AN EPR-DETECTABLE INTERMEDIATE IN THE CYTOCHROME OXIDASE–DIOXYGEN REACTION

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1. Introduction

Chance and his associates were the first to describe a number of spectrophotometric intermediates in the reaction between reduced cytochrome *c* oxidase and O_2 by kinetic measurements at low temperatures [1]. Later our group, by following the reaction both by optical and by EPR spectroscopy [2], were able to show that 3 consecutive intermediates are formed at 173 K, and that these differ in the number of electrons that have been transferred to the cytochrome a_3–Cu_B–O_2 unit from reduced cytochrome *a* and Cu_A (see [3] for a definition of the prosthetic group metal ions). The first intermediate (I) has O_2 in the peroxide state, if the metals are considered oxidized. Intermediates II and III are then formed by two successive transfers of one electron. This means that the a_3–Cu_B–O_2 unit in intermediate II must have at least one unpaired electron and be paramagnetic.

A disturbing observation in our earlier investigation was the absence of any EPR signals other than those from cytochrome a^{3+} and Cu_A^{2+}. We have now renewed our search for a new EPR-detectable species by the use of more extreme conditions for the EPR measurements (lower temperature, higher microwave power) and have found a new copper EPR signal with unusual properties. The species giving this signal is formed and disappears with the same kinetics as intermediate II. The EPR properties suggest that it contains a Cu^{2+} ion in the vicinity of another paramagnetic group. A likely interpretation is that the EPR signal arises from Cu_B^{2+} interacting with cytochrome a_3 in the ferryl ion state, Fe^{4+}=O.

2. Materials and methods

Cytochrome oxidase was prepared from beef heart mitochondria [4]. Chemical and spectroscopic methods were the same as in [2]. In the EPR measurements the actual temperature at the sample was determined by the use of a carbon resistor.

3. Results

EPR spectra of a cytochrome oxidase sample were recorded at different times after the initiation of the reaction at 173 K between the reduced enzyme and O_2, as illustrated in fig.1. With the conditions of measurement given in the caption a new EPR signal, characterized by 4 peaks centered around *g* 2.25, was discovered. Its intensity at first increases continuously with the reaction time, reaching its maximal amplitude after ~200 s (fig.1). EPR spectra recorded at lower power showed that the cytochrome *a* signal at *g* 3.0 and the Cu_A signal at *g* 2.0 increase concomitantly with the new signal. On further reaction at 173 K the *g* 2.25 signal decays (third spectrum in fig.1), and it vanishes completely when the reaction is allowed to continue at 193 K (bottom spectrum in fig.1).

The conditions at which the new signal can be detected are quite different from those commonly used for recording EPR spectra of cytochrome oxidase. The effects on the EPR spectrum of changing the temperature and the microwave power are shown in fig.2. At 30 K (spectra C and D) the signals from Cu_A^{2+} (*g* 2.0) and a low-spin haem (*g* 2.54) are essentially non-saturated even at 100 mW, and the new signal cannot be observed. At 10 K, on the other hand,

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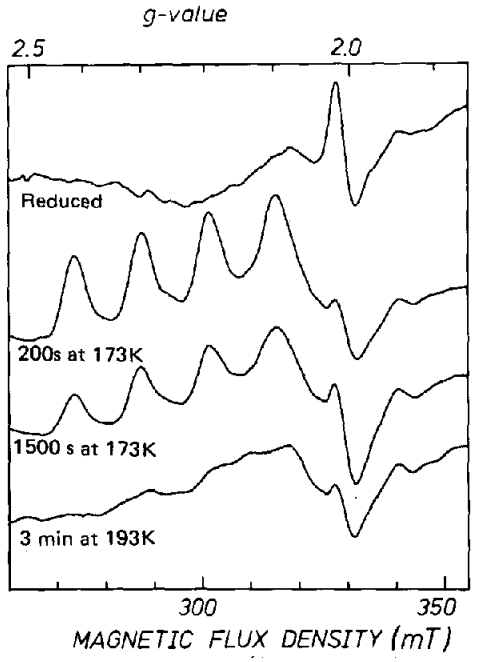

Fig.1. EPR spectra of a sample at different stages of reoxidation of fully reduced cytochrome oxidase with O_2. The sample contained 162 μM reduced cytochrome oxidase–CO complex in 40 mM Hepes buffer at pH 7.4 and 0.5% Tween 80, 28% ethylene glycol, 0.9 mM NADH and 4 μM phenazine methosulphate. From top to bottom are shown: the sample before reaction, at 200 s and at 1500 s after initiation of the reaction at 173 K, and after 3 min at 193 K. The conditions of EPR spectroscopy were: temp. 10 K; microwave power 100 mW; microwave frequency 9521 GHz; modulation amplitude 3.2 mT.

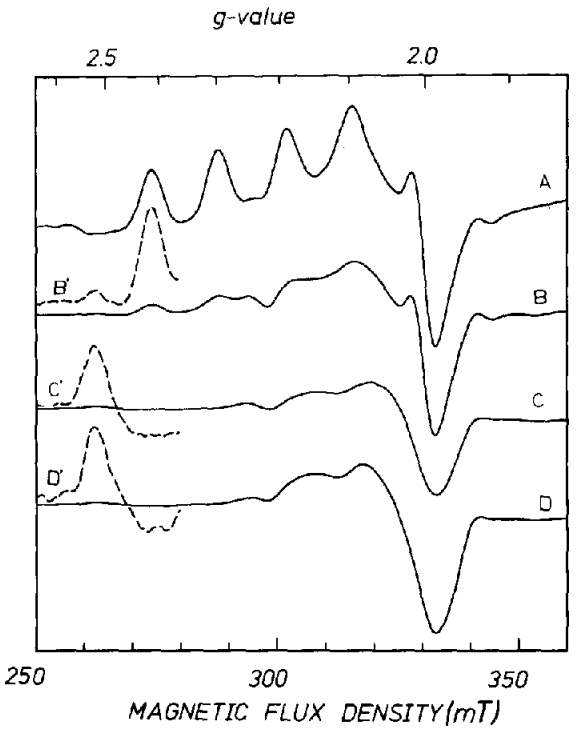

Fig.2. Temperature and microwave power dependence of the new Cu^{2+} signal. EPR spectra were recorded with a sample containing the same concentration of enzyme and other reagents as that of fig.1. (A,B) were recorded at 10 K and gain 1, with the microwave power 100 mW and 1 mW, respectively, (C) at 30 K, gain 0.1 and 100 mW, and (D) at 30 K, gain 1 and 1 mW. Conditions chosen for A, B′, C′ and D′ are such that a non-saturating signal with T^{-1} temperature dependence would have the same intensity.

the copper and haem signals are saturated, and the new signal appears, becoming more pronounced with increasing microwave power (spectra A,B in fig.2). From the position of the 4 hyperfine peaks the parameters $g = 2.25$ and $A = 13.8$ mT can be calculated. No features corresponding to the normal $g_\perp$ part of the spectrum can be detected. As the spectrum is not the normal type for Cu^{2+}, only a lower limit for its intensity can be given; this corresponds to 10% of the oxidase concentration.

4. Discussion

The results in fig.1,2 demonstrate the appearance of a new EPR-detectable species in the low-temperature reaction of fully reduced cytochrome oxidase with O_2. In [2] we could not detect any EPR signals other than those from cytochrome a^{3+} and Cu_A^{2+}, which was puzzling in view of the fact that intermediate II was predicted to be paramagnetic. Our earlier failure to observe this paramagnetic species by EPR finds its explanation in the unusual properties, making the spectrum detectable only with high microwave powers at temperatures well below 30 K, as shown in fig.2.

The new signal has an unusual shape, displaying essentially only 4 'absorption-type' peaks; it should be noted that the features in the region g 1.95–2.05 do not belong to the new spectrum, as they remain after the hyperfine structure has disappeared (bottom spectrum in fig.1). The magnitude of the hyperfine coupling shows, however, that the unpaired electron is mainly associated with a copper nucleus. The spec-

trum is unusual for Cu^{2+} also in being characterized by a very fast relaxation, which makes it essentially non-saturating at 10 K even with a power as high as 100 mW (fig.2).

The unusual shape and relaxation properties must be due to an interaction with a nearby paramagnetic ion. Such an interaction could result in a very anisotropic spectrum, in which the other parts are too weak to be detected. The coupling with another paramagnetic ion would also give a very short relaxation time, particularly if the neighbour itself has a short relaxation time.

The rate of formation and decay of the new signal (fig.1) demonstrates that it represents intermediate II, as this was earlier found [2] to have reached its maximum concentration after 200 s at 173 K and to have decreased to half the maximum after 1500 s. This conclusion is also consistent with the prediction [2] that intermediate II should be paramagnetic.

In [2] we could only assign an overall charge to the O_2-reducing $[Cu_B a_3]$ unit in the intermediates but could not specify the exact electron distribution among its components because of the lack of any EPR signals from cytochrome a_3 or Cu_B. The discovery of the new spectrum, with its unusual properties, makes possible, however, a more exact structural description of intermediate II.

The copper EPR signal must come from Cu_B^{2+}, as it rises concomitantly with the g 2.0 signal from Cu_A^{2+}. Its hyperfine splitting is very different from that of the Cu_A^{2+} EPR signal but is only somewhat larger than that of the Cu_B^{2+} signal in [5]. The slight difference must be due to the different structures of the $[Cu_B a_3]$ unit in the two species giving Cu_B^{2+} signals, the signal discovered earlier arising from a Cu_B^{2+}–a_3^{2+}–O_2 complex [6].

The overall charge of the $[Cu_B a_3 O]$ unit in intermediate II is +4 [2]. The identification of the EPR signal with Cu_B^{2+} means that the rest of the unit should be $[FeO]^{2+}$. There are at least 3 possible electron distributions in this ion: Fe^{2+}–O, Fe^{3+}–O^- or Fe^{4+}=O. The first two of these would, however, not be expected to be paramagnetic, whereas ferryl ion would be, S=0 being very rare for the d^4 configuration [7].

The ferryl ion would be expected to have a short relaxation time and consequently to have the observed effects on the Cu_B^{2+} spectrum. An analogous situation has, in fact, been described for horseradish peroxidase [8]. In its compound I a combination of magnetic

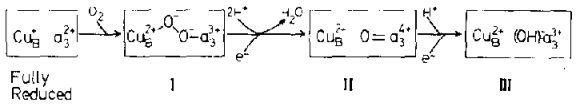

Fig.3. The intermediates in the reaction of fully reduced cytochrome oxidase with O_2 at 173 K.

susceptibility and Mössbauer results has demonstrated the presence of a ferryl ion–radical complex. The nearby ferryl ion gives the radical such a short relaxation time, however, that its EPR spectrum can only be detected below 10 K, and its central feature has an intensity corresponding to only 1% of the heme content [9].

In view of the analysis given here the intermediates observed in the reaction of reduced cytochrome oxidase with O_2 [2] can be written as in fig.3. The structure we have demonstrated for intermediate II had been predicted on the basis of chemical reasoning in [10].

Acknowledgements

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References

[1] Chance, B., Saronio, C. and Leigh, J. S. jr (1975) J. Biol. Chem. 250, 9226–9237.
[2] Clore, G. M., Andréasson, L.-E., Karlsson, B., Aasa, R. and Malmström, B. G. (1980) Biochem. J. 185, 139–154.
[3] Malmström, B. G. (1979) Biochim. Biophys. Acta 549, 281–303.
[4] Van Buuren, K. J. H. (1972) Binding of cyanide to cytochrome aa_3, PhD Thesis, University of Amsterdam, Gerja, Waarland.
[5] Reinhammar, B., Malkin, R., Jensen, P., Karlsson, B., Andréasson, L.-E., Aasa, R., Vänngård, T. and Malmström, B. G. (1980) J. Biol. Chem. 255, 5000–5003.
[6] Karlsson, B. and Andréasson, L.-E. (1981) Biochim. Biophys. Acta 635, 73–80.
[7] Orgel, L. E. (1960) in: An Introduction to Transition-Metal Chemistry: Ligand-Field Theory, pp. 41–52, Methuen, London.
[8] Schulz, C. E., Devaney, P. W., Winkler, H., Debrunner, P. G., Doan, N., Chiany, R., Rutter, R. and Hager, L. P. (1979) FEBS Lett. 103, 102–105.
[9] Aasa, R., Vänngård, T. and Dunford, H. B. (1975) Biochim. Biophys. Acta 391, 259–264.
[10] Brudvig, G. W., Stevens, T. H. and Chan, S. I. (1980) Biochemistry 19, 5275–5285.