Electron Paramagnetic Resonance-detectable Electron Acceptors in Beef Heart Mitochondria

REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE UBIQUINONE REDUCTASE SEGMENT OF THE ELECTRON TRANSFER SYSTEM*

(Received for publication, June 22, 1973)

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SUMMARY

The EPR-detectable (≥4.2 K) electron acceptors of the DPNH-ubiquinone reductase segment of the electron transfer system of beef heart mitochondria were studied in preparations of varying complexity. Four distinct iron-sulfur centers associated with this segment of the electron transfer system were identified. They occur in approximately equal concentrations, and this concentration approximates that of the bound flavin of DPNH dehydrogenase. Quantitative reductive titrations with DPNH and dithionite showed that the order of the oxidation-reduction potentials of the iron-sulfur centers is: Center 3 > 2 > 4 > 1, indicating that there are two centers of high and two of low potential. Complex I accepts approximately 20 electron neq per mg of protein from DPNH. In rapid reaction studies using the freeze-quench method the rates of reduction of the iron-sulfur centers by DPNH could not be resolved. With acetylpyridine DPNH, however, the rates of appearance of the signals were in this sequence: Center 2 > 3 + 4 > 1.

There have been numerous studies in the past on components of the electron transfer system of mammalian mitochondria. These studies have largely concentrated on those electron carriers that can be detected by optical methods. Although over 10 years ago it was shown by EPR spectroscopy that additional components are present which appear to participate in oxidation-reduction (1), their number was small and their functional significance was not generally accepted. More recently, however, through technical developments in the application of EPR spectroscopy to biological systems a number of additional, thus far unknown, oxido-reductively active components of mitochondria have been recognized. The majority of these components belongs to the family of iron-sulfur compounds, and it indeed appears that there are more iron-sulfur groups present in mitochondria than cytochromes, which were previously thought to be the principal type of electron carrier in mitochondria. Recent work also implicates iron-sulfur centers in energy conservation coupled to electron transfer (2, 3). Although there is now general awareness of these observations and notions derived therefrom, little systematic EPR work has been published which would make this knowledge more generally accessible and could provide a firm foundation for further studies.

The present series of papers represents an attempt to provide basic information on these electron carriers in mammalian mitochondria that are detectable by EPR spectroscopy at the present state of technical developments.

In the resolution of the EPR signals and assignment of these signals to the corresponding components mainly six approaches were used: (a) physical separation by fractionation of mitochondria into their constituent complexes and soluble proteins; (b) reductive titration, by which the signals of species of different oxidation-reduction potential can be made to appear in sequence; (c) rapid kinetic studies, by which electron acceptors may be functionally separated if their rates of reaction differ; (d) use of inhibitors to modulate the results obtained in (b) and (c) above; (e) variation of observation temperature, which will allow one to resolve the signals from components with different electron spin relaxation rates or different strength of magnetic coupling; (f) variation of incident microwave power to resolve signals from species with different spin relaxation rate.

The present papers report on attempts using these approaches to obtain spectra of individual components, identify and characterize them, and explore their gross functional roles. The first paper is concerned with the DPNH-ubiquinone reductase segment of the electron transfer system. A preliminary report of this work has appeared (4). The accompanying paper (5) will deal with the next segment of the electron transfer system, viz. that of ubihydroquinone-cytochrome c reductase. The accompanying paper also contains a discussion of the behavior...
and relationships of components dealt with in both papers and a discussion of some implications of the fact that the electron transfer system consists of, or is in equilibrium with, a much larger number of electron carriers than previously thought.

**MATERIALS AND METHODS**

The purification of Complex I (6) and Complex I + III (7) and the fractionation of Complex I (8) were carried out according to published procedures.

DPNH and 3-acetylpyridine DPNH were purchased from P-L Biochemicals, Milwaukee, Wis. The concentration of APDPNH solutions was calculated from the absorbance of the solution (pH 8) at 363 nm using the molar extinction coefficient (8.2 × 10^4) given in the analytical data provided with the APDPNH by the company. The concentration of DPNH solutions was assayed by the enzymatic method of Klingenberg (9).

EPR spectroscopy was carried out and the temperature was controlled as described (4). Unless otherwise stated the EPR spectra were recorded at 13.3 ± 0.15 K. Magnetic field was measured with a proton probe, since the frequency is not constant over a series of experiments, field positions of absorptions are not expressed in gauss but on the frequency-independent g factor scale. These are the values shown along the abscissa above or below the spectra. The values in the figures (except in Fig. 6) and in the text are in general arbitrarily rounded off for convenience, while those listed in the tables are given to that decimal which could be consistently reproduced in independent measurements at different times and on different preparations. Minima and maxima are designated as peaks. In many instances no quantitative evaluation of the EPR signals was attempted, instead heights of signals of the same kind were compared. Whenever feasible, peaks at low or high field were used for this purpose, because their height from the base-line can be estimated with less error than the height of peaks overlapping with other signals in the center at g = 2.

Quantitative determinations of the number of unpaired spins represented in a signal were obtained by double integrations using cubic EDTA as a standard, and applying a correction for the difference in g values (10). To determine the number of unpaired spins in the individual iron-sulfur centers, use was made of the different temperature dependence of their signals and their sequential appearance in reductive titrations, as outlined in Table II (4). In the case of Centers 3 + 4, which are never seen alone, three approaches were used: (a) a reasonable line shape for the center line was assumed by analogy to those of other iron-sulfur proteins, (b) the integration was based on the intensity of the low field line, and (c) the value was obtained by subtracting those found for Centers 1 and 2 from integrations of spectra comprising Centers 1 to 4.

Titrations with dithionite and DPNH were performed according to Ref. 10 and analogous to Ref. 11, respectively. All materials were titrated anaerobically. The rapid reaction technique of Dray (12, 13) was used with some modifications.

**RESULTS**

Resonances Observed in Complex I—Fig. 1 shows EPR spectra recorded at several stages of a titration of Complex I with DPNH. Table I lists the field positions on the g value scale (not implying that the values are g values) for the observed resonances. Scans covering a magnetic field of 100 to 5100 G at 9.2 GHz did not show resonances other than those described here (cf. Ref. 14) with the exception of the usual high spin ferric signal at g = 4.3.

Unidentified Components—The resonance centered at g = 2.01 has maximum amplitude in the oxidized sample (Fig. 1A) and disappears during the course of reduction. As reduction

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1 The abbreviations used are: Complex I, DPNH-ubiquinone reductase; Complex III, ubiquinone-cytochrome c reductase; APDPNH, 3-acetylpyridine DPNH; Complex I + III, DPNH-ubiquinone-cytochrome c reductase.

2 By an unpublished procedure of Dr. Tore Vängård. We are indebted to Dr. Vängård for his advice in this matter.

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![Fig. 1. EPR spectra between 2900 and 3900 G at 9.2 GHz (g = 2.3 to 1.7) from a titration of Complex I with DPNH. Complex I, 40.6 mg of protein per ml, was dissolved in 0.06 M sucrose, 1 mM histidine, and 0.05 M Tris chloride (pH 8.0). Two minutes after incubation with DPNH (see "Materials and Methods") the samples were frozen and stored in liquid nitrogen. The conditions of EPR spectroscopy were: microwave frequency, approximately 9.2 GHz; microwave power, 250 microwatts; modulation frequency, 100 KHz and amplitude, 6.3 G; temperature, 13.3 K; time constant, 0.5 s; scanning rate, 200 G per min. For the enlarged wings, the modulation amplitude was doubled and the amplifier gain was raised 6.4-fold for A to C and 3.2-fold for D and E. A, Complex I without DPNH; B to E, reduced with 2.1, 9.3, 11.9, and 65.2 neq, respectively, of DPNH per mg of protein.](http://www.jbc.org/content/242/4/1923 matrimonial/F1)

**Table 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Field positions</th>
<th>g average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron-sulfur Center 1</td>
<td>2.022, 1.938, 1.923</td>
<td>1.96</td>
</tr>
<tr>
<td>Iron-sulfur Center 2</td>
<td>2.054, 1.922</td>
<td>1.97</td>
</tr>
<tr>
<td>Iron-sulfur Center 3</td>
<td>2.100, 1.856, 1.862</td>
<td>1.95</td>
</tr>
<tr>
<td>Iron-sulfur Center 4</td>
<td>2.105, 1.864</td>
<td>1.864</td>
</tr>
</tbody>
</table>

* The numbers are the measured field positions of prominent peaks given on the g value scale.

* The average g values were calculated by assuming that the values measured at the low and high field peaks correspond to g and g2, respectively, and by interpolating or extrapolating a probable value of g3 from the position of the center line or peak.

* Since Center 4 is only seen in the presence of Center 3, i.e. the field position of the combined resonances is measured, these values may only be approximate. It is likely that the values differ somewhat more from those of Center 3.
proceeds (Fig. 1B) additional resonances emerge; one at \( g = 2.00 \), due to a free radical, and one with components at \( g = 2.036 \) and 1.986. These latter resonances disappear again on further reduction, a behavior typical of resonances from an intermediate state of a two electron acceptor. These three species are unidentified, although it is likely that the free radical signal largely represents flavin semiquinone. The unidentified species will be considered in the accompanying paper (5) since their signals are more prominent in the materials studied there.

**Resolution of Resonances from Iron-Sulfur Centers 1 and 2** — The other resonances observed in these spectra of Complex 1 were assigned to four iron-sulfur centers as follows. In Spectrum B of Fig. 1 the resonance at \( g = 2.05 \) is approximately 50% of its maximal value (cf. Spectra D and E) and in Spectrum C 70%. The line at \( g = 1.92 \) increases in a parallel manner up to Spectrum C but then increases further as the lines at \( g = 2.02 \) and 1.94 develop. It appears, therefore, that two iron-sulfur centers are reduced in sequence, one with axial symmetry showing lines at \( g_1 = 2.05 \) and \( g_2 = 1.92 \), and one with rhombic symmetry and lines at \( g = 2.02 \) (\( g_L \)), 1.94 (\( g_{\perp} \)), and 1.92 (\( g_z \)), such that \( g_L \) of the former overlaps with \( g_z \) of the latter. This assignment was confirmed by variation of temperature and microwave power. Fig. 2, A and B, shows the spectra of samples analogous to those of Fig. 1, B and D, at 42 K. The line at \( g = 2.00 \) is due to a free radical, presumably flavin. It is obvious that the lines of the iron-sulfur center with \( g_1 = 2.05 \) and \( g_2 = 1.92 \) are severely broadened at this temperature, whereas those of the other center are still quite distinct with \( g_z \) and \( g_{\perp} \) resolved. This latter iron-sulfur center observed in Spectrum D is therefore presumably the same that had been observed in previous EPR studies conducted at 95 K (15). This center has been called Center 1 and the center with the more temperature sensitive spectrum, Center 2 (4). In a similar manner the same relationship of the various lines can be demonstrated by increasing the microwave power at very low temperature. In Fig. 3 the spectrum of the sample of Fig. 2B, in which the lines of both Centers 1 and 2 are well developed, is shown at 2.7 and 270 microwatts of power at 7.7 K. It can be clearly seen that the line at \( g = 2.05 \) of Center 2 is somewhat diminished with high power while the lines at \( g = 2.02 \) and 1.94 are increased. Since the line at \( g = 1.92 \) also increases it can be concluded that it has a contribution from Center 1.

**Resolution of Signals from Iron-Sulfur Centers 3 and 4** — Thus far the resonances toward low and high field have not been considered. Their amplitude increases roughly in parallel during the titration (Fig. 1) and, as evident from Figs. 2 and 3, disappear with rising temperature, but these resonances are not

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**Figure 2.** EPR spectra of Complex I at 42 K. A and B are EPR spectra of samples analogous to those of Fig. 1, B and D, respectively. The conditions of EPR spectroscopy were as in Fig. 1, except for microwave power, modulation amplitude, and temperature which were 27 microwatts, 7.5 G, and 42 K, respectively. The amplifier gain in A was 1.56 times that in B. A and B, Complex 1 reduced with 1.4 and 17.3 neq of DPNH per mg of protein, respectively.

**Figure 3.** EPR spectra of Complex I at different microwave powers. The sample of Complex I is that used for Fig. 2B. The conditions of EPR spectroscopy were as in Fig. 1, except that the modulation amplitude was 7.5 G, the temperature 7.7 K, and for A the microwave power was 2.7 microwatts. The amplifier gain for A was 2.56 times that for B.

**Figure 4.** EPR difference spectrum of completely reduced Complex 1 (Center 1 + 2) minus partly reduced Complex 1 (Center 2). The samples used were those of Fig. 2, A and B, and their spectra were analogous to those of Fig. 1, C and E. The difference spectrum was obtained by use of a digital computer. The conditions of EPR spectroscopy were: microwave power, 9 microwatts; modulation amplitude, 7.5 G; temperature, 13.3 K; time constant, 0.25 s; and scanning rate, 100 G per min.

Subtraction of a spectrum showing mainly the lines of Center 2 (e.g. Fig. 1, B or C), suitably scaled, from a spectrum exhibiting the lines of both Centers 1 and 2 (e.g. Fig. 1, D or E) should yield the spectrum of Center 1, which at these temperatures is only observed with the more readily reduced Center 2. Thus, a spectrum approximating that of Center 1 was obtained by computer subtraction (Fig. 4; the lines outside the area of \( g = 2.05 \) to 1.90 are ignored for the present purposes). In turn, admixture of this spectrum with that of Center 2 at appropriate intensities yielded spectra simulating different degrees of reduction or presence of Centers 1 and 2 (Fig. 5). These simulated spectra were used in the quantitative evaluation of spectra obtained from experimental samples, where the centers were reduced to a different extent.

Resolution of Signals from Iron-Sulfur Centers 3 and 4 — Thus far the resonances toward low and high field have not been considered. Their amplitude increases roughly in parallel during the titration (Fig. 1) and, as evident from Figs. 2 and 3, disappear with rising temperature, but these resonances are not
readily saturated at high microwave power. Thus these resonances do not show the behavior of Center 1 or 2 and must be due to one or more additional species. On close inspection of Fig. 1 one can see that, as reduction proceeds, the line at $g = 2.10$ is slightly shifted downfield ($g = 2.100$ to $g = 2.103$); similarly, the line at $g = 1.886$ is shifted upfield. While these shifts occur, the intensity of the resonances at $g = 2.10$ and 1.862 increases, but that at $g = 1.886$ remains constant. Such behavior may be interpreted either as indicative of two separate, sequentially reduced iron-sulfur centers with partly overlapping signals, or as an indication that there is a change in the environment (e.g. change of magnetism in an adjacent acceptor) of the iron-sulfur center under observation. We conclude that there are two distinct but similar iron-sulfur centers represented by the lines at $g = 2.10$, 1.886, and 1.862, for the following reasons.

(a) The shifts of line positions do not occur after the signal has reached its full intensity, but when the signal is approximately at 50% of its maximal size. (b) During the second stage of the reduction (>$50\%$) when the shift of the lines occurs, only the lines at $g = 2.10$ and $g = 1.862$ increase, not the line at $g = 1.886$. (c) Double integrations of these lines account for as many iron-sulfur centers as do the lines of Centers 1 and 2 together, i.e. approximately two per flavin (cf. Table II). (d) When the position of the line at $g = 2.10$ is carefully measured and plotted against the quantity of reductant added and the combined signal height at $g = 2.10$ as in Fig. 6, sequential reduction of two components with different oxidation-reduction potentials is clearly indicated. Since in these measurements we were dealing with small changes in $g$ factor, we also measured for every sample the line position of the resonance at $g = 2.054$. For this resonance we found the field position to be constant to within ±0.0003 $g$. The center reduced first in the experiment of Fig. 6 we call Center 3, the other 4. The explanation that a shift of lines in a single center occurs could only be upheld, if gross heterogeneity in the preparation is assumed.

Sequence of Reduction of Iron-Sulfur Centers 1 to 4 in Titrations—The relationship of the degree of reduction of Complex I and the development of the lines of the individual iron-sulfur centers is shown in Fig. 7 for all four centers. From this plot it appears that in Complex I, Center 3 has the highest oxidation-reduction potential closely followed by Center 2. Center 4 is reduced before Center 1. If the contributions of Centers 3 and 4 are not resolved, the sequence 2 > 3 + 4 > 1 is obtained, as previously reported (4). This order is seen with more complex preparations when the poorer signal to noise of the spectra does not permit the resolution of Centers 3 + 4 (cf. Ref. 5).

Quantitative Relationships between Components of Complex I—Table II presents the results of integrations of the EPR signals as outlined under "Materials and Methods" and as indicated in the last column of this table. In a number of such integrations the ratio of total centers to flavin was found to be between 2.5 and 4 to 1 with the concentrations of the individual centers being approximately equal. Thus it appears that there is present one iron-sulfur center of each kind per flavin. If the centers were of the plant ferredoxin type, i.e. with 2 iron atoms per center, this would mean that we can account for close to one-half of the iron found by chemical analysis in Complex I, viz. 16 to 20 g atoms per mole of flavin; if there were 4 iron atoms per center, as in ferredoxins of the clostridial type, the centers represented in the signals would account for 80 to 100% of the iron.

We would like to correct here an error which occurred in our preliminary publication (4). It was stated there on p. 451 that the iron content of Complex I is 23 to 26 ng atoms per mole of flavin, which should have read: "per mg of protein."
Kinetic Studies on Reduction of Iron-Sulfur Centers—It was not possible to determine the time sequence of reduction of the iron-sulfur centers by an excess of DPNH, even when the reaction was carried out at 4°, because all centers were reduced within 6 ms (not corrected for freezing time), the earliest reaction time available with our apparatus. However, with APDPNH the sequence of signal appearance of Centers 1, 2, and 3 + 4 can be resolved, particularly when the reaction is carried out at 4°. The data from such an experiment are plotted in Fig. 8. A sample incubated with DPNH for 1 min was used as the 100% reduced reference. It is apparent that the potential of Center 1 is sufficiently low that APDPNH ($E'_{o} = -248$ meV) is unable to reduce this center more than 50%. The sequence of signal appearance is: Center 2, 3 + 4, and 1. We have attempted the resolution of the contributions from Centers 3 and 4. The data suggested that the signal of Center 3 appeared before that of Center 4, but the signal to noise ratio was not sufficient to consider this as established.

Resonances of Subfractions of Complex I—The EPR spectra after reduction of the three fractions obtained by resolution of Complex I according to the procedure of Hatefi and Stempel (8) are shown in Fig. 9, B to D together with the spectrum of reduced Complex I (A). Apparently the spectrum of the iron-protein fraction is largely that of a single species, similar to Center 2. The spectrum of the flavoprotein fraction (Fig. 9D), however, is that of at least three species as suggested by studies of both power and temperature dependence of the individual lines. One of these species is the iron-protein as represented in Fig. 9C. The fractions were analyzed for iron and labile sulfide and double integrations of the EPR signals were carried out. The number of unpaired electrons accounted for in the signals were 5 and 15% respectively, of the iron or labile sulfur atoms found in the iron-protein and the flavoprotein fraction. Thus, the iron-protein fraction seems to contain much more EPR undetectable labile sulfur and iron than the starting material, Complex I, in which the ratio of total labile sulfur or iron atoms to the number of unpaired electrons accounted for in the EPR signal lies between 4 and 6.5. Inasmuch as these findings point to the possibility that fractionation of particles may introduce artifacts, we have examined the EPR spectra of whole heart tissue frozen immediately after the death of the animal. Since these experiments also have a bearing on the material presented in the accompanying paper, these spectra are shown there in Fig. 6 (5). The major resonances in the range of $g = 2.2$ to 1.8 are indeed those of the iron-sulfur centers of DPNH.

**Table II**

<table>
<thead>
<tr>
<th>Iron-sulfur center</th>
<th>Ratio of concentration of iron-sulfur center to flavin concentration</th>
<th>Reference figure(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 + 2 + 3 + 4</td>
<td>4.0</td>
<td>1E</td>
</tr>
<tr>
<td>1</td>
<td>0.81</td>
<td>2B</td>
</tr>
<tr>
<td>2</td>
<td>0.89</td>
<td>3B</td>
</tr>
<tr>
<td>1 + 2</td>
<td>2.2</td>
<td>1E</td>
</tr>
<tr>
<td>3 + 4 by difference</td>
<td>1.8</td>
<td>1E-1E(^b)</td>
</tr>
</tbody>
</table>

\(^a\) This refers to one of the figures of this paper which shows a spectrum typical of the one integrated.

\(^b\) The outer lines at high and low field assigned to Centers 3 and 4 were omitted in this integration.

ubiquinone-reductase. Understandably, there are additional resonances present as will be discussed (5).

**Fig. 8.** Plot of data from a kinetic freeze-quench experiment on the anaerobic reduction of Complex I by APDPNH. Complex I, 94 mg of protein per ml and dissolved as for Fig. 1, was mixed with 39 mM APDPNH at a ratio of 5:1. The APDPNH was dissolved in the same buffer and 2 mM Tris base was added to bring the Tris concentration to 0.2 M and the pH to a value of 9.1. Each point on the abscissa corresponds to a separate sample. The data are plotted as percentage reduction (ordinate) versus time elapsed between mixing of Complex I with APDPNH and freezing of the sample (abscissa). The degree of reduction of the centers is evaluated as in Fig. 7. The notation used is that of Fig. 7.

**Fig. 9.** EPR spectra of Complex I and of proteins obtained by treatment of Complex I according to Ref. 8. A, Complex I reduced with an excess of DPNH, B, sediment obtained by low speed centrifugation of Complex I that had been treated with 2.5 M urea for 10 min at 37°. C and D, ammonium sulfate fractions of the supernatant from B obtained at 27.5% and 36.4 to 52.9% saturation, respectively. The protein concentrations were 13.0, 19.8, and 10.1 mg per ml for B, C, and D, respectively. The samples for B to D were reduced with an excess of solid dithionite. The conditions of EPR spectroscopy were as in Fig. 1.
FIG. 10. EPR spectra of Complex I exposed anaerobically to DPNH for various lengths of time. Complex I, 18.2 mg of protein per ml and dissolved as for Fig. 1, was reduced with 130 neq of DPNH and frozen at 1 min for A. B, the sample of A was kept anaerobically at 0° for 10 min and refrozen. C, the sample of B was thawed and left at 0° for another 30 min. D, the sample of C was thawed and left at 0° for a further 120 min. E, the spectrum of the sample at the stage of C above recorded at 42 K. The conditions of EPR spectroscopy were as for Fig. 1 except that for E the temperature was 42 K. 

(16) showed that these signals probably arose on deterioration of the protein. It may be recalled that Beinert et al. (17) had shown that soluble DPNH dehydrogenase deteriorates when exposed to DPNH for long periods. An alternative suggestion (18) that the unexplained signals were due to molybdenum in the enzyme was not substantiated (19). By spectroscopy at 13 K we have essentially confirmed the suggestion of Kawakita and Ogura (16) that the enzyme deteriorates when exposed to DPNH. Typical records are shown in Fig. 10. The spectrum of reduced Complex I exposed to DPNH for only one min before the sample was frozen is shown in A. When the sample was thawed and further exposed to DPNH (Curves B to E) the resonances due to Center 1 disappeared and new resonances appeared. Contrary to the resonances from the intact iron-sulfur centers, these resonances are readily saturated with high microwave power and can easily be detected at 90 K (6). It thus appears that Center 1 is the most labile structure in Complex I in the presence of substrate and that the previously observed sensitivity of DPNH dehydrogenase toward DPNH (17) is probably due to this lability. A possibly related observation is, that Complex I cannot be effectively reduced with dithionite. We found that only Centers 2 and 3 are reduced; we are not certain about Center 4, but the signal of reduced center 1 was never seen after addition of dithionite, so that the reduction appeared to go only to the state of Fig. 1C.

Acknowledgments—We are indebted to Dr. Y. Hatefi for helpful discussions and for a gift of Complex I and fractions derived from Complex I, for comparison to our own preparations. We also gratefully acknowledge the assistance of Mr. W. D. Hamilton with instrumentation and Eva Olson with preparative work.

REFERENCES
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