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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME aa_3

III. THE EPR SPECTRUM OF NO–FERROCYTOCHROME a_3

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SUMMARY

1. The reaction of hydroxylamine with oxidized cytochrome aa_3 results in the formation of NO–ferrocytochrome a_3.

2. NO–ferrocytochrome a_3 has an EPR spectrum with $g_x = 2.09$, $g_y = 2.0$, $g_z = 2.005$. g_z is split into nine lines, due to interaction with two nitrogen nuclei: $A_{N_1} = 21.1$ gauss; $A_{N_2} = 6.8$ gauss.

3. It is suggested from this spectrum that histidine is the fifth ligand of the iron in cytochrome a_3.

INTRODUCTION

The effect of hydroxylamine on cytochrome aa_3[1,2] and on Site 3 phosphorylation[3–5] has been investigated recently.

Although the EPR spectrum of cytochrome aa_3 and the effect of ligands have been studied extensively by BEINERT and co-workers[6,7], little attention has been paid to the effect of hydroxylamine[7] or NO. This paper describes the EPR properties of NO–ferrocytochrome a_3 and the effect of hydroxylamine on oxidized cytochrome aa_3**.

MATERIALS AND METHODS

Cytochrome aa_3 was prepared from beef heart by the method of FOWLER *et al.*[8], as modified by MACLENNAN AND TZAGOLOFF[9]. The precipitated enzyme was dissolved in Tris–H_2SO_4 buffer (pH 8), resulting in a solution of about 0.5 mM cytochrome aa_3 (1 mM haem *a*). The concentration of cytochrome aa_3 was calculated from $\Delta A_{605\ nm}$ (reduced — oxidized) using an absorbance coefficient of 24 $mM^{-1} \cdot cm^{-1}$ (ref. 10).

EPR spectra were measured with a Varian E-3 spectrometer. The instrument field set and scan range were determined by using diphenylpicrylhydrazyl. The field scan range was measured with a degassed benzene solution of diphenylpicrylhydrazyl, taking a hyperfine splitting of 9.0 gauss[11]. From the mean g value ($g = 2.0036$) of

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crystalline powdered diphenylpicrylhydrazyl[12] the field set was calculated, assuming the frequency to be correct.

The solution was made anaerobic by flushing the Thunberg cuvette with Ar, purified by bubbling through pyrogallol containing traces of iron and copper ions.

RESULTS

EPR spectra

The upper and lower tracings of Fig. 1 show the EPR spectra of isolated cytochrome aa_3 in the absence and presence of hydroxylamine, respectively. The spectrum in the presence of hydroxylamine shows, in addition to the spectrum of oxidized cytochrome aa_3 and a small $g = 6$ (cytochrome a_3) described by VAN GELDER AND BEINERT[6], lines in the copper region at $g = 2.09$ and at $g = 2.0$. The intensities at $g = 3$ (cytochrome a) and $g = 2.17$ ($g_{//}$ of copper) are 25% less in the presence of hydroxylamine, indicating a partial reduction of cytochrome a and of the EPR-detectable copper.

The effect of hydroxylamine is better resolved at lower power and a 10 times smaller scan range (Fig. 2). This spectrum has also been reported by BEINERT *et al.*[7] (*cf.* their Fig. 2E).

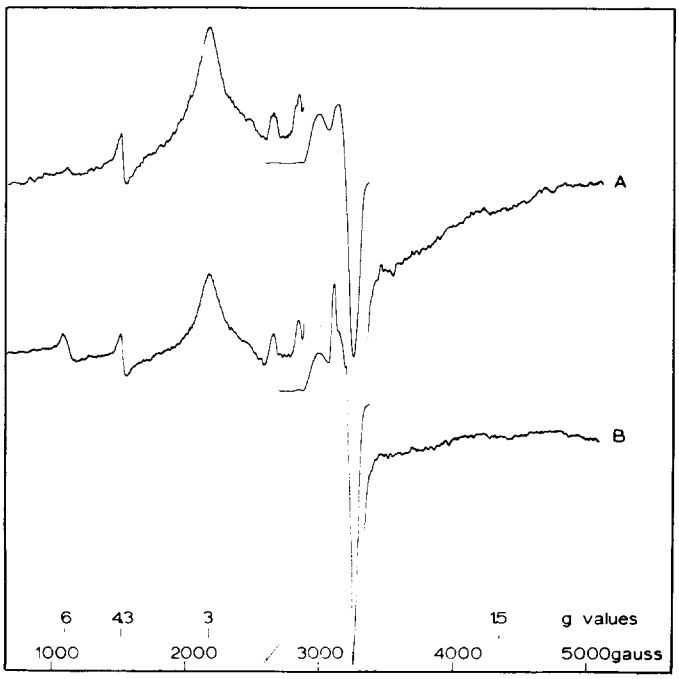

Fig. 1. EPR spectra of 0.3 mM cytochrome aa_3 under anaerobic conditions. A, isolated enzyme; B, enzyme in the presence of 0.6 mM hydroxylamine. Conditions of EPR spectroscopy were: microwave power, 100 mW; modulation amplitude, 16 gauss; scanning rate, 300 gauss/min; time constant, 0.3 sec; temperature, 80° K. The copper signal was recorded at a 20-fold lower receiver gain. The field markers are in gauss; the frequency was 9.130 GHz.

The extra signal due to hydroxylamine has three different g values, consistent with rhombic symmetry. The extremum of g_x has the value 2.09, the principal slope of g_y is about 2 and that of g_z 2.005. g_z is split into three sets of three lines. Taking the absorption of g_y into consideration, the nine lines of g_z are equal in intensity, so that the splitting of g_z must be caused by two non-equivalent nuclei of nitrogen with spin $I = 1$. The hyperfine constants are 21.1 and 6.8 gauss. Hyperfine splitting can also be seen in g_x, but the lines are not well resolved.

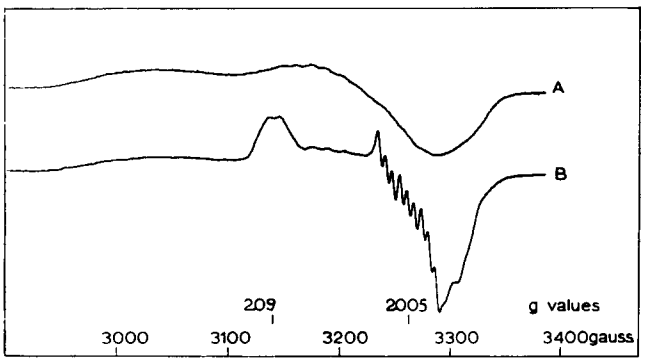

Fig. 2. EPR spectra of 0.3 mM cytochrome aa_3 under anaerobic conditions. A, isolated enzyme; B, enzyme in the presence of 0.6 mM hydroxylamine. The conditions of EPR spectroscopy were: microwave power, 10.0 mW; modulation amplitude, 1.25 gauss; scanning rate, 30 gauss/min; time constant, 0.3 sec; temperature, 80°K. The field markers are in gauss; the frequency was 9.130 GHz.

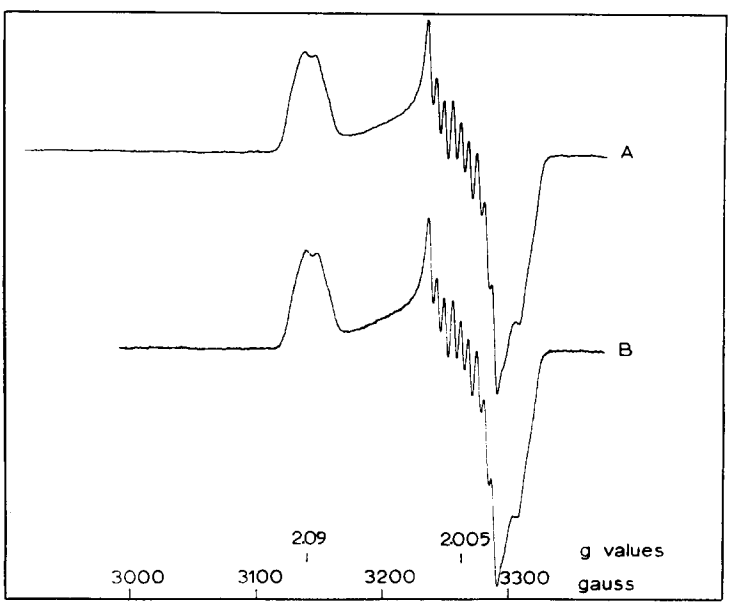

Fig. 3. EPR spectra of 0.3 mM cytochrome aa_3 under anaerobic conditions. A, the enzyme was allowed to react with 0.6 mM hydroxylamine, followed by the addition of $Na_2S_2O_4$; B, enzyme in the presence of $Na_2S_2O_4$ and NO. The conditions of EPR spectroscopy were those of Fig. 2, except for the temperature, which was 93°K.

Fig. 3A shows that the effect of hydroxylamine is still observed after reduction of the heme and copper of the enzyme by dithionite, added subsequently to the hydroxylamine. No signals are observed when NH_2OH is added to the reduced enzyme. Since the spectrum of dithionite-reduced cytochrome aa_3 *plus* NO (Fig. 3B) has exactly the same g value and hyperfine splitting constants as the spectra in Figs. 2B and 3A, it may be concluded that the reaction of cytochrome aa_3 with hydroxylamine leads to the formation of a NO–ferroheme compound, presumably as the result of oxidation of hydroxylamine to NO (ref. 5), with a concomitant reduction of part of the heme of cytochrome aa_3. Addition of hydroxylamine or of NO *plus* succinate to beef heart mitochondria gives the same NO–ferroheme signal (not shown), suggesting that the effect of hydroxylamine is not due to changes in the enzyme, introduced by purification.

Confirmation that the spectra obtained with hydroxylamine, followed by dithionite, and NO + dithionite, are due to the same compound is given by the identical saturation behaviour at 93°K, shown in Fig. 4.

Other hemoproteins tested (hemoglobin, methemoglobin, ferro- and ferricytochrome *c*), do not give NO–ferroheme signals on addition of hydroxylamine.

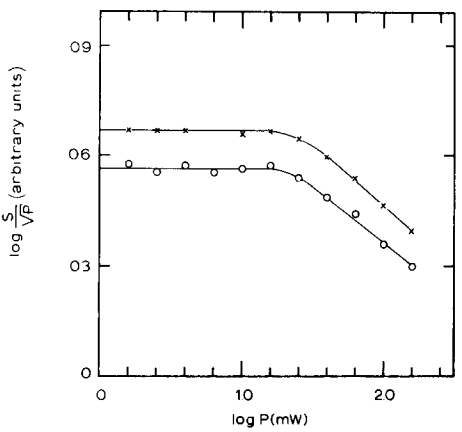

Fig. 4. Saturation curves of NO–ferrocytochrome a_3. ×—×, same conditions as in Fig. 3A; ○—○, same conditions as in Fig. 3B. P = microwave power; S = signal height of g_x.

Effect of hydroxylamine on the redox components of cytochrome aa_3

The maximal NO–ferroheme signal is observed after 10 min incubation at 0°C, under anaerobic conditions, when about 2 moles hydroxylamine are added per mole cytochrome aa_3, and the signal is stable at room temperature. NO–ferroheme is also formed by hydroxylamine under aerobic conditions, but the signal decreases in time and disappears completely after 45 min standing at 25°.

The amount of NO–ferroheme calculated by double integration of the maximal signal formed, using copper–EDTA as standard and correcting for transition probability[13], corresponds with 80% of cytochrome aa_3, *i.e.* 40% of the total heme *a* in the enzyme. The same intensity was obtained by addition of hydroxylamine to the oxidized enzyme or of NO to the reduced enzyme.

According to the definition of KEILIN AND HARTREE[14] NO is a ligand of cytochrome a_3. This is illustrated in Fig. 1, which shows that 2 moles of hydroxylamine reduce only 25 % of cytochrome a, while the NO–ferroheme signal represents 80 % of one of the heme components of cytochrome aa_3. When larger concentrations of hydroxylamine are used, cytochrome a and the EPR-detectable copper are further reduced, but no increase of the NO–ferroheme signal is observed.

The large high-spin $g = 6$ or the low-spin $g = 2.6$ signals of cytochrome a_3, observed at partial reduction of the enzyme by VAN GELDER AND BEINERT[6], are not seen in the presence of hydroxylamine. Moreover, the signals obtained after partial reduction of the enzyme with NADH and catalytic amounts of phenazine methosulphate, are abolished by the subsequent addition of hydroxylamine.

DISCUSSION

It has been clearly shown that hydroxylamine reduces cytochrome a_3 under aerobic and anaerobic conditions. Hydroxylamine itself is oxidized to NO, after which NO–ferrocytochrome a_3 is formed. In the presence of O_2 the NO–ferrocytochrome a_3 compound is unstable, probably due to the oxidation of cytochrome a_3.

This effect of hydroxylamine is specific for cytochrome aa_3, since under the same conditions neither methemoglobin nor ferricytochrome c form a NO–ferroheme compound. The structure of the porphyrin ring of heme a may play an important role in the mechanism of reduction of the iron by hydroxylamine.

The spectrum of NO–ferrocytochrome a_3 resembles those of NO–hemoglobin and NO–ferrocytochrome c (not shown). Our spectra of NO–hemoglobin and NO–ferrocytochrome c are identical to those of KON[15,16]. It is interesting to note that the hyperfine lines of g_z are much better resolved in NO–ferrocytochrome a_3 and NO–ferrocytochrome c (at pH 12) than in NO–hemoglobin (at pH 8).

In NO–ferrocytochrome c and NO–hemoglobin the larger hyperfine splitting of the line with g_z was shown by KON AND KATAOKA[17] to be due to the nitrogen nucleus of NO, while the superimposed hyperfine splitting is attributed[17] to the nitrogen of the fifth ligand of the iron, which is histidine in both cases. Therefore, in NO–ferrocytochrome a_3 the hyperfine splitting of 21.1 gauss has to be ascribed to the nitrogen of NO and the smaller hyperfine splitting of 6.8 gauss to a nitrogen of the fifth ligand of the iron. Candidates for the fifth ligand of the heme in cytochrome a_3 are, then, the basic amino acids (lysine, arginine, histidine) and tryptophan.

Since the g values and hyperfine-splitting constants are almost equal for NO–ferrocytochrome a_3 and NO–ferrocytochrome c (pH 12) (*cf.* ref. 16) and since the saturation behaviour is identical (not shown), it is probable, that the fifth ligand of the iron in cytochrome a_3 is histidine.

VAN GELDER AND BEINERT[6] have explained the undetectable signal of half of the copper and of the heme of cytochrome a_3 by an antiferromagnetic coupling of electron spins, by heme–heme, copper–copper or copper–heme interaction. Our spectra (Fig. 1) show that no extra copper signal is observed while cytochrome a_3 is visible. This seems to indicate that a copper–heme interaction does not occur. However, although cytochrome a and the EPR-detectable copper are only slightly reduced by hydroxylamine, it is possible that the EPR-undetectable copper is completely reduced under the conditions used.

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