EPR STUDIES OF THE CYTOCHROME b-c_1 SEGMENT OF THE MITOCHONDRIAL ELECTRON TRANSFER SYSTEM*

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SUMMARY: Previously unreported resonances (X-band) were observed in beef heart mitochondrial fragments (ETP_H, Complexes II and III). They have characteristics of low field lines of low spin ferric heme compounds. On the basis of their behavior during reduction, as also monitored optically, they are attributed to cytochromes c_1 (g=3.33), b_K (g=3.44) and b_T (g=3.78).

In view of current interest in the cytochrome b-c_1 (b-c_1) region of the mitochondrial electron transfer system (1-3), a report on our attempts to characterize the EPR spectra of the electron carriers involved appears timely. To date we are aware only of reports on the resonances of the reduced iron sulfur protein (ISP) of this segment (4). Fig. 1A shows the EPR spectrum of the oxidized form of ubiquinone-cytochrome c reductase (Complex III (5)). In addition to resonances described previously (g=4.3; g=2.019 and 2.005, see (6)), and a small amount of reduced ISP (g=2.026; 1.887; 1.809), there are two prominent peaks at g=3.778 and 3.427. On addition of an excess of ascorbate (Fig. 1B) the peak at g=3.4 decreases and, as known (4), the ISP signal rises. With dithionite both peaks (g=3.8 and 3.4) disappear (Fig. 1C). In the EPR spectrum of succinate-ubiquinone reductase (Complex II (7), Fig. 1D) we find, in addition to the known signals at g=6; 4.3; and 2.019 and 2.005, a prominent peak (g=3.509) in the same region as with b-c_1. These new resonances of Complexes II and III at g=3.4 to 3.8 have the general appearance and properties of low field resonances (g_z) of low spin ferric heme compounds.** It should then be

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** As low field g-value g=3.8 appears unusually high; if this resonance is indeed to be interpreted as g_z of a low spin heme, g_y and g_x would be expected to be ≤ 1 and <1, respectively, and should be difficult to detect at the prevailing concentrations. If the g=3.8 resonance were a component of a high spin heme signal, we would expect to be able to observe an additional low field component for this signal (8). Since we failed to do so, we are inclined to consider the g=3.8 line as part of a low spin signal.

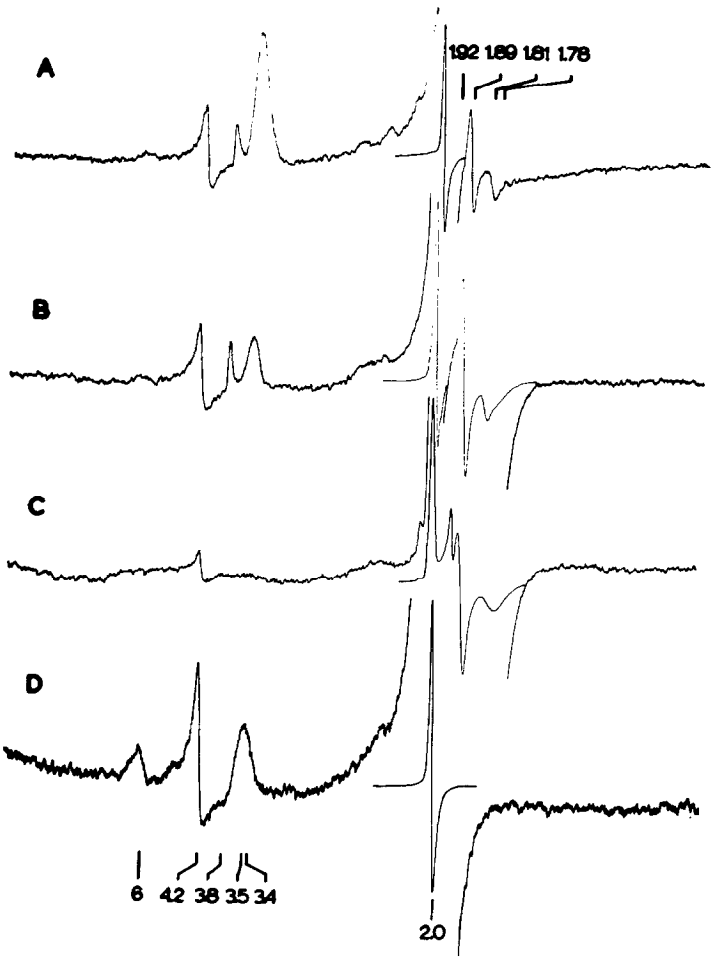

Fig. 1: EPR spectra of Complexes III and II. 13 mg of Complex III were dissolved in 0.4 ml of 0.66 M sucrose, 1 mM histidine and 0.05 M Tris chloride (pH 8.0) and 6 mg of Complex II were dissolved in 0.4 ml of 0.25 M sucrose. A, Complex III untreated; B, reduced with solid potassium ascorbate; C, with solid dithionite; D, Complex II untreated. Conditions of EPR spectroscopy: microwave frequency, 9.16-9.17 GHz, microwave power, 3 mW; modulation frequency, 100 KHz and amplitude, 6 gauss; temperature, 13°K; time constant, 0.5 sec; and scanning rate, 1000 gauss/min. The insets at g $\sim$ 2 in A and B were recorded at 1/25, in C at 1/10, and in D at 1/200 the amplification used for the main spectrum. The insets at g=1.8 to 1.9 in B and C were recorded at 1/10 the amplification of the main spectrum. The positions of prominent peaks are given on the scale of g values but are not to be considered as such.

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possible to detect at least the central line (g_y) of the resonances at g=3.4 to 3.5. The spectra of Fig. 1 as well as other records we obtained leave little doubt that g_y is at g 2.0 to 2.1, where a broad line appears superimposed on the sharper resonances at g $\sim$ 2. According to theory (9,10) the high field

resonance associated with g_z = 3.4 to 3.5 and $g_y \sim 2$ should be located at g <1 and might be hard to detect because of its width.

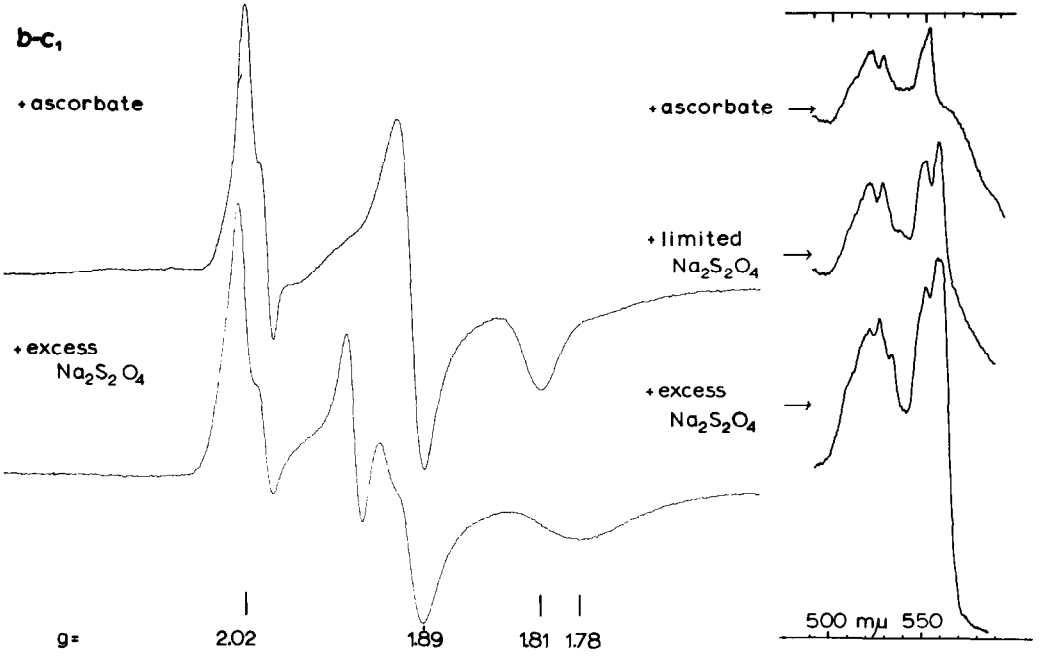

Fig. 2: EPR spectra (g ~ 2 region) and optical reflectance spectra of Complex III. The samples were analogous to those of Fig. 1 but more dilute (~1/2) for better optical resolution. The oxidation state of the samples was as indicated in the figure. The EPR spectrum of the sample partially reduced with dithionite is not shown since it is simply a superposition of the other two spectra. Contamination by a small amount of DPNH dehydrogenase is indicated by the peak at g=1.92. Conditions of EPR spectroscopy: microwave frequency, 9.16-9.17 GHz, microwave power, 0.3 mwatt; modulation frequency, 100 KHz and amplitude, 7 gauss; temperature, 32°K; time constant, 0.5 sec; and scanning rate, 200 gauss/min. For an evaluation of the relative intensities of the reflectance spectra cf. ref. 18.

Fig. 2 shows details of the EPR spectra of ISP at 32°K and corresponding reflectance spectra recorded at 95°K. According to these, ascorbate reduces ISP and $\underline{c}_1$ only, indicating that the resonance at g=3.427 is due to, or has a major contribution from $\underline{c}_1$. With an excess of dithionite the optical spectra clearly show reduction of both $\underline{b}_K$ and $\underline{b}_T$ (cf. (11)), while both EPR signals (g=3.4 and 3.8) have disappeared (Fig. 1C). With a limited amount of dithionite a state is reached with $\underline{c}_1$ and only $\underline{b}_K$ reduced (Fig. 2). Under these conditions the resonance at g=3.4 is decreased but that at g=3.8 undiminished. We would also

like to draw attention to the change in the ISP resonance from the ascorbate- to the dithionite-reduced state (Fig. 2). This had been observed previously

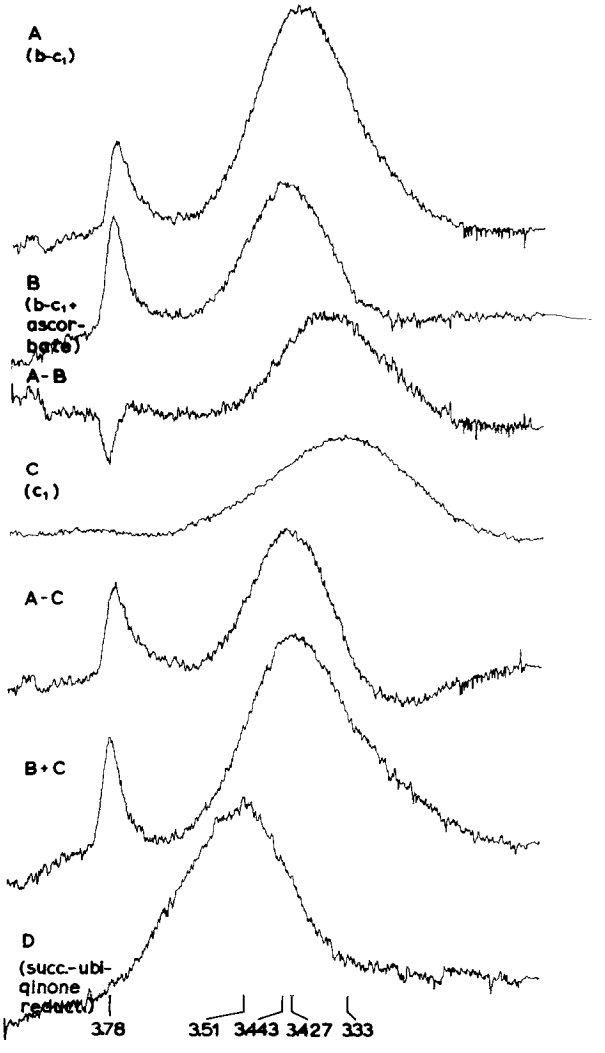

Fig. 3: EPR spectra (g ∿ 3 to 4 region) of Complexes II and III and of purified cytochrome c_1. Samples analogous of those of Fig. 1 were employed. 4 mg of Complex II were dissolved in 0.25 ml 0.25 M sucrose; 15 mg of Complex III, in 0.25 ml 0.66 M sucrose, 1 mM histidine, 0.05 M Tris chloride (pH 8.0); and 25 nmoles of cytochrome c_1, in 0.25 ml of 0.05 M phosphate buffer (pH 7.4). c_1 was purified according to Yu, Yu and King (13). Nine spectra of each sample were accumulated in the Varian C-1024 computer of average transients under the conditions given in Fig. 1, except that the time constant was 0.25 sec and the scanning rate was 200 gauss/min. The resultant spectra were recorded on tape and played back into the C-1024 for addition and subtraction. In this step the signal height of C was adjusted to match the height of A-B. Note that the peak at g=3.78 is somewhat larger and of different shape after addition of ascorbate, for reasons not understood at present. The difference spectrum A-B has therefore a negative peak at g=3.78.

(4) but it was thought that this may be an effect of dithionite. Since, however, the integrated area under the absorption curve of ISP remains constant within ± 20% and the same transition from the ascorbate to the dithionite-type occurs on progressive reduction of DPNH-cytochrome c reductase by DPNH, we conclude that this transition is coupled to the reduction of another component, presumably b_K according to our more complete titration data (12), of which Fig. 2 shows excerpts.

Fig. 3A shows an enlarged record of the g=3 to 4 region of the EPR spectrum of Complex III. It can be clearly seen that the peak at g=3.427 is asymmetric and that after addition of ascorbate a more symmetric and more narrow signal remains with the peak shifted by 9 gauss (to g=3.443; Fig. 3B). Subtraction of B from A results in a line with $g \sim 3.3$ and this obviously represents the component reduced by ascorbate. We conclude from experiments such as those of Figs. 1-3 that at X-band the resonances of c_1 and b_K are not separated and that the g=3.443 peak in the spectrum of Fig. 3B represents b_K, the peak in Fig. 3 (A-B; $g \sim 3.3$) c_1, and that at g=3.778 b_T. We were able to support this conclusion by comparison of the first three spectra of Fig. 3 with the corresponding spectra obtained with functionally competent, purified c_1 (Fig. 3C), which was kindly provided by Drs. C.A. Yu and T.E. King (13). Thus A, B and A-B in Fig. 3 should be compared to B+C, A-C and C, respectively. Although we consider the agreement satisfactory, some discrepancies are seen because of the larger line-width of purified c_1 which might be due to microheterogeneity or slight modifications on purification. For comparison we also present an enlarged record of Complex II (Fig. 3D), showing that its resonance differs considerably from that of b_K in Complex III.

Analogous EPR spectra can be observed with DPNH cytochrome c reductase (14) and sonicated particles (ETP_H (15)). The ETP_H had been treated with 4, 5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB (16)) to prevent reduction by endogenous reductants, and frozen. Therefore, the extent of coupling in these particles is uncertain and was not considered in this prelim-

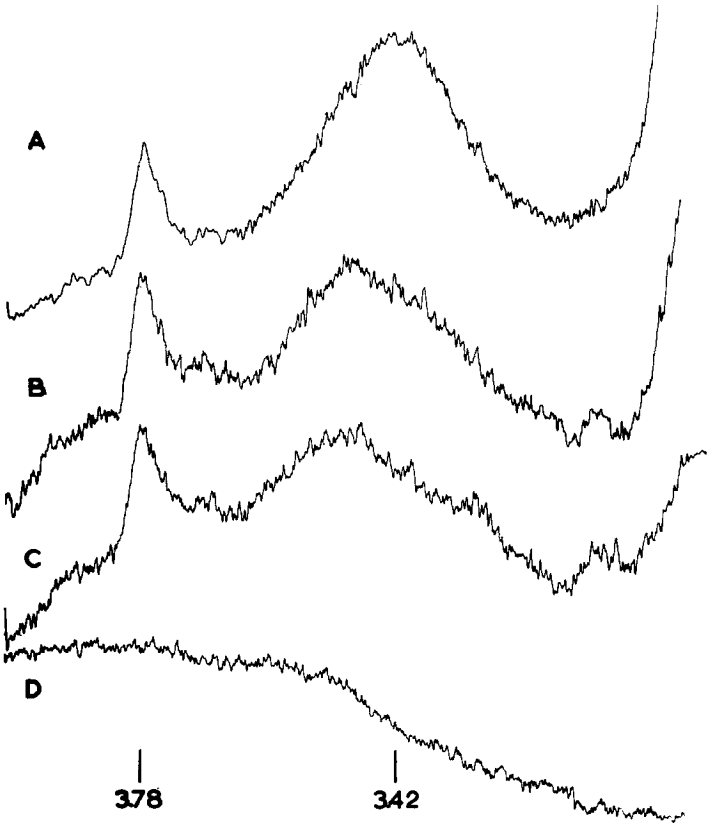

Fig. 4: EPR spectra (g ∿ 3 to 4 region) of ETP_H. 36 mg of TTFB (16) treated ETP_H (15) were suspended in 0.5 ml of 0.25 M sucrose, 0.01 M Tris chloride (pH 7.8). A, untreated; B, reduced with 80 mM potassium ascorbate; C, as B, and in addition 80 mM succinate; D, with dithionite (baseline). Addition of antimycin to C does not bring about additional reduction by more than 20%. The conditions of EPR spectroscopy were those of Fig. 3. 16 Spectra were accumulated in the C-1024.

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inary work. Fig. 4 shows the resonances corresponding to those discussed above in ETP_H as obtained and ETP_H reduced with ascorbate, succinate or dithionite. The position of the g=3.4 peak is at somewhat higher field (g=3.417). This could be due to the presence of a mixture of $\underline{b}_K$ and $\underline{c}_1$ of somewhat different proportions or to a slight modification of $\underline{b}$-$\underline{c}_1$ on isolation. We are also unable to say whether in any of the particles examined a resonance of low intensity of the kind given by Complex II (g=3.509) is superimposed on those at g=3.4. In experiments not shown here we observed that with DPNH as reductant in the presence of rotenone all resonances in the g=3 to 4 region remained un-

changed whereas in the presence of antimycin both the g=3.4 and the g=3.8 resonance disappeared completely.

A number of experiments have been proposed in the course of the years which should permit differentiation between various species of b (cf. 1-3,17). In a number of these we have not been able to see components other than those described above, except in a series using cyanide. As shown in Fig. 5 two new species appear in the presence of cyanide, one with g=3.516, possibily related to the g=3.509 species of Complex II, and another species at g=3.608. The former appears on addition of cyanide and is reduced by succinate only in

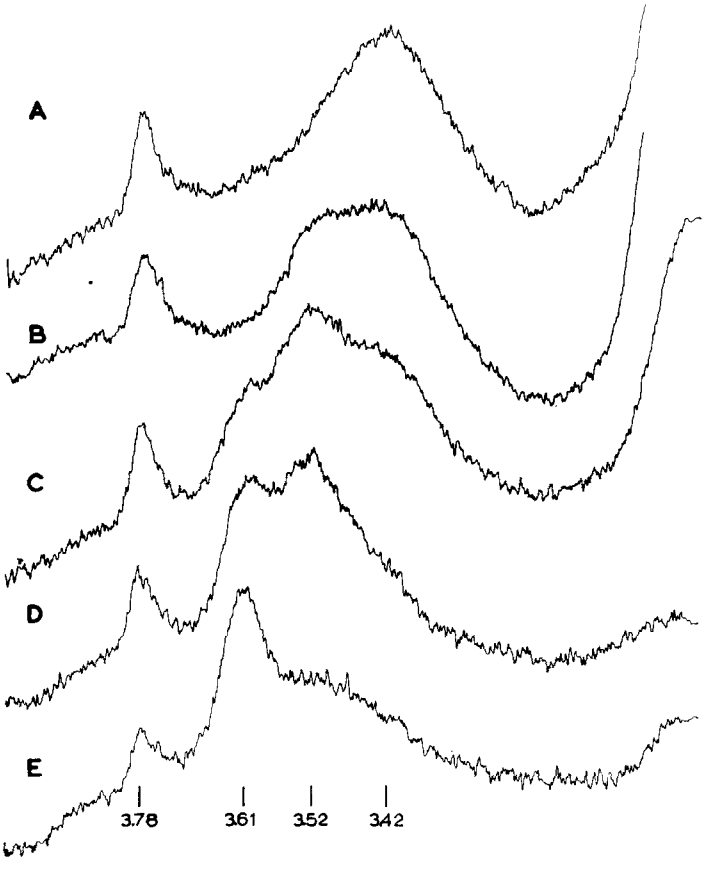

Fig. 5: EPR spectra of ETP_H in the presence of cyanide. 34 mg of TTFB treated ETP_H were suspended in 0.4 ml 0.25 M sucrose, 0.01 M Tris chloride (pH 7.8). A, untreated; B, with 5 mM cyanide; C, as B, in addition 85 mM ascorbate; D as C, and in addition 80 mM succinate; E, as D and in addition 595 µg of antimycin A/gm protein. Note that in the presence of antimycin and succinate (E) b_T is partly reduced. EPR spectroscopy as for Fig. 4, with 9 spectra accumulated in the C-1024.

the presence of antimycin, the latter, arising on addition of ascorbate, is not reduced under these conditions. We are not certain of the identity of the corresponding species which exist in the absence of cyanide. These observations caution against the assumption that species made detectable through special conditions are always naturally occurring. EPR appears more suited than light spectroscopy to detect subtle modifications.

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