A MITOCHONDRIAL IRON PROTEIN WITH PROPERTIES OF A HIGH-POTENTIAL IRON-SULFUR PROTEIN*

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SUMMARY: A brown, soluble protein which, in its oxidized form, has a highly temperature sensitive EPR signal centered at g=2.01 with a width of 25 gauss at 9.2 GHz, was purified from the supernatant of sonicated beef heart mitochondria to apparent homogeneity by criteria of protein chemistry. No other signals were observed in the oxidized or reduced state. Two g atoms each of iron and labile sulfur were found per mole (MW 97,000). Signal intensity and reductive titration account for 3 electrons per molecule. The protein is labile and autooxidizable. In view of the stoichiometric presence of iron and labile sulfur and the magnetic properties, it is proposed that this protein belongs to the class of high-potential iron-sulfur proteins. Heterogeneity in the state and distribution of iron-sulfur groups, but not at the level of gross protein structure, is assumed to explain the observed stoichiometry. On fractionation of mitochondria an EPR signal similar to that of the purified protein is concentrated in succinate-ubiquinone reductase. Aspects of the function of the corresponding substance in electron transfer are dealt with in a companion report (Beinert, Ackrell, Kearney, and Singer, Biochem. Biophys. Res. Commun. this issue).

In a number of laboratories (1-6) studying mitochondria or microorganisms an EPR signal has been observed, which is centered at g=2.01 with a peak to peak width of $\sim$ 25 gauss, is detectable only at <25°K and is characteristic of the oxidized state. The behavior of this signal on oxidation-reduction indicates that it represents a component, which is in equilibrium with known electron carriers, either by exchanging electrons or by changing its state such that the EPR signal is affected. Some high spin ferric complexes of high symmetry may show narrow signals at g $\sim$ 2 (7) and in view of the described properties of the material, it was thought that the signal may originate from an iron compound (1). Signals from ferric complexes of this type should, however, be detectable at room temperature. Highly temperature sensitive EPR signals at g $\sim$ 2, representing an oxidized state, have, however, been observed

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with the high potential type of iron-sulfur (Fe-S) protein (Hipip) (8, 9). To date Hipip has only been found in three strains of bacteria (10). If the unknown signal were due to a Hipip-type of Fe-S structure, the presence of labile S, stoichiometric with Fe should be demonstrable. Since numerous Fe-S proteins of the ferredoxin type are present in the source materials, purification of the unknown material is necessary to decide this.

On fractionation of mitochondria signals of the type described are mainly recovered in Complex II (11) and in the supernatant. Considerations of solubility made us choose the latter source for purification. While this work was in progress, DerVartanian et al. (5) showed by substitution of ^{57}Fe that a similar signal in azotobacter does indeed originate from Fe.

MATERIALS AND METHODS.

Beef heart mitochondria were prepared according to Crane et al. (12). All purification procedures were carried out at 0-4°. EPR spectroscopy and anaerobic titrations were carried out and the data were evaluated as described (2). The molecular weight was estimated by three independent techniques: approach to equilibrium in the ultracentrifuge, gel filtration with Sephadex G200, and sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis. Disc electrophoresis was carried out according to Davis (13) and SDS-polyacrylamide electrophoresis according to Fairbanks et al. (14). Fe and labile S were determined as in previous work (15).

RESULTS.

Mitochondria were washed twice in 0.25 M sucrose, 0.01 M Tris-chloride (pH 7.4) containing 1 mM dithiothreitol (DTT), suspended in the same buffer, containing in addition 1 mM succinate, and frozen in liquid nitrogen. After thawing the protein concentration was adjusted to 50 mg per ml with the same solution. Aliquots, 150 to 200 ml, were treated at 90 watts with a Branson sonifier four times for 1 min in the cold. The suspensions were centrifuged at 78,000 x g for 2 hrs. The typical EPR signal was found largely in the

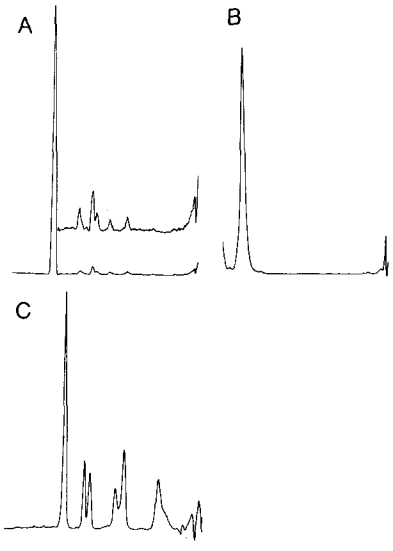

FIGURE 1. Optical scans at 560 nm of polyacrylamide gels stained with Coomassie Blue following electrophoresis of soluble Fe-S protein and Complex II. Protein added per tube and current requirements were: SDS-polyacrylamide electrophoresis, 1.5 µg soluble Fe-S protein (A, enlarged portion x 3.3); 5 µg Complex II, 5 mA per tube (C); disc polyacrylamide electrophoresis, 3 µg soluble Fe-S protein, 2 mA per tube (B). The cathode is to the left.

supernatant. This solution was passed through a TEAE cellulose column, equilibrated with 0.01 M Tris chloride (pH 7.4) containing 1 mM DTT. The effluent containing the signal was immediately passed through a column of CM-cellulose equilibrated with the same solution. The effluent containing the signal was concentrated under nitrogen to 10 ml. Approximately 600 mg protein were recovered from 200 ml of mitochondrial paste. This solution was dialyzed for 3 hrs against 500 ml of 8 mM potassium phosphate buffer of pH 7.4 containing 1 mM DTT and then applied to a column of CM-Sephadex C50 equilibrated with the same solution. The material containing the signal was held by this column and was then eluted by the use of a linear gradient produced by equal volumes each of 8 mM and 25 mM potassium phosphate of pH 7.4 containing 1 mM DTT. The material containing the signal is eluted between 10 and 22 mM phosphate. The eluate was concentrated and stored in 0.01 M Tris chloride (pH 7.4), 1 mM DTT, frozen in liquid nitrogen. The yield of Fe-S protein from 200 ml paste at this stage was 50-75 mg. Speed in processing the material is

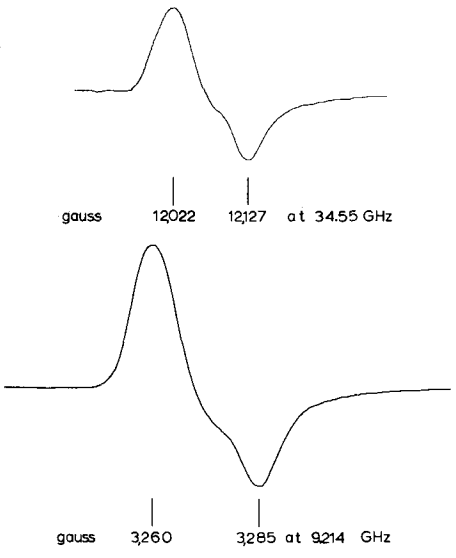

FIGURE 2. EPR spectra at 34 and 9 GHz of the oxidized form of the purified Fe-S protein from beef heart mitochondria. Protein, 15 mg per ml, was dissolved in 0.05 M Tris chloride of pH 7.4. The conditions of EPR spectroscopy were at 34.55 and 9.214 GHz, respectively. Power, 10 mwatt and 10 μwatt; modulation amplitude, 3.2 and 4 gauss; scanning rate 400 and 100 gauss per minute; temperature 13°K and time constant 0.5 sec in either case.

essential as the protein exhibiting the signal is labile loosing Fe and labile S readily.

Figure 1 shows that neither by the conventional method of polyacrylamide disc gel electrophoresis at pH 8.9 (B) nor by the method incorporating SDS (A) was there any significant heterogeneity in the preparation as described. This agrees with the results of analytical ultracentrifugation. An optical scan of the SDS-gel of Complex II (C) is included for comparison. No protein of molecular weight similar to that of the soluble species was found in Complex II. Estimates of the molecular weight of the purified protein indicate that it is a relatively large protein, MW $\sim$ 100,000.

The EPR signal at 9.2 and 34 GHz of the purified protein is shown in Fig. 2. The splitting (shoulder) in the center of the signal indicates that the signal may be due to the superposition of signals from two species. This is supported by the spectrum observed under rapid passage conditions

TABLE I

Properties of a Soluble Fe-S Protein From Beef Heart Mitochondria

Preparation #	A	B	C	D	E	F	G	H	I
	Fe	$S^=$	e^- in signal	e^- Required for Reduction	Fe / e^- A/C	Fe / e^- A/D	Fe / Mole†	e^- / Mole† from C	e^- / Mole† from D
	ngat or neq per mg dry weight								
1	20.2	-	7.5	-	2.7	-	1.96	0.73	
3	21.9	24.5	7.6	8.5	2.9	2.6	2.12	0.74	0.82
5,6	22.7	21.2	6.7	7.3	3.4	3.1	2.20	0.65	0.71
7	20.2	26.5	6.9	7.6	2.9	2.7	1.96	0.67	0.74
9*	26.6	28.3					2.58		
10	20.2	26.0	6.9		2.9		1.96	0.67	
11*	18.5	21.3					1.79		

*Anaerobic preparations

†Based on a MW of 97,000

where the envelopes of two signals are separated and by the observation that during titrations the low field species is reduced before the high field one.

The signal of the purified protein is not as temperature-sensitive as that seen in particles or whole tissue and can be observed up to $\sim$ 50°K. The temperature sensitivity appears to be due to relaxation phenomena only, i.e., the signal behaves down to 1.5°K in a manner compatible with that of a spin 1/2 system. No other signals were observed in the oxidized or reduced forms from 1.5° or 6°K upwards, respectively.

The protein is brown and is partly bleached on reduction. Similar to the light absorption spectra of bacterial Hipip the spectrum of the oxidized protein has shoulders at 400 and 460 nm and the reduced protein at 370-400 nm. The absorptivities on an Fe basis are also similar.

The protein is sufficiently autooxidizable so that titrations have to be performed anaerobically. It is not reduced by ascorbate or DPNH plus phenazine methosulfate. In titrations with dithionite the protein was capable of taking up one electron for approximately 3 Fe. Double integrations of EPR spectra of the fully oxidized species yielded similar values for the concentration of unpaired spins relative to Fe. These relationships are summarized in Table I.

DISCUSSION.

The principal facts in support of our contention that the iron compound in question is an Fe-S compound of the Hipip type are the following: 1) Fe and labile S occur at a stoichiometric ratio in the purified protein; 2.) The protein shows a signal, i.e., is paramagnetic, in the oxidized state and has no signal when reduced; 3.) The EPR signal at g=2.01 is the only signal observed in the oxidized or reduced state, indicating that there are no EPR detectable Fe-S proteins of the ferredoxin type present, which contain the Fe and labile S. 4.) The light absorption spectrum of the purified protein shows features of the spectra of the known Hipip type Fe-S proteins.

We use the term "high potential" in this context to indicate a certain structural type of Fe-S protein, without implying that the purified protein has or ought to have a particular redox potential. The redox potential of the purified protein was not determined, but estimates of the mid-point potential of the corresponding material in particles indicate that the material is not homogeneous and has components in the potential range from -60 to + 150 mVolt (4). The titration behavior of the component with the signal at g=2.01 in Complex II (16) indicates that its potential lies at $\geq$ 0 volt.

In analogy to the work on bacterial Hipip (8-10) which contains a 4-Fe center (17) one would have expected to find 4 Fe per molecule and a relationship of one electron taken up for every 4 Fe atoms present. Despite attempts to eliminate possible deficiencies in our purification or analytical procedures, we have consistently found the stoichiometry reported in Table I. If differences in source material, deterioration of the protein during isolation or

analytical work, or random errors in our determinations had played a significant role, we would have expected to find a wider scatter of data. Preparations from fresh mitochondria, or completed within 24 hrs were not significantly different.

Although conventional criteria of protein chemistry showed no significant heterogeneity, heterogeneity of a more subtle nature may exist. The EPR spectra indicate that there are two species in approximately equal quantities, which have somewhat different redox potentials.

Heterogeneity at the level of Fe-S groups not necessarily expressed in gross protein structure could explain our findings. We consider the following possibilities, which, in combination among each other and with a certain latitude of error in the various determinations entering the stoichiometric relationships, could be responsible for the observed stoichiometries: (1) admixture of apoprotein; (2) presence of 2-Fe-S as well as 4-Fe-S centers (3) admixture of reducible or non-reducible EPR undetectable Fe-S centers (4) reducible groups other than Fe-S. It should also be considered that the EPR signal of Hipip is physically not understood and that the assumption of a spin 1/2 system rests on empirical grounds (18).

Our work raises the question whether Hipip type structures with 2-Fe-S centers are possible, although, if heterogeneity is assumed as outlined above, this is not required by our data.

It is not possible at this time to say whether there are more than one Hipip species present in mitochondria and what exactly the relationship is of the protein we have purified to that or those species in mitochondria. Some aspects of the function of the mitochondrial Hipip are dealt with in the companion report (16).

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