TWO IRON–SULFUR CENTERS IN MITOCHONDRIAL OUTER MEMBRANES FROM BEEF HEART AS PREPARED BY FREE-FLOW ELECTROPHORESIS

Hans-G. HEIDRICH, Simon P. J. ALBRACHT+ and Dan BÄCKSTRÖM*

Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, FRG, +Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands and **Department of Biophysics, University of Stockholm, Arrhenius Laboratory, S-106 91 Stockholm, Sweden*

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1. Introduction

Mitochondrial outer membranes from rat liver were shown [1] to give rise to an EPR signal analogous to that of [2 Fe–2 S] centers, when NADH, NADPH or dithionite was added. As 2 Fe and 2 acid-labile S atoms were found per free spin, the presence of a [2 Fe–2 S] center in these outer membranes was postulated [1]. A similar EPR signal in preparations enriched in outer membranes from beef-heart mitochondria was found [2,3]. Recently a Fe–S protein with similar EPR properties has been partially purified from beef-kidney cortex mitochondrial outer membranes [4].

On close inspection of the line shape of the EPR signal, we felt that the signal could not be due to a single $S = \frac{1}{2}$ system. We report here the results of an EPR lineshape analysis using high-purity outer membranes from beef-heart mitochondria, which were isolated by means of free-flow electrophoresis [5]. The isolation and the properties of these membranes are also reported. The EPR line shape of the beef-heart mitochondrial outer membranes was compared with the line shape of the partially purified Fe–S protein from the outer membranes of beef-kidney cortex mitochondria [4].

2. Materials and methods

2.1. *Preparation of beef-heart mitochondria*

Fresh beef hearts were freed from fat and connective tissue and ground in a meat grinder. To 1 part of ground meat was added 0.5 part of isolation medium (10 mM triethanolamine, 10 mM acetic acid and 0.3 M sucrose adjusted to pH 7.4 with 2 M NaOH) and 0.5 part of isolation medium (pH 10.5). The suspension was kept at 4°C and mixed 3 times for 10 s in a blender, then adjusted to pH 7.4. The mixture was then diluted with 5 parts of isolation medium (per 1 part meat) and centrifuged for 10 min at 500 × *g*. The supernatant was decanted through a double layer of cheese cloth and then centrifuged for 15 min at 13 200 × *g*. The pellet was resuspended in isolation medium and this suspension was first centrifuged for 10 min at 500 × *g* and then for 15 min at 13 200 × *g*. The pellet of this last step, containing the mitochondria, was washed once with isolation medium then assayed for total protein.

2.2. *Preparation of outer membranes*

Swelling and shrinking of the mitochondria were performed as in [6]. The pellet obtained after centrifugation for 30 min at 50 000 × *g* was suspended in isolation medium. The suspension (150 mg total mitochondrial protein in 100 ml medium) was first centrifuged for 10 min at 15 000 × *g*, then for 30 min at 50 000 × *g*, and finally for 20 min at 148 000 × *g*. The pellets of the last two runs were combined and suspended in the isolation medium (without sucrose). The suspension of crude outer membranes thus obtained was layered on top of an aqueous 25% sucrose solution (w/w) and centrifuged for 12 h at 64 000 × *g* in a swing-out rotor. The

resulting white layer at the entrance of the gradient was washed twice with the isolation medium, resuspended in 2.5 ml and then injected into a FFV-apparatus (Bender and Hobein, Munich). The fractions were tested for total protein and for activities of cytochrome *c* oxidase (EC 1.9.3.1), monoamine oxidase (EC 1.4.3.4) and NADPH-cytochrome *c* reductase (EC 1.6.2.3) as in [2,7]. Outer membrane protein (4–5 mg) was obtained from 4–5 g mitochondrial protein, starting from 2.7 kg beef heart.

The Fe–S protein from beef-kidney cortex mitochondrial outer membranes was prepared as in [4].

EPR spectra were recorded on a Varian E-3 EPR spectrometer equipped with a 60 dB microwave attenuator or on a Varian V-4502 apparatus. Cooling of the sample was performed with a flow of cold nitrogen gas. The digitizing and simulation of EPR spectra were as in [8].

3. Results and discussion

3.1. *Preparation of mitochondrial outer membranes from beef heart by free-flow electrophoresis and their characterization*

The mitochondrial outer-membrane preparation from beef heart used in this study was a high-purity preparation obtained by using a combination of differential and density sedimentation techniques with free-flow electrophoresis. As has been shown [2,7,9] by means of enzyme analyses and electron microscopy such membrane fractions are homogeneous. This was also true for the mitochondrial outer membranes of beef heart used here. Fractions 21–27 from the electrophoresis runs (fig.1) contained no inner-membrane matrix enzyme and no endoplasmic reticulum enzymes (not shown). Mitochondrial contamination was removed by density sedimentation, that of inner-membrane matrix particles by free-flow electrophoresis. The high specific activity of monoamine oxidase and the very low activity of cytochrome *c* oxidase in the outer-membrane fraction are shown in table 1. The electron micrographs (fig.2) also show only outer membranes and no contamination with other particles. It should be pointed out that the isolation procedure here was designed to obtain homogeneous high-purity outer membranes and that less stress was laid on the yield.

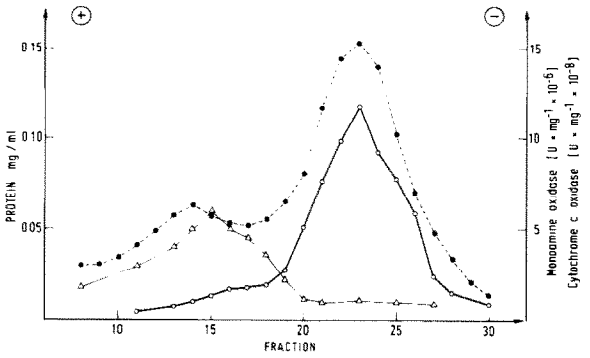

Fig.1. Free-flow electrophoresis of crude outer membranes from the density sedimentation run. The conditions in the FFV-apparatus were: 150 mA, 133 V/cm, buffer flow 2.25 ml/fraction/h, sample injection 1.5 ml/h above fraction 65, T = 4°C. 90 fractions were collected at 0°C. Protein distribution is (●- - -●). The left peak contains cytochrome *c* oxidase activity (△——△) and represents inner-membrane matrix particles. The right peak has a high activity of monoamine oxidase (○——○) and contains the mitochondrial outer membranes.

3.2. *EPR spectra*

In fig.3 it can be seen that the line shape of the signal as present in purified beef-heart mitochondrial outer membranes is exactly the same as that from the partially purified preparation of the Fe–S protein from beef-kidney cortex mitochondrial outer membranes. The shape in the $g = 2$ region is power-dependent, however, as a small radical signal is superimposed [4]. This is not seen from the spectra of fig.3 as the positive part of the radical signal coincides with the top of the line at $g = 2.007$ and the negative part of the radical signal lies under the top of the line at $g = 1.996$. A power plot (fig.4) makes this clear.

Table 1
Monoamine oxidase and cytochrome *c* oxidase activities in the outer-membrane fractions used for the EPR measurements

Fraction	Monoamine oxidase	Cytochrome *c* oxidase
Beef-heart mitochondria	0.22	0.8
Outer membrane	9.1	0.02

Activities are expressed in μmol/min/mg protein

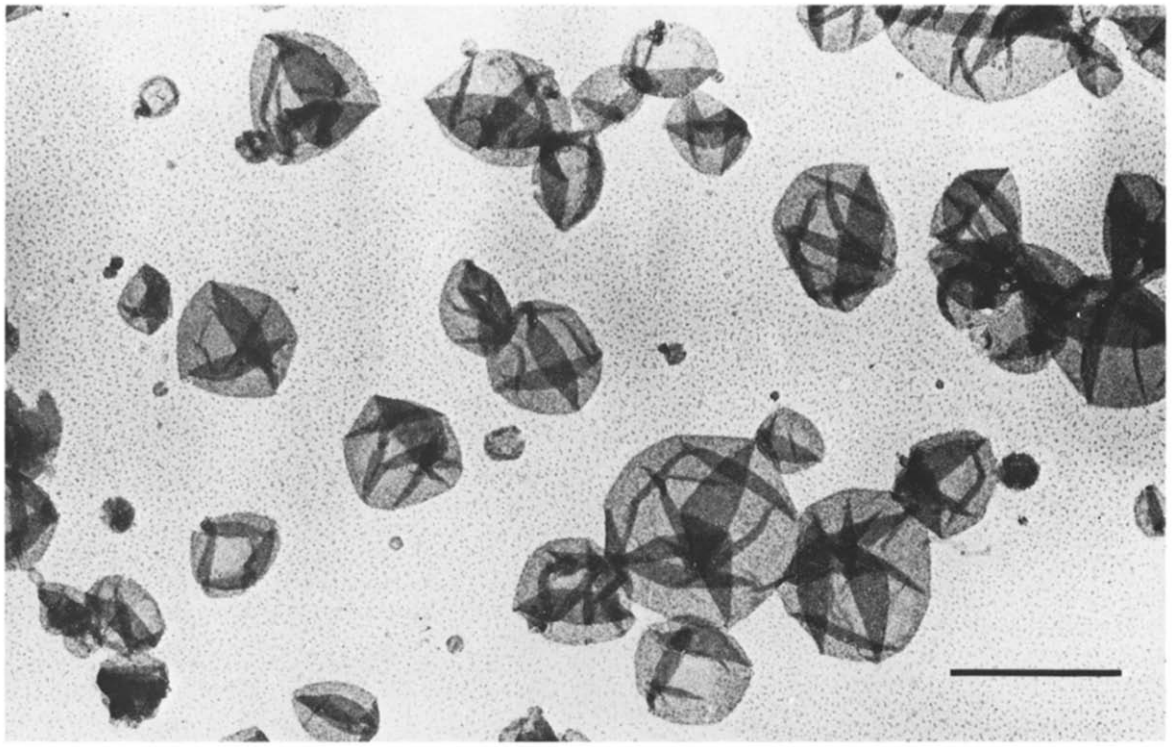

Fig.2A

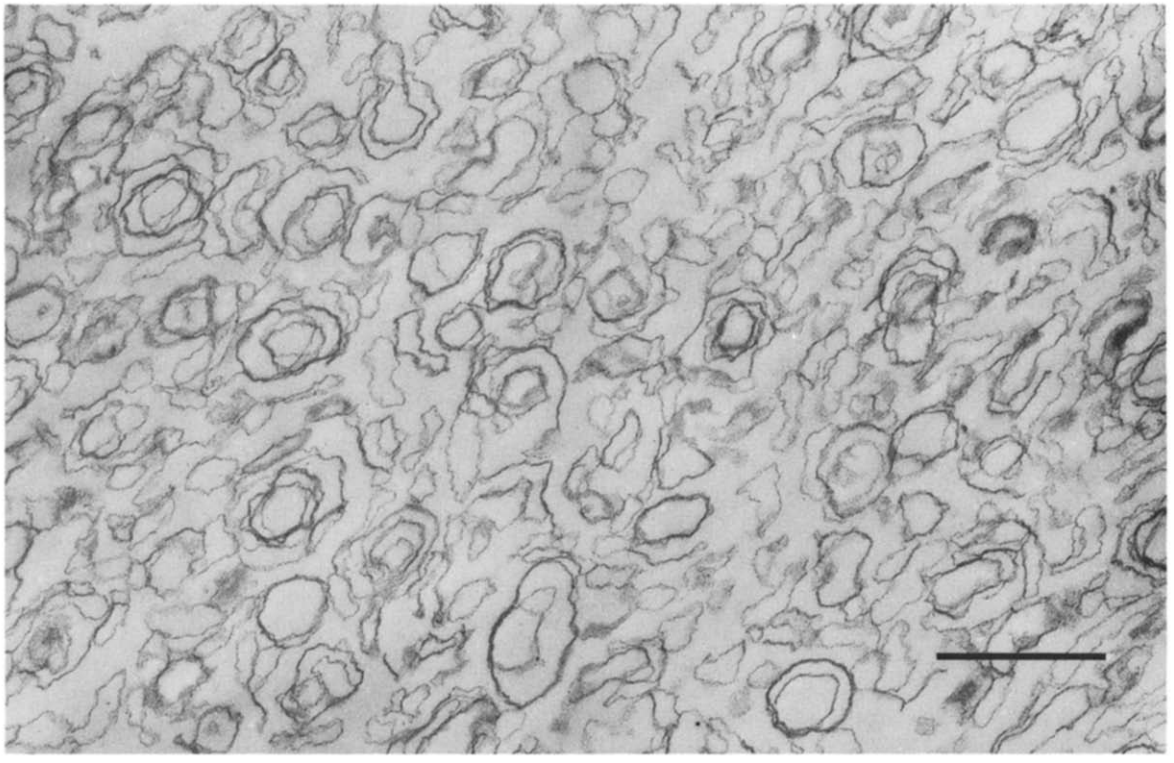

Fig.2B

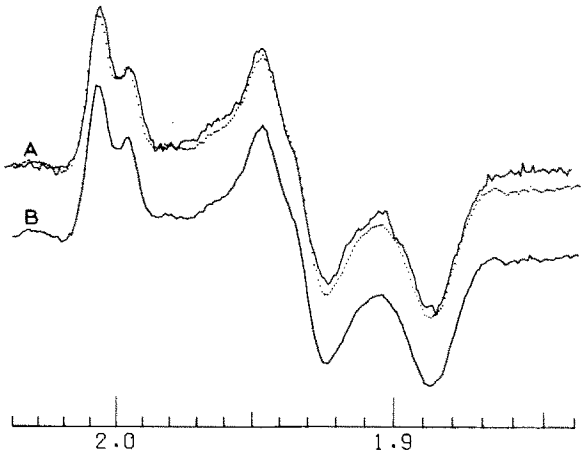

Fig.3. Comparison of EPR signals of the outer-membrane Fe–S protein from two different sources. Trace A (——): purified outer membranes from beef-heart mitochondria (25 mg protein/ml in the isolation medium in section 2, reduced with 5 mM NADH and a few grains of dithionite for 1 min at 293 K. The EPR spectrum was run on a Varian E-3 spectrometer. EPR conditions: microwave frequency (*F*) 9150 MHz; temperature (*T*) 93 K; microwave power (*P*) 26 mW; modulation amplitude (*MA*) 0.63 mT; scanning rate (*SR*) 12.5 mT/min; modulation frequency (*MF*) 100 kHz. Trace A (- - -) and trace B: purified Fe–S protein fraction from beef-kidney cortex mitochondrial outer membranes in 20 mM glycine–NaOH buffer, 1 mM EDTA and 1 mM 2-mercaptoethanol (pH 9.6) reduced with a few grains of dithionite for 1 min at 293 K. The EPR spectrum was run on a Varian V-4502 spectrometer. EPR conditions: F = 9222 MHz but the trace has been converted [8] to a 9150 MHz g-value scale; T = 90 K; P = 28 mW; MA = 0.75 mT; SR = 5 mT/min; MF = 100 kHz. The y-axis scaling was adjusted to best fit trace A, (——). The x-axis scale refers to g-values.

The amplitude of the positive peak at g = 2.007 first decreases with increasing power, due to the saturation of the radical, then a constant level is reached. At still greater powers saturation sets in at the same power at which the other lines at g = 1.935 and g = 1.888 start saturating. Thus at low power the line at g = 2.007 is relatively too high and the line at g = 1.996 is relatively too low. The power used in fig.3 is such that the radical signal is saturated to give a negligible

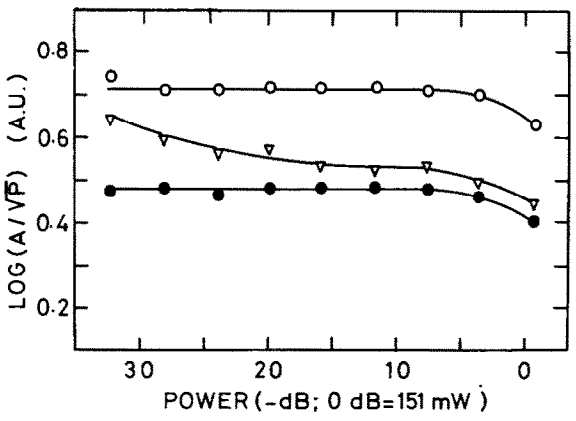

Fig.4. Power saturation behaviour of the signal in fig.3, trace A (——). (○——○) line at g = 1.935; (▽——▽) line at g = 2.007; (●——●) line at g = 1.888). *Abbreviations:* A, line amplitude; P, microwave power; A.U., arbitrary units.

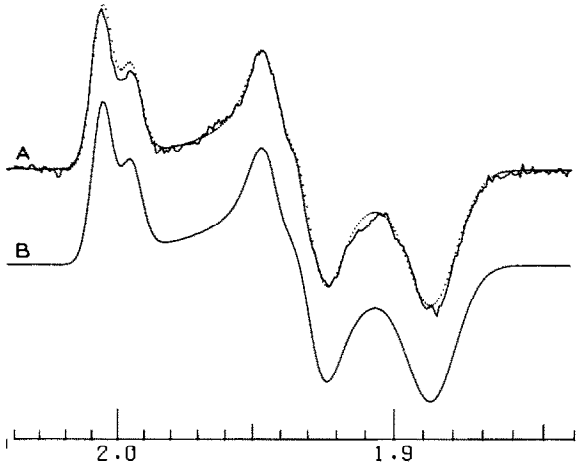

Fig.5. Computer simulation of the EPR signal of mitochondrial outer membranes. Trace A (——) same as fig.3 trace A (——). Trace A (- - -) and trace B: computer simulation as the sum of two rhombic S = ½ signals. Parameters used: signal 1 (corresponding to the [2 Fe–2 S] center defined as 1) $g_{x,y,z}$ = 1.8874, 1.93, 2.0033 and width (x,y,z) = 3.05 mT, 2.1 mT, 1.4 mT; signal 2 (corresponding to the [2 Fe–2 S] center defined as center 2) $g_{x,y,z}$ = 1.8874, 1.9417, 1.993 and width (x,y,z) = 3.05 mT, 1.65 mT, 1.45 mT. The two signals were added in a ratio signal 1 : signal 2 = 2:1. The g-values used for the simulation do not coincide precisely with the maximum, zero-crossing and minimum of the g_z, g_y and g_x lines, respectively, for reasons described in [10].

Fig.2. Electron micrographs of the mitochondrial outer membranes from beef heart. (A) is the membrane pool (fractions 21–27 from fig.1) negatively stained with 1% uranylacetate. (B) is a thin section from the same pool obtained with routine embedding and staining procedures. The bar represents 1 μm.

spectral contribution. The rest of the lines are not saturated. Sometimes the partially purified preparations of the Fe–S protein from beef-kidney cortex contained a much larger radical signal [4] and such spectra could not be used for line shape studies of the g_z region. A field modulation of 0.32 mT gave no better resolution than that used in fig.3.

What is obvious from fig.3 is that two lines are present in the $g = 2$ region and that the line at $g = 1.935$ has a distinct bend. Such a line shape cannot be due to a single $S = ½$ system. Figure 5 shows that the signal can be very well simulated as a sum of two $S = ½$ line shapes in a 2:1 ratio. Figure 6 shows the line shapes of the individual signals.

The conclusion then is that mitochondrial outer membranes from rat liver, beef-kidney cortex and beef heart contain 2 different Fe–S centers: centers 1 and 2 in a ratio 2:1. From the Fe and acid-labile S analysis in [1,4] it can be concluded that both centers are of the [2 Fe–2 S] type. These two centers differ from other [2 Fe–2 S] centers in that their g_z values are very close to the free electron g-value (g_e) and that the g_z value of center 2 is even less than g_e. Both centers still give EPR-signal intensity at 200 K [1,4].

The fact that both signals co-purify [4], suggests that they are part of a protein complex containing one center 2 and two centers 1 per unit. This complex probably also contains flavin [4]. The molecular weight of the partially purified Fe–S protein complex (mol. wt >200 000, [4]) also supports this suggestion. Such a complex is then comparable to the complexes I and II bound to the mitochondrial inner membrane which also contain flavin and several Fe–S centers [3]. The function of this outer membrane-bound complex is not yet clear [4].

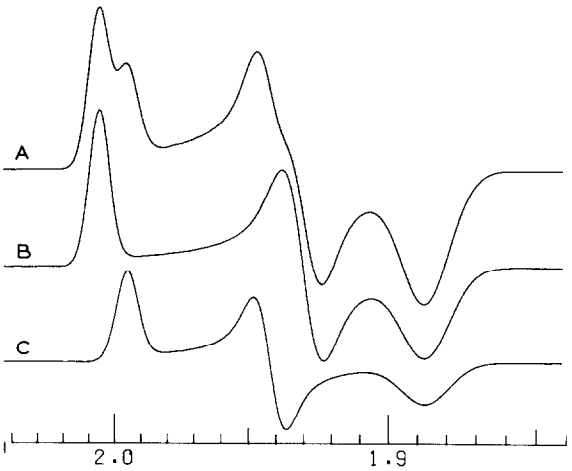

Fig.6. Computed line shape of the individual EPR signals of the Fe–S centers 1 and 2. Trace A, sum of traces B and C. Trace B, simulation of the signal of center 1. Trace C, simulation of the signal of center 2. The two signals are plotted and added in a ratio signal 1 : signal 2 = 2:1. Parameter as in fig.5.

Acknowledgements

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