide electrophoresis of preparations made by different procedures. One preparation, obtained by fractionation of sonic fragments of mitochondria, showed different impurities than those prepared from mitochondria directly, and those impurities that were present in both types of preparations occurred in different proportions. There is thus little chance for a separate flavoprotein occurring as major component in both types of preparations at the same ratio to the Fe-S protein, as indicated in Table I.

We have paid particular attention to the question whether this protein could be membrane-bound glycerol-3-phosphate dehydrogenase, an enzyme known to be present in heart mitochondria. This enzyme is thought to contain iron, flavin or both (14,15), but has not been thoroughly characterized. Since we did neither find reduction of the Fe-S center of our purified protein by glycerol-3-phosphate nor significant activity in assays, we conclude that glycerol-3-phosphate is not the substrate for this protein. Considering the function of the new Fe-S flavoprotein, we must remember that the majority of the components of the electron transfer system of mitochondria do not have a substrate of low molecular weight but are electron carriers between proteins. The observations reported above indicate that the Fe-S flavoprotein which we describe in this report may be the natural link between the fatty acyl dehydrogenation pathway and the electron transfer system. It seems surprising that nature should utilize here for electron transfer a relay system of three flavoproteins. Perhaps it is the Fe-S character of the last one in the series, which is necessary for efficient communication with the system which might not be readily accomplished by a metal-free flavoprotein.

According to Ohnishi *et al*. (1) the behavior of the EPR signal of "center 5" (i.e., of the Fe-S flavoprotein we describe here) on oxidation-reduction in submitochondrial particles from pigeon heart indicates an apparent midpoint oxidation-reduction potential of 40 mV at pH 7.2. In submitochondrial particles it is reduced after addition of NADH or succinate, indicating that it equilibrates with the corresponding dehydrogenase systems. According to our observations that the protein may be a contaminant in soluble NADH dehydrogenase preparations (16) it is likely that the FAD originating from this protein led to the difficulties in identifying the flavin component of NADH dehydrogenase (17). According to a survey of whole tissue and mitochondrial preparations from mammalian heart of various species which we have made (18), the concentration of the protein is particularly low in beef heart and relatively high in pigeon heart and breast muscle. These differences are, however, not much larger than 2-fold.

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