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EFFECTS OF ATP, ANTIMYCIN AND CYANIDE ON THE EPR SPECTRA OF CYTOCHROMES IN PHOSPHORYLATING SUBMITOCHONDRIAL PARTICLES

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Summary

1. Treatment of phosphorylating ATP-Mg particles with antimycin in ethanolic solution results in a shift of the signal of low-spin ferric haem of cytochrome *b*-562 from $g = 3.44$ to 3.48. This shift is not caused by the ethanol.

2. The addition of ATP to particles treated with ascorbate and antimycin results in a shift of the signal at $g = 3.48$ back to $g = 3.44$. This effect of ATP is sensitive to uncoupler. In the absence of antimycin, ATP has no effect on the signal.

3. Neither ATP nor antimycin has any effect on the intensity of the signal due to cytochrome *b*-566.

4. Ethanol (5%, v/v) has marked effects on the EPR spectrum of cytochrome *c* oxidase which have to be taken into account when interpreting the effects of antimycin in ethanolic solution. It causes a shift of the signal of low-spin iron from $g = 3.01$ to $g = 3.05$ and a change of height and shape of the signal of high-spin iron at $g = 5.85$.

5. ATP added to particles treated with ascorbate and antimycin causes an increase in intensity of the signal at $g = 3.05$. This is correlated with an increased oxidation of cytochrome *c* and c_1 and cytochrome *c* oxidase observed spectroscopically.

6. The addition of cyanide to particles or isolated cytochrome *c*

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

oxidase, in the presence of reducing agent, results in the appearance of a low-spin signal at $g = 3.58$ in the absence of ethanol and at $g = 3.62$ in the presence of 5% (v/v) ethanol. This is ascribed to low-spin ferric haem of the cyano compound of cytochrome *c* oxidase.

In a previous communication [1] we reported that treatment of isolated Complex III (ubiquinol—cytochrome *c* reductase [2]) with antimycin results in a shift of the signal of the low-spin ferric haem of cytochrome *b*-562 from $g = 3.44$* to 3.48. In the present communication, the same effect of antimycin has been confirmed with phosphorylating submitochondrial particles. In addition, effects of ATP and cyanide on EPR resonances from low-spin ferric haems of cytochromes *b*-562, *b*-566, c_1 and *c* oxidase are described.

Phosphorylating submitochondrial particles (ATP-Mg particles) were prepared from heavy beef-heart mitochondria according to the method of Löw and Vallin [3]. EPR spectra were measured in Varian spectrometers (V-4501 A and E-9) with an Airco Cryo-Tip unit with automatic temperature control as previously described [1,4]. Other experimental conditions are specified in legends to figures.

Fig. 1A shows the EPR spectrum of phosphorylating particles without any additions. The EPR resonances observed are signals from low-spin ferric haem of cytochromes *b*-566 ($g = 3.78$), *b*-562 ($g = 3.44$), c_1 ($g = 3.35$) and the signal at $g = 3.01$ originating from the haem components of cytochromes *c* and *c* oxidase. Also observed is a signal at $g = 5.85$ (Fig. 1B) in the low field originating from high-spin ferric haem of cytochrome *c* oxidase. Treatment of the particles with 5% (v/v) ethanol results in a pronounced low-field shift (by approx. 32 G) of the signal at $g = 3.01$ to $g = 3.05$ (not shown). It also affects the axial component of the signal at $g = 5.85$, so that the rhombic component is better resolved (cf. ref. 5). It is noteworthy that the same effect was also observed when ethanol was added to purified cytochrome *c* oxidase. These results indicate that the ligand field of the haems is very sensitive to changes in the medium, and further gives a warning that effects of ethanol must be taken into consideration when EPR resonances are studied in the presence of ethanolic solution of uncouplers or antibiotics.

As previously observed in isolated Complex III [1], treatment with antimycin and ethanol of the particles induces a shift of the signal of ferricytochrome *b*-562 from $g = 3.44$ to 3.48 (Fig. 1C). The shift is more clearly seen when the particles are first reduced with ascorbate, thereby eliminating the interference with the signal of cytochrome c_1 at $g = 3.35$ (Fig. 1E). Since the spectral shift at $g = 3.44$ does not occur in the presence of ethanol alone, it can be concluded that the shift is brought about by antimycin. This sug-

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*g_z values are presented as the maximum of the first derivative of the EPR absorbance spectrum.

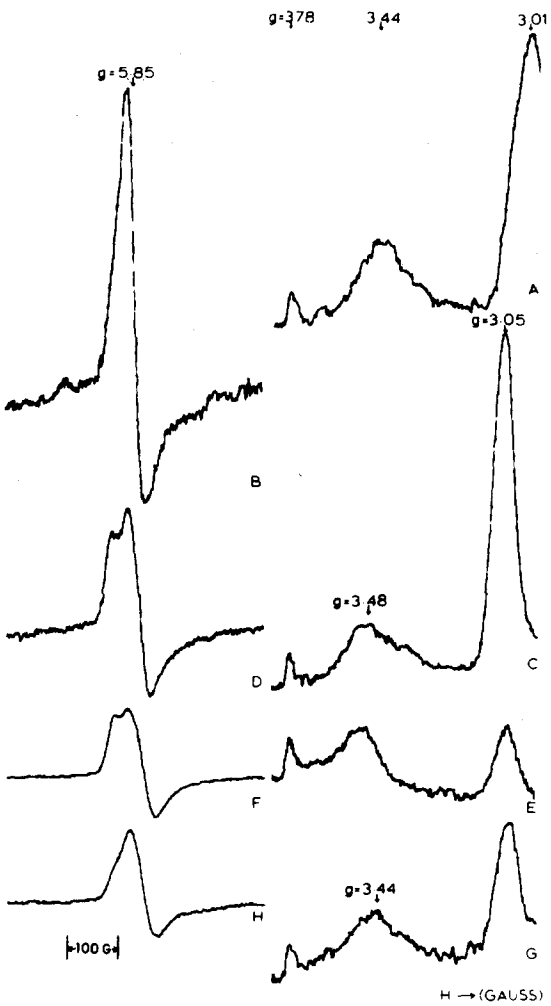

Fig.1. Comparison of effects of antimycin, ethanol and ATP on EPR spectra of cytochromes in phosphorylating ATP-Mg particles. The traces show EPR spectra of the particles in the absence of any addition (A,B); treated with antimycin (3 μg/mg protein) in ethanolic (5%, v/v) solution (C,D); treated with antimycin in ethanolic solution and 85 mM ascorbate (E,F); and as E and after addition of 24 mM ATP (G,H). The concentration of ethanol in the samples shown in Traces C—H was kept constant (5%, v/v). The particles were suspended in 167 mM sucrose, 50 mM Tris-acetic acid buffer (pH 7.4) and 7.5 mM $MgCl_2$ at a final concentration of 60 mg/ml. The samples were frozen after incubation with various reagents for 5 min at 20 °C. EPR spectra were measured at 12 °K, with microwave power of 10 mW and modulation amplitude of 5.9 G and microwave frequency of 9.163 GHz. Twelve spectra of each sample were accumulated in a Data General Corporation Nova computer and averaged spectra were recorded with time constant 0.1 s and scanning rate of 1000 G/min. Normalized receiver gain: 100 (Traces A,B,C,E,G); 50 (Trace D); 5 (Traces F and H).

gests that the binding of antimycin to the mitochondrial membrane elicits an alteration in the ligand field of the ferric haem of cytochrome *b*-562, and that the shift in the EPR resonance may serve as a useful probe for detecting subtle changes at the vicinity of the haem. The shift in the $g = 3$ region seen in Figs 1C and 1E and the improved resolution of the rhombic $g = 6$ component seen in Figs 1D and 1F are due to the effect of ethanol and not to antimycin.

Fig. 1G shows the effect of ATP on the EPR spectra of phosphorylating particles treated with ascorbate and antimycin. Two salient effects of ATP may be pointed out. First, it causes a shift of the signal of ferricytochrome *b*-562 from 3.48 to 3.44, thereby reversing the effect of antimycin on the signal. The subsequent addition of uncoupler, FCCP, abolishes the effect of ATP, the signal being shifted back to 3.48 (not shown). It is noteworthy that neither ATP nor FCCP affects the signal of ferricytochrome *b*-566 at $g = 3.78$. No effect of ATP on the signals of ferricytochrome *b*-562 or *b*-566 was observed in the absence of antimycin (not shown). Secondly, addition of ATP increases the height of the signal at $g = 3.05$ and that of the signal at $g = 3.35$ of ferricytochrome c_1, both in presence (Fig. 1G) and absence (not shown) of antimycin. This effect is also reversed by addition of uncoupler (not shown).

Optical studies at room temperature under conditions comparable with those of the EPR studies show that, in the presence of antimycin, addition of ATP to phosphorylating particles treated with ascorbate (or NADH) causes oxidation of both cytochrome *b*-562 and cytochrome *c* oxidase, whereas in the absence of antimycin ATP causes reduction of cytochromes *b*-562 and *b*-566 and oxidation of cytochrome *c* and *c* oxidase [6,7]. Thus, the ATP-induced increase in height of signal at $g = 3.05$ originating from ferricytochrome *c* and oxidized *c* oxidase can be directly related with the ATP-induced oxidation of these cytochromes by an energy-driven reversal of electron transport [8]. On the other hand, the ATP-induced shift of the signal at $g = 3.44$ originating from ferricytochrome *b*-562, without change of intensity, does not correlate with redox changes of this cytochrome. Similarly, the lack of effect of ATP on the signal of ferricytochrome *b*-566 bears no relationship with the optical data which show an ATP-dependent reduction of this cytochrome. Thus, it appears that EPR-undetectable haem components are involved in the energy-dependent redox changes. If this is the case, then the amount of EPR-detectable ferric haems of cytochromes *b*-562 and *b*-566 does not represent the total amount of oxidized cytochrome *b*, as has also been found for the heme of cytochrome *c* oxidase [9].

At present it remains uncertain whether the opposing effects of antimycin and ATP on the signal of ferricytochrome *b*-562 represent interactions in the direct vicinity of the haem. Neither optical nor potentiometric studies have yet demonstrated that ATP reverses the effect of antimycin on cytochromes *b*.

Fig.2 illustrates the effect of cyanide on the EPR spectrum of phosphorylating particles and that of isolated cytochrome *c* oxidase. When the particles are treated with cyanide in the absence of added substrate, a weak

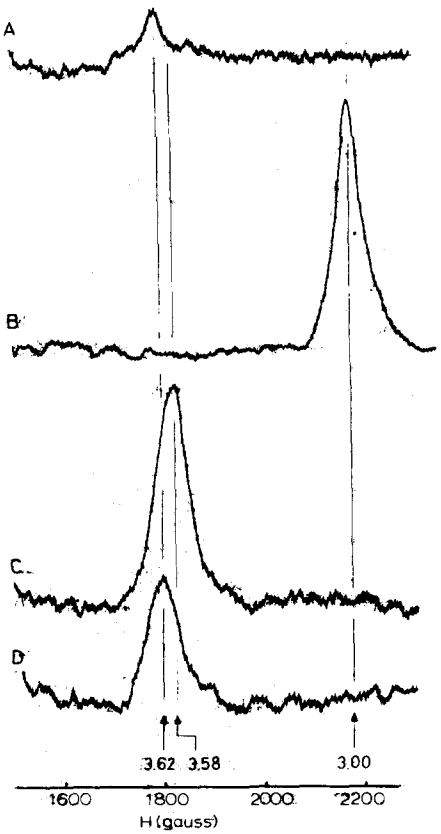

Fig. 2. Effect of cyanide (5 mM) on the EPR spectra of cytochromes in phosphorylating ATP-Mg particles and isolated cytochrome *c* oxidase. A, cyanide-treated phosphorylating particles reduced with ascorbate (85 mM) and succinate (80 mM) for 5 min at 20 °C. The concentration of ethanol was 5% (v/v). B, cyanide-treated cytochrome *c* oxidase in the absence of added substrate. C, cyanide-treated cytochrome *c* oxidase in the presence of dithionite. D, same as C in the presence of ethanol. EPR spectra were measured in a Varian E-9 at 12 °K, with microwave power, 10 mW, modulation amplitude, 10 G, microwave frequency, 9.119 GHz. The traces were recorded with a time constant of 1 s, scanning rate of 125 G/min and receiver gain of $2 \cdot 10^4$ (Traces A, C and D) and $4 \cdot 10^3$ (Trace B). Other conditions are similar to those described in Fig. 1.

signal appears at $g = 3.62$ (not shown) which becomes markedly intensified upon addition of ascorbate and succinate (Fig. 2A). This is in agreement with the previous observation of Orme-Johnson et al. [10] that treatment of electron-transferring particles(ETP_H) with cyanide induces an appearance of a signal at $g = 3.608$ that is not removed by either ascorbate or succinate plus antimycin. There is no correlation in the changes in intensity of the line at $g = 3.62$ in cyanide-treated phosphorylating particles with those of cytochromes *b*-562 and *b*-566 (see ref. 10, Fig. 5, Traces B and C). It is also unlikely that the signal at $g = 3.62$ represents a low-spin ferric haem of cyano-cytochrome *c* (or c_1) complex, since cyanide does not react with membrane-bound

cytochrome *c* [11]. Evidence that the line at $g = 3.62$ originates from the oxidized cyano-cytochrome *c* oxidase has been obtained with the cyanide-treated isolated oxidase. When the isolated oxidase is treated with cyanide, it shows a very weak line at $g = 5.86$ (not shown) from high-spin ferric haem and an intense line at $g = 3.00$ from low-spin ferric haem of the oxidase (Fig. 2B). The subsequent addition of dithionite to the oxidase, which is known rapidly to reduce only one of the hemes in the presence of cyanide,results in a decrease of the signal at $g = 5.85$ (not shown) and the complete disappearance of signal at $g = 3.00$ while a new line appears at $g = 3.58$ (Fig. 2C). These findings demonstrate that, as has been found with azide [9], cyanide hardly affects the EPR spectrum of isolated cytochrome *c* oxidase in the oxidized state, but that a specific signal occurs upon partial reduction of the enzyme.

Upon addition of ethanol in an amount equal to that added to the particles (5%, v/v), the signal is shifted from $g = 3.58$ to 3.62 with a concomitant halving of the height of the signal (Fig. 2D). We conclude, then, that the appearance of the line at $g = 3.62$ in cyanide-treated phosphorylating particles is directly associated with the formation of a cyano-cytochrome *c* oxidase complex, and that the line originates from a low-spin ferric haem of the cyano compound, since under reducing conditions the other haem of the oxidase is ferrous [12] and thus diamagnetic. Our interpretation is consistant with the earlier observation by Orme-Johnson et al. [10] that the signal is not reducible by ascorbate and succinate, since cyano-cytochrome a_3 is extremely slowly reduced by these substrates. A similar observation has been noted with the four-haem (and four-electron acceptor) cytochrome c_3 [13].

The experimental findings presented in this communication testify to the usefulness of EPR spectroscopy in detecting subtle changes in the vicinity of haems of various cytochromes and support the validity of using less complex biological systems (isolated cytochrome *c* oxidase and Complex III) for exploring molecular events in highly intricate biological systems such as phosphorylating mitochondrial membranes.

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