# EPR DETECTABLE ELECTRON ACCEPTORS IN SUBMITOCHONDRIAL PARTICLES FROM BEEF HEART WITH SPECIAL REFERENCE TO THE IRON-SULFUR COMPONENTS OF DPNH-UBIQUINONE REDUCTASE*

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An attempt is made to identify and assign, for sonic fragments from beef heart mitochondria at various oxidation states, all electron magnetic resonances which have been resolved thus far at observation temperatures ≥4.2° K. Novel features of this work are the resolution of four different iron sulfur centers of the DPNH-ubiquinone reductase fragment and the observation of an unidentified electron acceptor - maximally evident at intermediate oxidation states - which is concentrated in the DPNH-cytochrome c reductase fraction.

In the course of the past 12 years it was shown by EPR spectroscopy that mitochondria contain a number of electron acceptors in addition to those known from spectrophotometry. Most of those recognized by observation at liquid nitrogen temperature have been identified and assigned to specific components (1,2). This is a preliminary report on our attempts to sort out the resonances which are observable in beef heart mitochondria at temperatures between 4.2 and 77° K.

Figure 1 shows typical spectra obtained at 13° K on submitochondrial particles ($ETP_H$) (3) at different oxidation states produced by measured quantities of dithionite. Although all resonances referred to below can be recognized in these spectra, EPR spectroscopy at defined oxidation states of particle fractions prepared from mitochondria (4-6) furnished valuable clues to an understanding of the relationships of these resonances.

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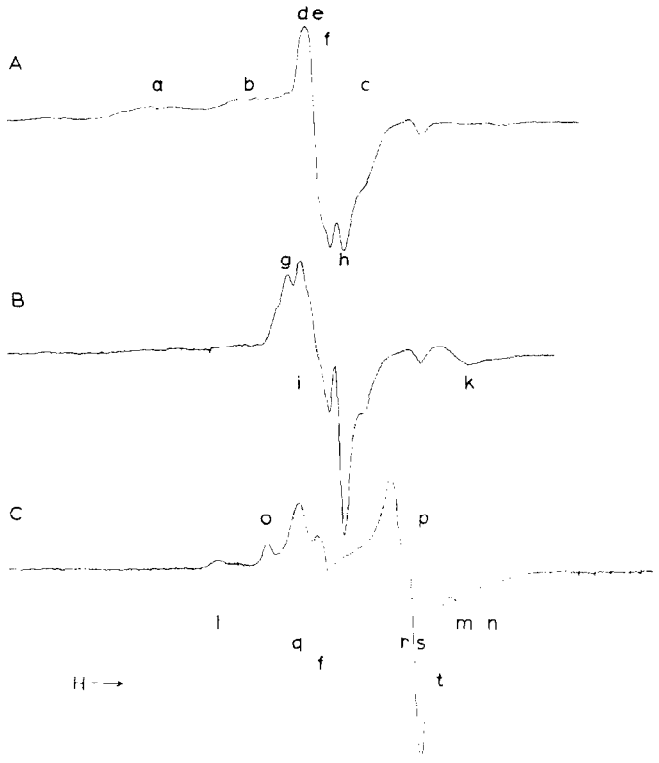


Figure 1: EPR spectra recorded on submitochondrial particles from beef heart (3). The particles, 40 mg/ml, were suspended in 0.25 M Sucrose 0.01 M Tris, pH 7.8. These particles had been treated with 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole (12) to prevent reduction by endogenous reductants. For each particular state of reduction 0.60 ml of particle suspension were placed in a special cell (13), which was repeatedly evacuated and filled with nitrogen (<0.25 ppm of oxygen). The particles were reduced with the desired amount of solid dithionite (13). Three minutes after contact with the reductant the samples were frozen and stored in liquid nitrogen. Particles in A, as prepared; B, reduced with 4.9 electron nanoequivalents (neq); and C, with 210 neq of dithionite. The samples were cooled by a flow of cold helium gas. The temperatures were measured with a carbon resistor below and an iron-doped gold thermocouple immediately above the sample. They are corrected to the latter position of the measuring device. The microwave frequency was approximately 9.2 GHz for all experiments; the power 0.3 mwatt, the modulation amplitude 7.5 gauss, the temperature 13° K, the time constant 0.5 sec and the scanning rate 200 gauss per min. The abscissa indicates magnetic field linearly increasing to the right, the ordinate the first derivative of the microwave absorption in arbitrary units. Small roman letters are placed along the spectra vertically above or below field positions of resonances typical for various components. Letters referring to a particular set of lines are found on the same horizontal and are consecutive letters of the alphabet. The small letters used throughout the Figures always refer to the same lines and components. The letters a, b, c refer to lines due to parts of the copper signal of cytochrome oxidase. This signal is saturated under the conditions used. At t a shoulder due to the high field part of the resonance of succinate dehydrogenase is observed; f indicates free radicals.

Resonances previously known, but in mitochondria or particles derived therefrom only poorly or not at all resolved at liquid nitrogen temperature, are the following: 1.) those arising from the oxidized forms of cytochromes a and $a_3$ (7,8) and 2.) a signal with derivative peaks at g = 2.019 and g = 2.005 (Fig. 1A, d,e,), presumably due to high spin ferric iron in a cubic environment. Signals of this type have been found in a number of proteins by several workers[1]. On fractionation this resonance is found to some extent in all fractions but is concentrated in ubiquinone-cytochrome c reductase (complex III) (6).

The resonances which we have been able to resolve and assign are four iron sulfur centers associated with the DPNH-ubiquinone reductase. On fractionation these resonances clearly follow this fraction (complex I). On partial reduction

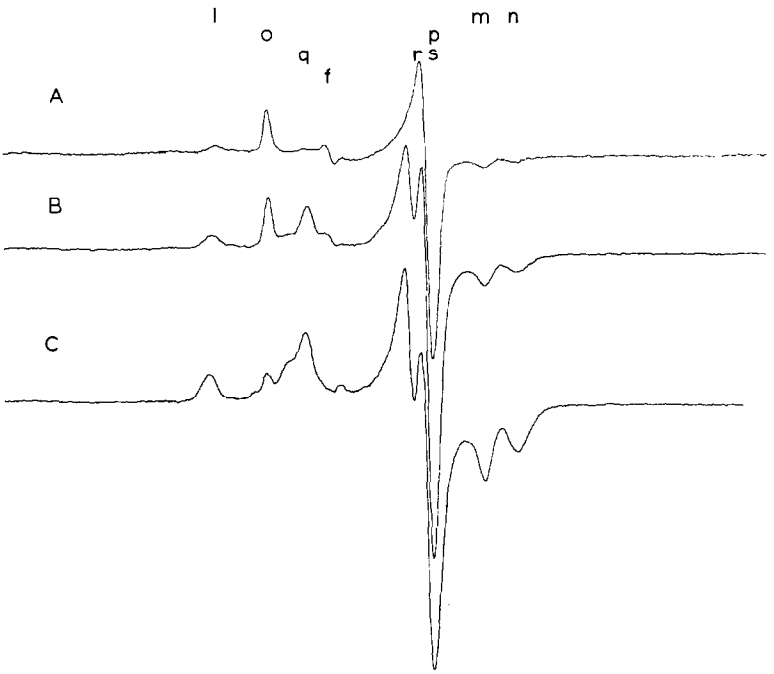


Figure 2: EPR spectra of DPNH-ubiquinone reductase, 18.2 mg protein/ml dissolved in 0.66 M Sucrose, 1 mM histidine and 0.05 M Tris, pH 8. 0.4 ml of this solution was treated in a special cell with sidearms as described (8) and was reduced with the desired amount of DPNH. A: reduced with 10.6 electron neq of DPNH; B and C: with 127 neq. Conditions of EPR spectroscopy as for Figure 1, except that C was recorded at 7.7° K.

1 Personal communication from Drs. G. Palmer and J. Peisach.

with DPNH or addition of dithionite only the resonances of iron-sulfur centers 2 and 3 appear (Fig. 2A, o,p and l,m,n). On further reduction centers 1 (Fig. 2B, q,r,s) and 4[2] (Fig. 3) can be seen. The spectrum of center 1 (Fig. 4) can be obtained by subtraction of the spectrum at partial reduction (Fig. 2A) from that at full reduction (Fig. 2B). The resonances of centers 3 plus 4[2] are particularly emphasized at high power and low temperature (Fig. 2C). The signal of center 2 is most readily saturated. In spectra of $ETP_H$ or DPNH-cytochrome c reductase (complex I + III) (5) these resonances overlap those of the iron-sulfur protein of the cytochrome b, $c_1$-region (Fig. 1B, i,k) (6) which is not present in DPNH-ubiquinone reductase. This iron-sulfur protein is concentrated in the ubiquinone-cytochrome c reductase fraction and its EPR signal can be observed at liquid nitrogen temperature, as previously shown (6). In this fraction and at this temperature the signals of centers 3 and 4 of DPNH-ubiquinone reductase are not observable. The resonance of center 1 is that seen largely at liquid nitrogen temperature in a number of DPNH dehydrogenase preparations. Center 1 appears to be the most labile component. Its signal

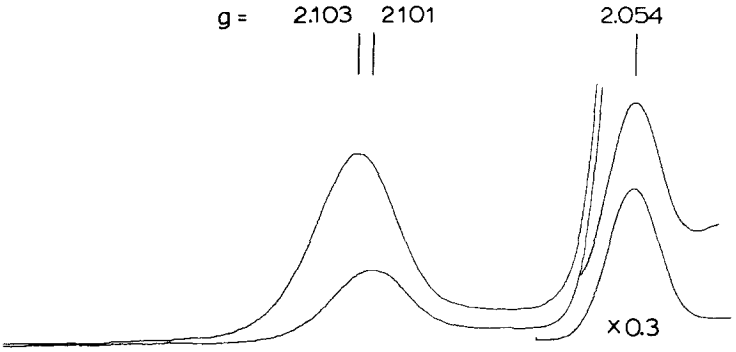


Figure 3: Enlarged recordings of peaks 1 and o of centers 3 and 2, respectively, of samples similar to, but more concentrated, than those of Figure 2, indicating the appearance of the signal of center 4 at the low field side of that of center 3. Note that the peaks at g = 2.05 (o) were recorded at 1/3 the amplification and coincide, while that at g = 2.10 (1) shifts. Microwave power, 3 mwatt; modulation amplitude 14 gauss, scanning rate 100 gauss per min, time constant 1 sec, temperature 13° K and frequency 9.1758 GHz for both samples.

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[2] Because of overlap of resonances only two lines (at l and n, Fig. 1) can be clearly assigned to center 4. An average g-value is therefore not calculated, but it may be close to that of center 3.

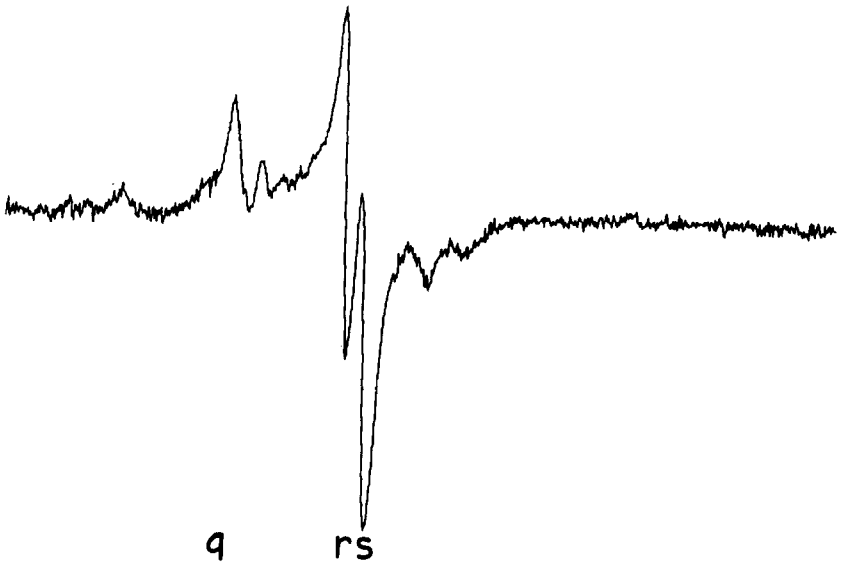


Figure 4: Difference spectrum of Figure 2B and 2A, approximating the spectrum of iron-sulfur center 1. As there was also an increase in the signals of iron-sulfur centers 3 and 4 on progressive reduction from the state of sample A to that of B, corresponding signals from iron-sulfur centers 3 plus 4 are also seen in this difference spectrum at high and low field. The difference spectrum was obtained by the use of a digital computer. Microwave power 0.01 mwatt, modulation and temperature as for Figure 1, time constant 0.25 sec and scanning rate 100 gauss per minute.

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disappears on exposure to DPNH for more than five minutes and new unexplained signals appear (cf. 9,10). These are not due to Mo, since neither $ETP_H$ nor DPNH-ubiquinone reductase contain more than 0.03 atoms of Mo per acid-extractable flavin molecule, according to chemical analyses.

Double integrations of the signals of DPNH-ubiquinone reductase show that each of centers 1-4 takes up a number of electrons approximately equal to the molarity of flavin present. The field positions of the prominent peaks for the centers are (average g values in parenthesis are calculated from measured peaks at $g_x$ and $g_z$ and from $g_y$ values interpolated from the position of the center peak): 1, 2.022, 1.938, 1.923 (1.96); 2, 2.054, 1.922 (1.97); 3, 2.101, 1.886, 1.864, (1.95); 4, 2.103, 1.863[2].

The iron-sulfur centers are therefore probably of the ferredoxin type, which means that each center contains 2 or 4 iron atoms, which are capable of taking up one electron. Thus DPNH-ubiquinone reductase is one of the most

complex ferredoxin-type structures known thus far. Twenty-three to 26 iron and labile sulfur atoms per flavin are found associated with this fraction. The centers recognized thus far could maximally account for $4 \times 4 = 16$ of these.

It should be noted that resonances similar and probably analogous to those designated here as o,p and m have been observed by Ohnishi *et al* (11) in submitochondrial particles from yeast and assigned to a single iron-sulfur component of DPNH dehydrogenase.

In addition to the described resonances which we were able to tentatively identify and assign, we have also observed a strong resonance with peaks at $g = 2.036$ and $g = 1.986$ (cf. Fig. 1B, g,h) at X band frequency, which appears only at intermediate oxidation states and disappears again on reduction. The origin of this resonance is not clear at this time. On fractionation it follows the DPNH-cytochrome *c* reductase complex and retains the property of appearing only on partial reduction. It is also clearly seen in partly reduced whole mitochondria indicating that it is not due to an artefact.

On titration of $ETP_H$ with DPNH or dithionite the first equivalents reduced-cytochromes *a* and $a_3$, and the high spin non-heme iron (Fig. 1A, d,e); additional eqs reduce copper, cytochromes *b*, and *c* + $c_1$ and the associated iron sulfur center (6), with the iron sulfur centers of the dehydrogenase reduced last. Among these the gradation of potentials is clearly in the order: center 2>3>4>1. The unidentified electron acceptor appears to occur in 3 states (which may be 3 different states of oxidation), as its signal initially increases on reduction with dithionite (or with DPNH in DPNH-cytochrome *c* reductase) and then disappears. Kinetic experiments on the course of reduction of the components of DPNH-cytochrome *c* and DPNH-ubiquinone reductases by DPNH or acetyl-pyridine DPNH show the following sequence of appearance of the signals of the iron sulfur centers: 2, 3 + 4, 1. With DPNH at 20° all four centers are reduced within 10 msec. From these experiments a sequence of electron transfer between the iron-sulfur components cannot be deduced, but the observed sequence of signal appearance poses certain restrictions on the ratios of rate constants of individual steps. Where present,

cytochromes b and $c_1$ and the associated iron-sulfur center are reduced thereafter. The high spin non-heme iron and the unidentified electron acceptor are reduced only within several seconds and, therefore, may not be of significance as catalysts, although they must be reckoned with in titrations and measurements of potentials. Although we think that all of the signals attributable to known functional components of mitochondria occur in each individual molecular unit of such components, heterogeneity of these units cannot be ruled out on the basis of available information.

The identity and significance of the unknown electron acceptor and that of the ferric iron will be the subject of further study as will be the interrelationships of the iron-sulfur components of the DPNH dehydrogenase region. It is obvious that all efforts to prove or disprove the essentiality of "non-heme iron" associated with the DPNH dehydrogenase region of the electron transport system must take account of every single one of the iron sulfur components now shown to be present and active in oxidation-reduction.

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