AN IRON SULFUR PROTEIN IN THE MITOCHONDRIAL OUTER MEMBRANE, REDUCIBLE BY NADH AND NADPH

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Summary

On addition of NADH or NADPH to the mitochondrial outer membrane fraction from rat liver, an electron paramagnetic resonance (EPR) spectrum is observed which is characteristic of a protein, containing an iron-sulfur center. The g-values are 2.01, 1.94 and 1.89. Quantitation of the EPR absorption and analysis of the acid labile sulfur content suggest that the paramagnetic center contains two iron and two acid labile sulfur atoms. The concentration of the center in the outer membrane is about 0.5 nmoles/mg protein.

Introduction

On addition of NADPH or $Na_2S_2O_4$ to the rat kidney cortex microsomal fraction, the presence of an iron-sulfur protein was revealed by EPR spectroscopy (1). From the g-values and the absence of sensitivity towards rotenone this center appeared distinct from all known iron-sulfur centers found in the mitochondrial inner membrane. In fact the best resolution of the EPR spectrum was obtained when the respiratory chain inhibitors rotenone or thenoyltrifluoroacetone were present. The improved resolution in these experiments was interpreted as a result of inhibited reduction of succinate dehydrogenase impurities.

Further experiments showed that treatment of whole rat kidney cortex mitochondria with NADPH and rotenone could give rise to the same iron-sulfur spectrum as seen in rat kidney cortex microsomes. This prompted us to make a closer examination of the mitochondrial outer membrane.

Materials and Methods

Fractionation of rat livers

Male Spraque-Dawley rats were killed by decapitation and the livers

were removed and placed in ice-cold, 0.25 M sucrose. The livers were cut into small pieces and homogenized in four volumes of 0.25 M sucrose. The liver homogenate was fractionated by differential centrifugation. The first fraction was collected at 500 x g for 10 min. The pellet was saved and the supernatant was centrifuged at 4500 x g for 20 min; the mitochondria were collected in the precipitate. The mitochondria were washed twice with 0.25 M sucrose and resedimented at 4500 x g. The supernatant from the first centrifugation at 4500 g was spun at 17,500 x g for 15 min and the pellet was saved. The supernatant was centrifuged at 123,000 x g for 60 min and microsomes were collected in the pellet and washed once with 50 mM TRIS-Cl buffer, pH 8.0. The first supernatant at 123,000 x g was collected and concentrated in a collodion bag.

Preparation of mitochondrial outer membrane

Mitochondrial outer membrane was prepared essentially according to the method of Parsons and Williams (2). A 15 ml solution of mitochondria containing about 100 mg protein per ml was suspended slowly in 230 ml of 20 mM phosphate buffer, pH 7.2, containing 0.02% bovine serum albumin. After swelling for 20 min, the released outer membrane was concentrated by differential centrifugation. The suspension was first spun for 20 min at 35,000 x g. The supernatant was saved and concentrated in a collodion bag as the intermembrane fraction. The pellet was resuspended for another 20 min period in 230 ml swelling medium. The swollen inner membrane was collected at 1900 x g for 15 min and saved as a crude inner membrane fraction. The supernatant was centrifuged at 35,000 x g for 20 min and the pellet containing the outer membrane was suspended in a small volume of 0.25 M sucrose; the final concentration was 25 mg protein per ml.

The different fractions were characterized by their EPR spectra at 77 K using a Varian V-4502 spectrometer with 100 kHz field modulation. The g-values were determined from simultaneous measurements of the magnetic field and the microwave frequency by means of a simple proton resonance field meter and a frequency counter (Hewlett-Packard 5245L and 5255A, respectively).

Monoamine oxidase was used as a marker enzyme (3) for the mitochondrial outer membrane and was assayed according to Tabor et al. (4) at 30°C by observing the formation of benzaldehyde from benzylamine. The final assay mixture of 3 ml was made up in a 1 cm light path cuvette and contained 0.067 M phosphate buffer, pH 7.2. The reaction was started by adding 10 μmoles of benzylamine. The increase of absorbance at 250 nm was measured on a Cary 14R spectrophotometer.

The concentration of the iron-sulfur protein was determined by double

integration of its EPR spectrum and compared to a Cu(II)-EDTA standard solution after g-value correction (5). Acid labile sulfur was determined according to King and Morris (6). Protein concentration was determined by both the biuret (7) and Lowry (8) methods.

Results and Discussion

The relative concentration of iron-sulfur protein, cytochrome P-450, and monoamine oxidase in the fractions from the liver homogenate and mitochondrial subfractions is seen in Table I.

The amplitudes at g = 2.25 were taken from spectra of untreated samples and represent a relative measure of the cytochrome P-450 concentration in the various fractions, provided no major change of the high-spin low-spin equilibrium occurs in P-450. The amplitudes at g = 1.94 and g = 1.89 were obtained after the addition of NADPH to a final concentration of 10 mM and a reaction time of 3 min. The intensity at g = 1.94 is a relative measure of the total amount of several Fe-S proteins, whereas the amplitude at g = 1.89 measures the amount of the new Fe-S protein alone (1).

EPR spectra from whole mitochondria and from the mitochondrial subfractions after addition of NADPH are seen in Fig. 1. Fig. 2 shows the development of the EPR signal from the mitochondrial outer membrane after addition of different reducing agents.

The upper portion of Table I shows that the EPR signal at g = 1.89 does not follow the strong signal at g = 2.25 of P-450 in the microsomes, but is most intense in the mitochondria. The last two columns of Table I further demonstrate that there is a strong correlation between the amplitude at g = 1.89 and the monoamine oxidase activity. This is strong evidence that the Fe-S protein with g = 1.89 is located in the outer membrane of the mitochondria.

In Fig. 1 the top spectrum shows that the g = 1.89 signal can be distinguished also in whole mitochondria. The amplitude at g = 2.01 does not only originate from the Fe-S protein of the outer membrane. Some contribution must also come from free radicals with the midpoints of their derivative spectra centered on the high field slope of the g = 2.01 signal. This is particularly evident from the large negative excursion in the spectrum of the crude inner membrane.

The spectrum of the intermembrane fraction is devoid of the Fe-S signal. The weak resonances which are observed are probably saturated and overmodulated radical signals and Mo signals from sulphite oxidase (9).

The outer membrane fraction shows, in addition to the Fe-S absorption, a signal at g = 2.25 which probably is from P-450. Whether this is due to microsomal impurities or belongs to the outer membrane is presently not known.

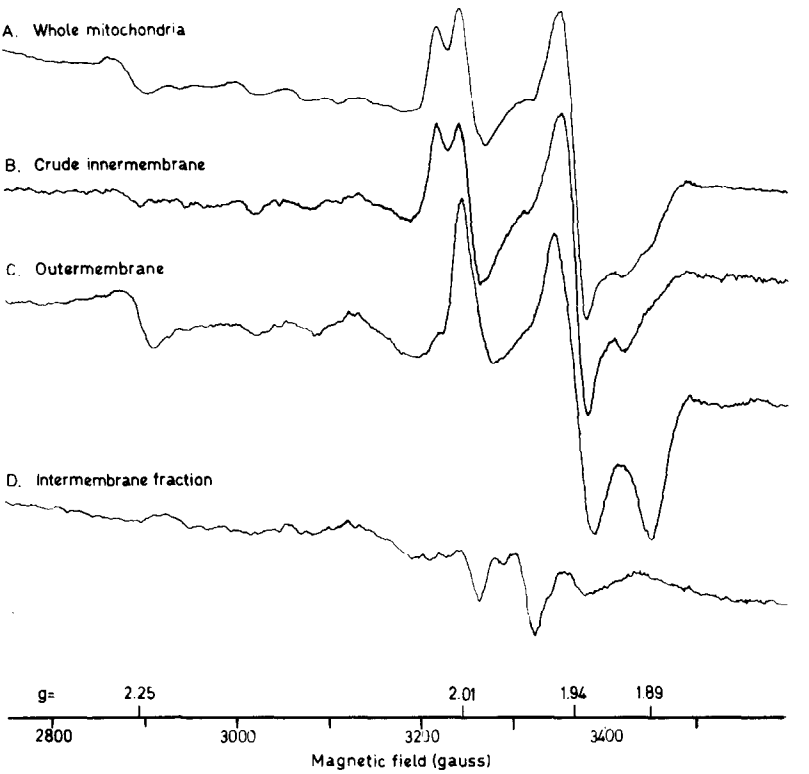

Fig. 1 EPR spectra from mitochondria and mitochondrial subfractions at 77 K after reduction with NADPH to a final concentration of 10 mM.
A. Whole mitochondria, 105 mg protein per ml.
B. Crude inner membrane fraction, 40 mg protein per ml.
C. Mitochondrial outer membrane, 24 mg protein per ml.
D. Mitochondrial intermembrane fraction, 21 mg protein per ml.
The sample tubes had 3.5 mm inner diameter, and the sample volume was 0.2 ml. EPR conditions were: microwave frequency, about 9.0 GHz; time constant, 1 sec; scanning rate, 100 G min^{-1}; microwave power, 100 mW. The gain of spectrum A is half that of the other spectra.

Fig. 2B shows that the outer membrane preparation contains only minor succinate reduced impurities from the inner membrane. From the lower spectra it can be seen that the Fe-S protein of the outer membrane is reduced by either NADPH or NADH.

The EPR spectrum of the reduced outer membrane is very similar to that of a reduced iron-sulfur protein. The g-values were determined to be 2.008, 1.937 and 1.891 ± 0.004.

The number of paramagnetic centers, determined by the double integration of the EPR spectrum of the reduced outer membrane and assuming $S_{eff} = 1/2$, was 0.52 ± 0.05 nanoequivalents per mg protein. The content of acid labile sulfur was determined to be 1.4 ± 0.1 nanomoles per mg protein.

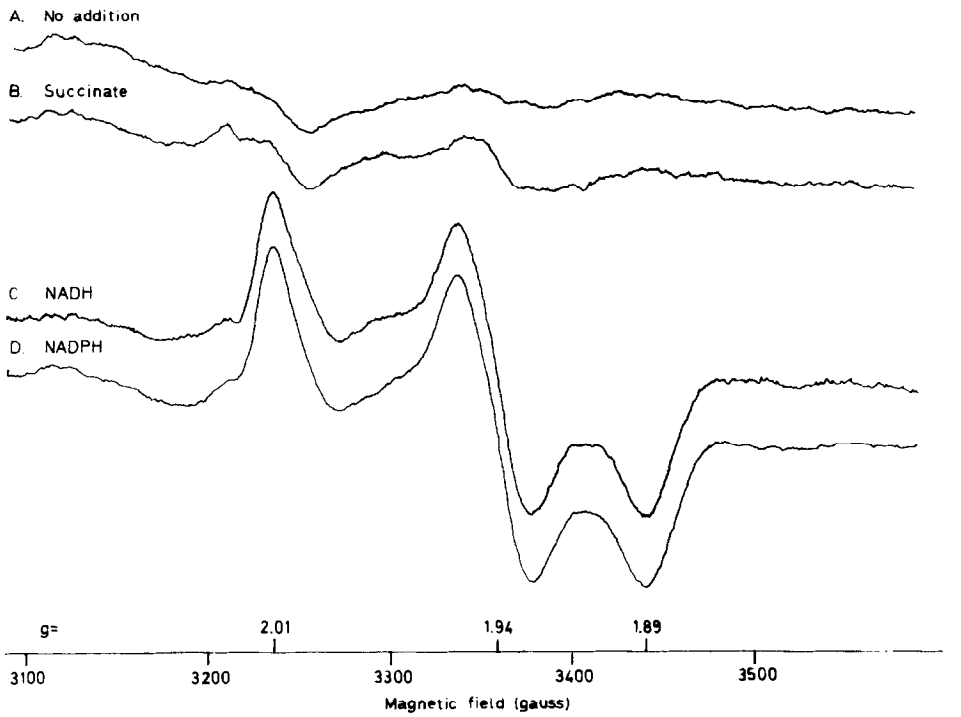

Fig. 2 EPR spectra from mitochondrial outer membrane, 24 mg protein per ml at 77 K.
A. After no addition.
B. After addition of succinate disodium salt to a final concentration of 50 mM.
C. After reduction with NADH to a final concentration of 10 mM.
D. After reduction with NADPH to a final concentration of 10 mM.
The EPR conditions were the same as in Fig. 1, spectra B - D.

Iron-sulfur proteins that give EPR spectra of the type obtained here may contain centers with 2 or 4 Fe atoms and the equivalent number of acid labile S. The experience is that each center may take up one electron and then give S_{eff} = 1/2 EPR absorption. Hence the ratio of EPR intensity to acid labile sulfur equivalents should in the two cases be 0.5 and 0.25, respectively. In our case the experimentally determined amount of acid labile sulfur is an upper limit since there might be some Fe-S protein impurities which do not give an EPR signal under the conditions used. Hence the ratio between EPR intensity and acid labile sulfur must be > 0.37. The new Fe-S protein described here thus most probably contains a center with 2 Fe and 2 labile S.

The function of the Fe-S protein described is at present unknown. Experiments are under way in this laboratory to test whether the Fe-S protein is linked to the rotenone-insensitive NADH-cytochrome c reductase or to the monoamine oxidase activity of the outer membrane. Preparations of the latter are known to contain considerable amounts of very tightly bound iron (10).

Table I

Fraction	EPR signal heights at g-values 2.25	1.94	1.89	Specific activity of monoamine oxidase units/mg protein
Ratliver				
500 x g pellet	26	46	6	15
Mitochondria	13	134	29	47
17,500 x g pellet	64	45	14	21
Microsomes	237	35	10	10
123,000 x g supernatant	0	0	0	0
Mitochondria				
Crude inner membrane	9	153	18	28
Outer membrane	52	259	111	391
Intermembrane	0	22	0	0

All EPR signal heights were measured in mm from single sample scans relative to base line scan, normalized to the same instrumental gain, and corrected for differences in protein concentration.

Specific activity figures are averages of two determinations with less than 10% difference.

Preliminary experiments have shown that purified monoamine oxidase does not give rise to an EPR signal corresponding to the mitochondrial outer membrane Fe-S protein.

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