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The EPR spectrum of isolated Complex III

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SUMMARY

It has been found that antimycin shifts the EPR signal of oxidized Complex III at $g = 3.44$ (ferricytochrome *b*-562) to $g = 3.48$, while the signal at $g = 3.8$ (ferricytochrome *b*-566) sharpens. Antimycin also affects the optical spectrum of ferricytochrome *b* by sharpening the α-band and splitting the γ-band. It is shown that nitric oxide reacts irreversibly with the non-heme iron components of Complex III. A reaction of NO with ferrocytochrome *b*-566 is suggested, resulting in lines at $g = 2.10$, 2.07 and 2.01.

Orme-Johnson *et al.*[1] have reported lines at $g = 3.8$ and $g = 3.4$ in isolated Complex III (QH_2–cytochrome *c* reductase) and assigned them to ferricytochromes b_T and b_K (ref. 2), respectively**. We have confirmed these findings, but find that a pre-incubation with ferricyanide for 5 min at 0 °C is necessary for maximal development of the $g = 3.8$ line. Judging by the increase in intensity of this line, cytochrome *b*-566 was about 70% oxidized in the sample of freshly isolated complex used in these experiments. The asymmetric highly temperature-sensitive signal around $g = 2.00$, attributed to cubic iron[4], was also increased in intensity by 60% by this treatment, while the line at $g = 1.89$ due to reduced iron sulphur[5] disappeared entirely within 15 s at 0 °C. The intensity of the $g = 3.4$ line was unchanged.

It was found necessary to use low concentrations of ferricyanide (not greater than 0.15 mM) to avoid interference by an unusual line at $g = 2.7$, which disappeared at temper-

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** These cytochromes will be referred to in this paper as *b*-566 and *b*-562, respectively (*cf.* ref. 3).

atures above 35 °K. Since the intensity of this signal varied with different batches of ferricyanide, it is probable that it is due to an impurity, perhaps to molecules containing less than six cyanide groups. Exposure to 0.15 mM ferricyanide for longer than 5 min at 0 °C resulted in a gradual decline in the intensity of the lines.

Dodecyl sulphate (0.4% of the sodium salt) had no effect on the intensities of the lines given by the iron components after these had reached a maximum.

The EPR resonances of the *b* and c_1 cytochromes are quite temperature-dependent, broadening considerably above 25 °K. The lines due to the *b*-cytochromes could be observed without interference of that of c_1 ($g = 3.35$) by reduction of the latter by treatment with ascorbate for short periods. Reduction with $Na_2S_2O_4$ resulted in loss of the haem resonances, except for a small residual line at $g = 3.8$. Incubation with succinate for 5 min at 0 °C resulted in the disappearance of about 95% of the line due to *b*-562, but of only about 20% of that due to *b*-566. (Complex III contains traces of succinate dehydrogenase.) Prolonged incubation (*e.g.* for 5 min at room temperature) caused some further reduction of *b*-566. This is in agreement with previous observations made by absorption spectrophotometry[6].

After standing for 2 days at 0 °C in 36% satd $(NH_4)_2SO_4$ – 2% cholate a line at $g = 2.95$ appeared. A similar resonance has been previously noted in an aggregated form of cytochrome *b*, prepared in Dr T.E. King's laboratory (Beinert, H. and King, T.E., personal communication), as well as in cytochromes b_2 and b_5 (refs 7,8). Succinate caused a decline in the intensity of this line, indicating that the modified cytochrome *b* is able to accept electrons from succinate.

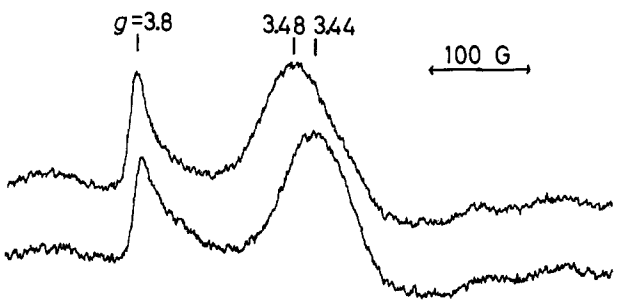

Fig.1. Effect of antimycin on the EPR spectrum of cytochrome *b* in Complex III at 11 °K. Complex III was concentrated with $(NH_4)_2SO_4$ in the presence of 1 mM ferricyanide and dissolved in 0.66 M sucrose, 50 mM Tris–HCl buffer (pH 8.0) and 1 mM histidine to a protein concentration of 80 mg/ml. 0.25 ml of the complex was mixed with antimycin (in ethanol, 6 nmoles antimycin/mg protein) in an EPR tube and the mixture was kept for 5 min at 0 °C. Then 5 mM ascorbate (pH 6.0) was added and after 5 s the mixture was frozen in liquid nitrogen. In a control experiment an equivalent amount of ethanol (2%, v/v) was used instead of antimycin. Upper trace, *plus* antimycin; lower trace, ethanol only. Spectra were taken on a Varian E-3 EPR spectrometer equipped with a helium flow system from Air Products. *g*-values were determined from field markers of prominent resonance peaks previously obtained on a Varian 4502-10A equipped with a proton probe and Hewlett–Packard frequency counter. These *g*-values are accurate to ± 0.03. A carbon resistor was placed just below the sample and was previously calibrated against a calibrated germanium resistor situated at the sample position. Spectrum conditions: microwave power, 10 mW; modulation amplitude, 12.5 G; modulation frequency, 100 kHz; temperature, 11 °K.

Antimycin, which has been shown to shift the α-absorption peak of ferrocytochrome *b*-562 about 1 nm to the red[9-11], also has an effect on the EPR spectrum of the ferricytochrome. This is most clearly seen when cytochrome c_1 is reduced by a short treatment with ascorbate. Two effects are obvious in Fig. 1: (i) an apparent sharpening of the line at $g = 3.8$ due to a decrease in intensity of the high-field shoulder; (ii) a shift of the line at $g = 3.44$ by 15 G to $g = 3.48$. Ethanol at the concentration used to dissolve the antimycin had no noticeable effect on the shape of the EPR spectrum or on the g values under the same experimental conditions, although it did cause an increase of the intensity of the lines at $g = 3.4$ (by 9%) and at $g = 3.8$ (by 38%). Ethanol was present in the control in the experiment shown in Fig. 1.

Under the same conditions as used for measurement of the EPR spectrum, except for the fact that the temperature was 77 °K instead of 12 °K, antimycin was found to bring about a multiple splitting of the Soret band of oxidized Complex III, as well as an increased resolution of the absorption bands in the visible region (Fig. 2).

The addition of succinate resulted in a greater decline of EPR absorption at $g = 3.8$ (ferric *b*-566) in the presence of antimycin than in its absence (Fig. 3). A part of the *b*-562 was not reducible by succinate, in the presence of antimycin, resulting in a residual line at $g = 3.48$. The high-field shoulder on the $g = 3.8$ line could be resolved as a $g = 3.71$ line in the presence of succinate and antimycin. This is presumably due to a modified non-reducible *b* species.

Nitric oxide was found to have a marked effect on the EPR spectrum of oxidized Complex III. After treatment with gaseous NO for 10 min at 20 °C, the intensity of the non-haem iron signal in the region of $g = 2$ declined greatly in intensity. The EPR spectrum (Fig. 4, upper) is dominated by two lines at $g = 2.04$ (top) and 2.02 (trough), visible at temperatures as high as −30 °C, where the signal begins to

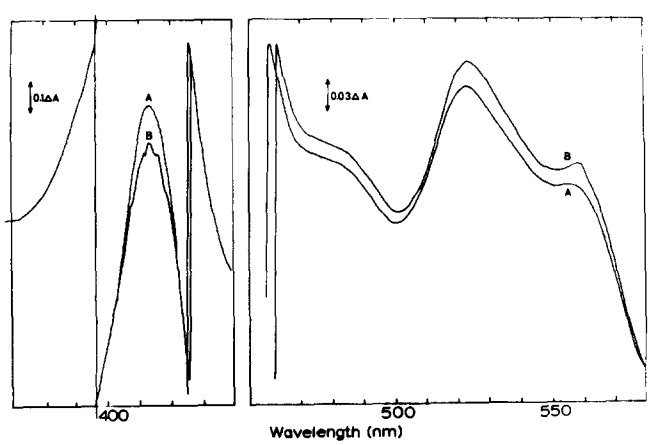

Fig. 2. Optical absorption spectrum of oxidized Complex III at 77 °K. Complex III (0.9 mg/ml containing 3.5 nmoles cytochrome c_1 per mg protein) was oxidized with 5 μM ferricyanide and the spectrum recorded at 77 °K with a Perkin–Elmer 356 spectrophotometer with buffer as reference. The light path was 3 mm. A, 1% (v/v) ethanol added; B, 5.5 nmoles antimycin/mg protein added.

become saturated at microwave powers above 1 mW. Lines at $g = 1.95$, studied only below 40 °K, and at $g = 2.07$, studied only at 33 °K or below, are also visible. At 12 °K the trough at $g = 2.02$ was much more easily visible when the microwave power was increased above 10 mW. These effects of NO resemble those described for various haemoproteins.

The reaction of NO with succinate- or dithionite-reduced Complex III appeared to be irreversible, since replacement of the unreacted NO by evacuation and refilling with nitrogen, repeated 8 times, did not result in a return of the original spectrum. In the spectrum of NO-treated, succinate-reduced complex (Fig. 4, middle), the trough at $g = 2.02$ is much broader, as the result of the appearance of extra lines at

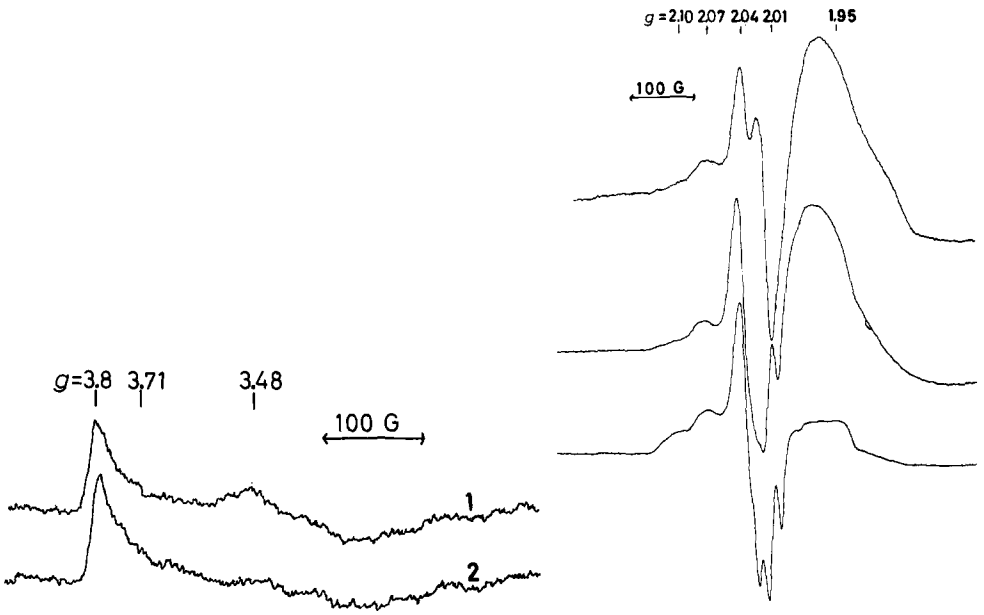

Fig.3. Reducibility of the *b* cytochromes, by succinate in the presence and absence of antimycin. 0.25 ml Complex III, prepared as for Fig.1, was mixed with antimycin (6 nmoles antimycin/mg protein) or an equivalent amount of ethanol (2%, v/v) in an EPR tube and the mixture was allowed to react 5 min at 0 °C. The tubes were then immersed in liquid nitrogen and the EPR spectra were scanned (not shown). After thawing, 10 mM succinate was added and the tube was kept for 5 min at 0 °C and then frozen in liquid nitrogen. Trace 1, *plus* antimycin; Trace 2, ethanol control. Spectrum conditions as in Fig.1.

Fig.4. Effect of NO on the EPR spectrum of Complex III in the $g = 2$ region at different redox states. Upper trace: 0.35 ml of Complex III as prepared for Fig. 1 was mixed with excess NO in an anaerobic EPR tube (Thunberg cell). After 10 min at room temperature the mixture was frozen in liquid nitrogen. Middle trace: same as upper trace except that before the reaction with NO, the Complex III was reduced with 55 mM succinate anaerobically for 10 min at room temperature. Lower trace: same as upper trace but now the complex was reduced with excess solid dithionite for 10 min at room temperature anaerobically prior to the NO treatment. Spectrum conditions: microwave power, 10 mW; modulation amplitude, 12.5 G; modulation frequency, 100 kHz; temperature, 14.5 °K except for the upper trace where it was 12 °K; The upper and the middle traces were scanned with a 3.3 times and 1.7 times higher gain, respectively, than the lower trace.

$g = 2.10$ (top) and $g = 2.00$ (trough). The lines at $g = 2.04$ and 2.07 increase in intensity. The broad resonance at $g = 1.95$ is more intense but of the same shape as in the oxidized complex. Situated on the major absorption at $g = 2.04$ is an unresolved triplet arising from nuclear hyperfine interaction due to ^{14}N (I = 1) from the liganded NO (^{14}N hyperfine structure equal to approx. 14 G). This triplet can be partially resolved by raising the temperature to 84 °K. This triplet is more clearly resolved after reduction with $Na_2S_2O_4$ (Fig. 4, lower curve). The lines at $g = 2.01$, 2.07 and 2.10 are more intense than with succinate as substrate.

The absence of resonance due to reduced iron–sulphur proteins suggests that NO reacts with such centres. Indeed, NO in the absence of O_2 bleaches and precipitates purified succinate dehydrogenase, with the liberation of much iron and the appearance of a line similar to that at $g = 1.95$ seen in Fig. 4. An identical signal was observed by saturation of the same buffer system with NO_2 gas (Matheson). We tentatively assign this resonance to a paramagnetic species of NO_2 and/or NO, the latter arising from the heterolytic cleavage of $(NO_2)_2$, *cf.* ref. 12.

Since the mean difference between succinate and dithionite-reduced Complex III is due to a greater reduction of *b*-566 by $Na_2S_2O_4$, it is suggested that the signals at $g = 2.10$, 2.07 and 2.01 are due to NO liganded to ferrocytochrome *b*-566. The appearance of different NO–heme derivatives attributable to the two different *b* species confirms the non-equivalence of these species.

These EPR studies indicate that there are only two major paramagnetic species of *b*-type cytochromes present in Complex III. The other *b*-species are present in much smaller amounts and are possibly modified forms. It is interesting to note that, when allowance is made for the interfering resonance of ferric c_1 the signal of *b*-562 is approx. 2–3 times as intense as that of *b*-566, in reasonable agreement with spectrophotometric measurements reported by Slater (*cf.* ref. 11, Table I).

These studies are being extended to phosphorylating particles.

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