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BEEF-HEART SUBMITOCHONDRIAL PARTICLES: A MIXTURE OF MITOCHONDRIAL INNER AND OUTER MEMBRANES

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SUMMARY

1. EPR spectra at 9 GHz and 83 °K of NADH-reduced anaerobic beef-heart submitochondrial particles, prepared from mitochondria by sonication and centrifugation, contain a signal ($g_z = 2.01$, $g_y = 1.94$, $g_x = 1.89$) due to an iron–sulphur center of the mitochondrial outer membrane.

2. The ratio of inner and outer membranes in submitochondrial particles is not greatly different from that in beef-heart mitochondria.

3. Beef-heart submitochondrial particles free from outer-membrane contamination have been prepared by free-flow electrophoresis. EPR spectra at 83 °K of such particles are presented.

INTRODUCTION

Recently a new signal ($g_z = 2.01$, $g_y = 1.94$, $g_x = 1.89$) was recognized [1] in EPR spectra at 9 GHz and 77 °K of NADH-reduced submitochondrial particles, prepared by sonication of beef-heart mitochondria. The signal could not be produced by reduction with succinate unless the artificial electron mediator phenazine methosulphate (PMS) was present. It is absent in reduced isolated Complex I. From these properties as well as from the shape and the g values of this signal it was concluded that it was probably due to the iron–sulphur protein in the mitochondrial outer membrane, discovered by Bäckström et al. [2], thus implying that submitochondrial particles obtained by the methods of Löw and Vallin [3] or Fessenden and Racker [4] contain mitochondrial outer-membrane fragments.

To test this hypothesis we investigated whether submitochondrial particles freed from outer-membrane fragments by free-flow electrophoresis [5, 6], contained species giving rise to this signal. It was found that no traces of this signal could be detected in these particles reduced with succinate in the presence of PMS, or in

Abbreviations: PMS, phenazine methosulphate; FFE particles, particles obtained by free-flow electrophoresis.

particles reduced with NADH. This clearly demonstrates that, in our hands, submitochondrial particles prepared from beef-heart mitochondria by sheer sonication and centrifugation are a mixture of inner- and outer-membrane fragments. Indeed, a comparison between EPR spectra from beef-heart mitochondria and Mg-ATP particles (prepared without purification in the electrophoresis system) indicates that the ratio of inner and outer membranes is approximately the same in the two preparations.

MATERIALS AND METHODS

Beef-heart mitochondria were prepared as described by Erdelt et al. [7] or Crane et al. [8]. The outer and inner membranes were separated from each other either by a swelling–shrinking process [5] or by treatment with digitonin [9]. From this inner membrane-matrix fraction, Mg-ATP submitochondrial particles were prepared according to Löw and Vallin [3]. These particles were then purified in a free-flow electrophoresis apparatus [10] in order to remove inner-membrane-matrix particles (mitochondria from which the outer membranes have been removed) and outer membranes. The conditions were similar to those described in ref. 6. The homogeneous submitochondrial particle fraction so obtained was used in some of the experiments reported here and will be referred to as FFE particles.

Cytochrome *c* oxidase (EC 1.9.3.1) was measured as in ref. 11, succinate dehydrogenase (EC 1.3.99.1) as in ref. 12, and monoamine oxidase (EC 1.4.3.4) as in ref. 5. Protein concentrations were determined in an automated system according to ref. 5.

For EPR measurements samples containing about 30–60 mg protein per ml were used.

EPR spectra at 9 GHz, with a field modulation frequency of 100 kHz, were recorded on a Varian E-3 spectrometer; low temperatures were obtained by a Varian N_2-flow cooling system, and the magnetic field strength was measured by means of an NMR probe. The microwave frequency was determined with a frequency counter. EPR conditions, as specified in the legends to the figures, are abbreviated as follows: F, microwave frequency; T, temperature; P, microwave power; MA, modulation amplitude; SR, scanning rate. The scale at the bottom of each figure is a *g*-value scale.

RESULTS AND DISCUSSION

Preparation of submitochondrial particles from beef-heart by free-flow electrophoresis and their characterization

In order to obtain a homogeneous preparation of submitochondrial particles, the method of free-flow electrophoresis was used for removing these particles (FFE particles) from contamination with inner-membrane-matrix particles and outer membranes. As has been demonstrated with submitochondrial particles from rat liver mitochondria [6], these particles have a much lower electrophoretic mobility than the other mitochondrial membrane systems, thus allowing for a clear separation by electrophoresis. An identical electrophoretic behaviour was expected in beef-heart mitochondria. Indeed, electron micrographs of negatively stained specimen of the fractions 35–38 of the electrophoresis run of Fig. 1 show typical submitochondrial particles with the 90-Å subunits. The membrane vesicles (FFE particles) from these

TABLE I

CYTOCHROME *c* OXIDASE AND MONOAMINE OXIDASE ACTIVITIES IN THE MEMBRANE FRACTIONS USED

Activities are expressed in μmoles/min per mg protein. The membrane fractions from the electrophoresis run were sonicated with a Branson B-12 sonicator (90 W, microtip setting 7) for 10 s at 0 °C and then assayed for enzymic activity and protein.

Fraction	Cytochrome *c* oxidase	Monoamine oxidase
Beef-heart mitochondria	0.8	2.2
FFE particles	1.3	0.4

pooled fractions were free of monoamine oxidase, i.e. of outer membranes, but were enriched in the inner-membrane marker enzymes cytochrome *c* oxidase (Table I) and succinate dehydrogenase activity (not shown here), showing that these vesicles are submitochondrial particles and are free of outer membrane contaminations. Intact mitochondria (and outer membranes) are concentrated into Fractions 28–32 of the electrophoresis experiment (Fig. 1).

The two methods used for preparing inner-membrane-matrix particles, the swelling–shrinking and the digitonin procedure, yielded preparations with similar properties. Both methods were used here for isolating the FFE particles.

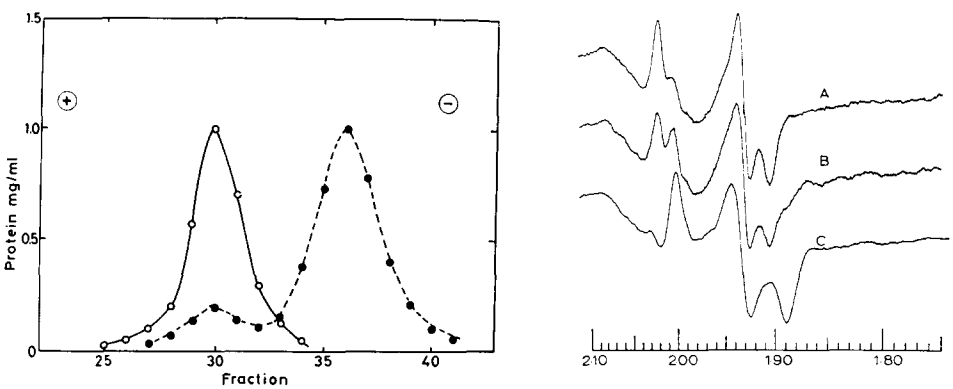

Fig. 1. Protein distribution in electrophoresis runs of intact beef-heart mitochondria (○—○) and submitochondrial particles (●—●) prepared as described in Materials and Methods. The conditions in the run were: 100 mA, 110 V/cm, buffer flow 2 ml per fraction per h, sample injection 2.5 ml/h above Fraction 60, temperature = 4 °C. 92 fractions were collected at 0 °C. The main peak in the right-hand curve (●—●) shows the submitochondrial particles (FFE particles), the smaller shoulder contains the contaminating outer membranes.

Fig. 2. Comparison of EPR spectra of mixtures of beef-heart mitochondrial inner and outer membranes. The preparations were suspended in 0.25 M sucrose and 20 mM Tris-HCl buffer (pH 7.5). A. Succinate (25 mM) was added to a partially purified FFE-particle preparation. After 3 min at 20 °C the suspension was frozen in liquid N_2 and a spectrum was recorded. B. The tube used for A was thawed and PMS (0.15 mM) was added; it was frozen after 3 min at 20 °C. C. A crude mitochondrial outer membrane preparation was treated as under B. EPR conditions: F, 9.1 GHz; T, 83 °K; P, 130 mW; MA, 12.5 G; SR, 250 G/min. The gain for traces A and B is the same.

EPR spectra of mixtures of beef-heart mitochondrial inner and outer membranes

In Fig. 2 EPR spectra at 9 GHz and 83 °K are shown of mitochondrial membrane preparations containing both inner- and outer-membrane fragments. Trace A is the spectrum of a partially purified FFE-particle preparation, that still contained monoamine oxidase activity. The particles were reduced with succinate. A rather high microwave power was used in order largely to saturate the radical signal due to flavin and ubiquinone [13]. The main signal is therefore of the succinate dehydrogenase type. When PMS was added, Trace B was obtained. New absorption lines are seen at $g = 2.01$ and 1.89, and the shape of the $g = 1.93$ line changes. The amplitude of the lines at 2.03, 1.93 and 1.91 is decreased by 15–25 % on addition of PMS. This decrease, the reason for which is unknown was noticed in four different preparations. Trace C shows the spectrum of a crude outer-membrane preparation reduced with succinate in the presence of PMS. Although the succinate dehydrogenase type of signals could be detected in the absence of PMS (not shown) they are too weak to be seen when the outer-membrane iron–sulphur center is also reduced by addition of PMS. Trace C is similar to the spectrum shown by Bäckström et al. [2] for reduced outer membranes from rat-liver mitochondria.

EPR spectra of pure FFE particles

In Fig. 3 EPR spectra of pure beef-heart FFE particles at 9 GHz and 83 °K are presented. Trace A, for which succinate was used as reducing agent, is the same as that of Fig. 2, Trace A. Addition of PMS (Trace B) now has no effect on the shape of the spectrum, although the amplitude of all lines is somewhat decreased. There are also no signs of the presence of the outer-membrane iron–sulphur center in Trace C, obtained with NADH as substrate. Reduction of center 1 of NADH dehydrogenase [14] is responsible for the relative increase of the amplitude of the line around $g = 1.94$.

These experiments show that the presence of the lines at $g = 2.01$ and 1.89 is coupled to the presence of monoamine oxidase activity and that these lines together

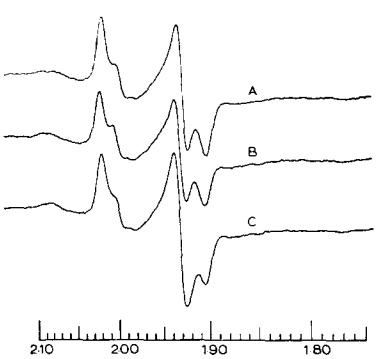

Fig. 3. EPR spectra of pure FFE particles. The particles were suspended in 0.25 M sucrose and 20 mM Tris–HCl buffer (pH 7.5). Substrate was added and after 3 min at 20 °C the mixture was frozen in liquid N_2. A, 25 mM succinate; B, 25 mM succinate plus 0.15 mM PMS; C, 6 mM NADH. EPR conditions: F, 9.1 GHz; T, 83 °K; P, 130 mW; MA, 12.5 G; SR, 250 G/min. The gain was the same for all traces. Calibrated EPR tubes were used.

with a line at $g = 1.94$ belong to a center from the outer membrane and not from the inner membrane. EPR spectroscopy of a submitochondrial particle preparation can therefore be used as a diagnostic tool for the presence of outer-membrane fragments.

Comparison of EPR spectra of beef-heart mitochondria and submitochondrial particles

In Fig. 4 EPR spectra at 9 GHz and 83 °K of reduced anaerobic beef-heart mitochondria (Trace A) and Mg-ATP particles, not purified by free-flow electrophoresis (Trace B), are compared. It can be clearly seen that the shapes of these two spectra are practically the same and that the relative amplitudes of the lines around $g = 2.01$ and 1.89 are not greatly different in the two traces. This means that the ratio of inner and outer membranes in this routinely made Mg-ATP particle preparation is nearly the same as in whole beef-heart mitochondria. It is obvious that this might complicate the interpretation of experiments with such particle preparations.

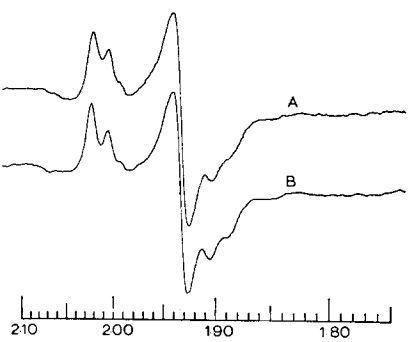

Fig. 4. Comparison of EPR spectra of beef-heart mitochondria and Mg-ATP particles derived from them by sonication and centrifugation [3]. A. Beef-heart mitochondria suspended in 0.25 M sucrose were mixed with 1.3 mM glutamate, 1.3 mM malate, 4 mM sodium phosphate buffer (pH 7.5) and 4 mM NADH. After 3 min at 20 °C the mixture was frozen. B. Mg-ATP particles, prepared from the same batch beef-heart mitochondria as used for A and suspended in 0.25 M sucrose and 10 mM $MgCl_2$ were mixed with the same substrates as used for A and also frozen after 3 min at 20 °C. EPR conditions: F, 9.1 GHz; T, 83 °K; P, 130 mW; MA, 12.5 G; SR, 250 G/min.

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