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THE IDENTITY OF A NEW COPPER(II) ELECTRON PARAMAGNETIC RESONANCE SIGNAL IN CYTOCHROME *c* OXIDASE

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Summary

In reoxidation experiments with cytochrome *c* oxidase (EC 1.9.3.1) in the presence of both reducing substrate and molecular oxygen, a new EPR signal from Cu^{2+} has been observed. The new signal corresponds to 0.45 Cu per functional unit. It is concluded that the new EPR signal originates from Cu_B^{2+}, the copper which is EPR-nondetectable in the resting enzyme.

Optical absorption changes in the 500—700 nm region accompanies the decay of the new Cu^{2+} EPR signal.

Based on the results in this investigation a catalytic cycle for cytochrome oxidase is proposed.

Introduction

The minimum functional unit of mammalian cytochrome *c* oxidase (EC 1.9.3.1), the terminal component in the respiratory chain, is thought to contain two A-types haems, cytochrome *a* and a_3, and two copper atoms, Cu_A and Cu_B. Cu_A and cytochrome *a* are magnetically isolated and detectable by EPR in their oxidized states, giving rise to the two dominating features in the EPR spectrum, the so called g 2 and g 3 signals, respectively; while Cu_B and cytochrome a_3 are antiferromagnetically coupled [1] and nondetectable by EPR in the resting, fully oxidized enzyme. On partial reduction a high-spin haem signal at g 6, attributed to cytochrome a_3, appears [2].

The maximal total intensity of the haem EPR signals corresponds in general to about 50% of the haem present [3]. However, under certain experimental

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfphonic acid.

conditions, strong high- and low-spin Fe^{3+} signals have been observed, together accounting for about 80% of the total haem content, i.e. the major parts of both cytochrome *a* and a_3 are, simultaneously, detectable by EPR [4].

The intensity of the EPR signals from copper in cytochrome oxidase have previously never been reported to exceed 50% of the total copper content (all of which is ascribed to Cu_A). Until recently [5,6], no EPR signals with certainty attributable to Cu_B^{2+} have been observed in any state of the enzyme [2,3,7].

The present paper describes the generation of a metastable form of the enzyme in which the integrated intensity of the EPR-detectable copper amounts to as much as 70% of the total copper. At room temperature the shape and intensity of the EPR spectrum eventually return to that of the resting, oxidized enzyme.

Materials and Methods

The characteristics of the enzyme, prepared from beef heart, and the chemical and spectroscopic methods used, were essentially the same as described in Ref. 8. Total iron and copper contents were determined by the methods in Refs. 9 and 10, respectively. The iron/copper ratio was 1.1, and the haem/copper ratio was 0.85, with extraneous copper corresponding to less then 10% of the total EPR-detectable copper of the resting enzyme.

EPR spectra at 9 GHz were recorded at 77 K and at 20 K with Varian E-3 and E-9 spectrometers, respectively. Integration of EPR spectra were made according to Aasa and Vänngård [11].

Optical absorption spectra were recorded on a Johnson Foundation DBS-2 dual wavelength spectrometer [12].

Sample preparation

The samples for both the optical and EPR measurements were prepared as follows: A solution of 220 μM cytochrome oxidase (in 46 mM Hepes and 0.5% Tween 80 at pH 7.4), 12 μM phenazine methosulphate and 0.9 mM NADH was made anaerobic and then placed under a stream of CO for at least 1 hour with occasional agitation to ensure full reduction and CO-saturation [8]. 200 μl of the fully reduced, CO-saturated enzyme solution was transferred anaerobically to an EPR tube, previously cooled to 250 K. 50 μl O_2-saturated ethylene glycol in water (30%) was added in the dark, the solutions were rapidly mixed and then immediately frozen in liquid N_2 at 77 K. The frozen reaction mixture was exposed to flash photolysis at 77 K and submitted to rapid thawing in a water bath at room temperature; The sample was saturated with oxygen by bubbling pure O_2 gas through the solution.

For optical measurements, a sample with an EPR spectrum as that in Fig. 1B, was thawed and transferred to a 0.1 cm cuvette, and the baseline was recorded at room temperature (20°C) within 2 minutes. The sample was allowed to react at room temperature and optical difference spectra were recorded repeatedly.

Parallel with the optical spectra, the corresponding EPR spectra were recorded, on an identically treated sample.

Results

EPR spectra

The EPR spectrum in Fig. 1A, obtained after thawing an oxygenated sample, is characteristic for a partially reduced sample with a signal from Cu_A^{2+} at g 2, high-spin haem signals at g 6 and a low-spin haem signal at g 3. The peaks at g 2.6, 2.16 and 1.86 arise from another low-spin haem species, whereas the signal at g 4.3 originates from impurities in the spectrometer cavity and a small amount of extraneous non-haem Fe^{3+} [3].

By bubbling pure O_2 gas into the sample, the spectrum in Fig. 1B was obtained, which, over a period of several hours at room temperature, was transformed into a spectrum identical to that of the resting enzyme (Fig. 1 C and D).

The EPR signal around g 2 in Fig. 1 B and C, arises from at least two copper species. Apart from the usual Cu_A^{2+} signal present in oxidized, resting enzyme, a new Cu^{2+} signal is seen. Integration of the low-field line at 0.271 T revealed

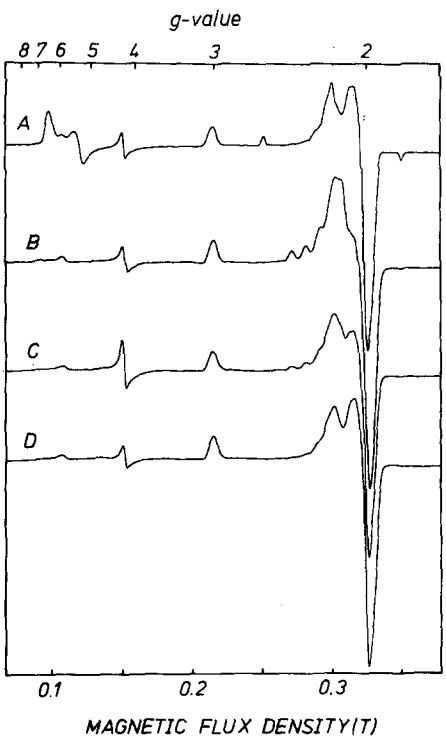


Fig. 1. Wide-field EPR spectra of cytochrome *c* oxidase demonstrating the presence of a new Cu^{2+} signal. A. Cytochrome oxidase, frozen as an oxygenated solution of the fully reduced CO-complex, photolyzed and thawed at room temperature. B. Sample A thawed and saturated with O_2 gas. C. Sample B thawed and kept at room temperature for 2 h and 10 min. The initial reaction mixture in A contained 177 μM cytochrome oxidase, 37 mM Hepes (pH 7.4), 0.4% Tween 80, 20% ethylene glycol, 0.7 mM NADH, 10 μM phenazine methosulfate, CO (~1.0 mM) and O_2 (~0.4 mM). D. Native resting enzyme, 180 μM in 50 mM Hepes buffer (pH 7.4) and 0.5% Tween 80. EPR spectra were recorded under the following conditions: microwave frequency, 9.182 GHz; microwave power, 2.0 mW; modulation amplitude, 2.0 mT; temperature, 20 K.

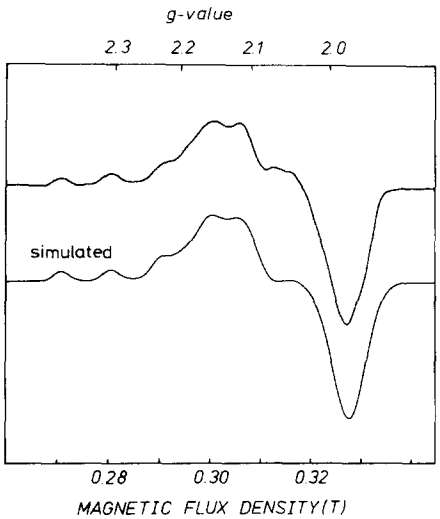


Fig. 2. EPR spectrum of the g 2 region of cytochrome oxidase with the new Cu^{2+} signal present. The upper trace shows the spectrum of the sample in Fig. 1B. Conditions for EPR measurements: microwave frequency, 0.17 GHz; microwave power, 20 mM; modulation amplitude, 2.0 mT; temperature 77 K. The simulated spectrum (lower trace) was obtained as a sum of two different components (Cu_A and new Cu^{2+}), with g_x 1.99, g_y 2.03, g_z 2.18 [3] and g_x 2.052, g_y 2.112, g_z 2.278, respectively. The corresponding line-widths are 6.0, 10.0, 10.5 mT and 7.5, 5.0, 3.0 mT with a hyperfine splitting constant A_z = 10.2 mT for the latter. The relative weight is 1.0 for the Cu_A component and 0.4 for the new component.

that the new signal corresponds to about 0.45 Cu/functional unit (two haems, two Cu). The total amount of EPR-detectable copper (Cu_A + new signal) in Fig. 1B was about 150% of the intensity in the resting enzyme, or 1.3 Cu/functional unit.

In EPR spectra with the new Cu^{2+} signal present, the high-spin haem signal at g 6 is identical in shape and intensity to that in the resting enzyme, corresponding to 1—2% of the total haem content. Judged from an expanded EPR spectrum of Fig. 1B, the intensity of the low-spin haem signal at g 3 is about 80% developed, compared to the g 3 signal in the resting enzyme, immediately after the O_2 gas bubbling, and reaches full intensity within 10 min.

The g 2 region of the EPR spectrum with the new Cu^{2+} signal is shown in Fig. 2. The simulated spectrum in Fig. 2 was obtained as the sum of the usual Cu_A^{2+} signal of resting oxidized cytochrome oxidase and a Cu^{2+} signal with parameters given in the figure legend.

Optical spectra

Fig. 3 shows the optical absorption changes in the 500—700 nm interval, occurring during the decay of the new Cu^{2+} species at room temperature. The spectra exhibit a sharp trough at 605 nm, ascribed to oxidation of haem, and are characterized by the gradual appearance of a broad absorption band at 655 nm, present in the resting, oxidized enzyme [13]. The total absorbance changes at 605 nm in Fig. 3 corresponds to 30% of the difference between the reduced and oxidized enzyme. Most of the absorbance changes at 605 nm occur simultaneously with the increase of the EPR signal at g 3 to full intensity, but before

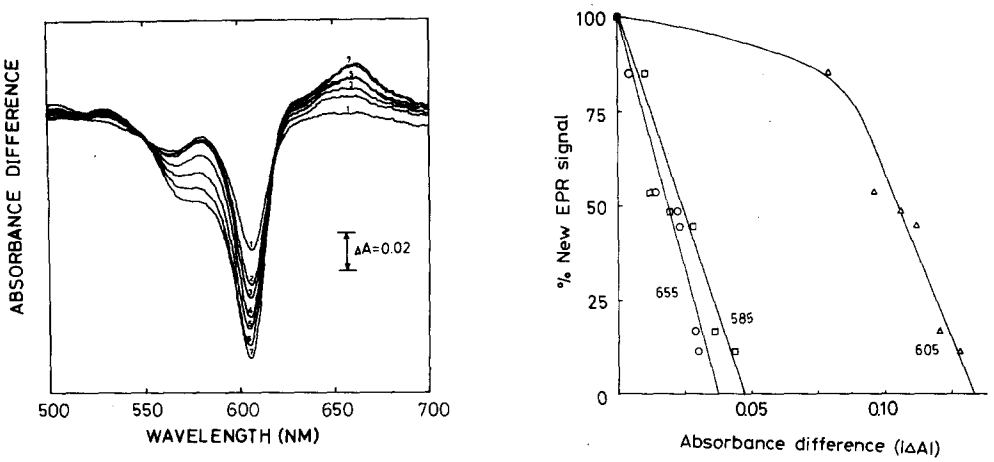


Fig. 3. Optical difference spectra in the visible region obtained during the decay of the Cu_B^{2+} EPR signal at room temperature (20°C). The reference spectrum (baseline not shown) was recorded immediately after thawing of the frozen sample (see Materials and Methods). The spectra were recorded 10 min (1), 25 min (2), 45 min (3), 1 h and 5 min (4), 2 h and 10 min (5), 3 h (6) and about 5 h (7) after thawing.

Fig. 4. Correlation between the integrated intensity of the low-field EPR signal at 271.0 mT and the absorbance differences at 605 nm (△), 585 nm (□) and 655 nm (○). The intensity of the EPR signal was calculated from EPR spectra recorded at 77 K. The optical data were obtained from the spectra in Fig. 3.

there is any noticeable change in the intensity of the new Cu^{2+} EPR signal.

The correlation between the integrated low-field line at 0.271 T and optical absorption changes at 585, 605 and 655 nm is shown in Fig. 4. In contrast to the absorption changes at 605 nm, there is a linear correlation between the optical absorbance at 585 and 655 nm and the intensity of the new EPR signal.

The absorbance changes at 655 nm in Fig. 3, corresponds to about 0.42 of the absorbance difference between the resting and the fully reduced enzyme at this wavelength.

The optical spectra in Fig. 3 also reveal a broad band centered around 585 nm, which decreases in intensity simultaneously with the decay of the new EPR signal (Fig. 4).

Discussion

In an earlier investigation from this laboratory it was suggested that the new EPR signal, present during reoxidation of reduced cytochrome *c* oxidase with molecular oxygen, originates from Cu_B [5]. It could not be entirely excluded that the new signal represented a different form of Cu_A; since the sum of Cu_A (0.8 Cu/functional unit) and the new copper signal did not exceed 50% of the total copper content.

In this investigation it is shown that the total intensity of the EPR-detectable copper may amount to as much as 70% of the copper content. This means that Cu_B must contribute to the EPR spectra shown in Fig. 1B and Fig. 2.

The broad optical absorption band around 655 nm (Fig. 3) has been suggested to originate from ferric cytochrome a_3 coupled to Cu_B [13]. The linear

correlation between the absorption increase at 655 nm and the decay of the new EPR signal (Fig. 4) and the similar figures for the changes in 655 nm chromophore (from 60 to 100%) and EPR-detectable Cu_B (0.45 equivalents) is strong evidence that Cu_B is involved. The disappearance of the Cu_B^{2+} EPR signal is not likely due to the reduction of this site, since the enzyme is transformed into a product indistinguishable from the resting oxidized enzyme, in which Cu_B is oxidized. The decay of the EPR signal and the concomitant appearance of the 655 nm band should therefore represent the restoration of the a_3-Cu_B pair in support of the earlier hypothesis by Beinert et al. [13].

Our optical data do not allow the unambigous assignment of the broad absorption band around 585 nm, but since the absorption at this wavelength decreases linearly with the appearance of the 655 nm band and the decay of the new Cu^{2+} EPR signal, this band may originate from a magnetically isolated Cu_B^{2+}, with a calculated molar absorptivity at 585 nm of about 4500 $M^{-1} \cdot cm^{-1}$.

The intensity of the g 3 signal in the EPR spectrum of Fig. 1B shows that cytochrome a is at least 80% oxidized initially, but that full oxidation is attained within a few minutes. It is reasonable to assume that the early optical changes with the prominent 605 nm band is mostly associated with reoxidation of cytochrome a. The amount of haem oxidized, calculated from the initial absorbance change at 605 nm, agrees well with the concomitant increase in the EPR signal at g 3.

The fact that no signal attributable to cytochrome a_3 is observed in the EPR spectra in which the new Cu^{2+} signal is present, and that the reaction mixture contains excess molecular oxygen, may indicate that oxygen is bound to cytochrome a_3 in a way analogous to that in oxyhaemoglobin [14]. The state in which Cu_B is EPR detectable may be represented by the following configurations: $a^{3+}Cu_A^{2+}Cu_B^{2+}a_3^{2+}O_2$ or $a^{3+}Cu_A^{2+}Cu_B^{2+}a_3^{3+}O_2^-$, in which the antiferromagnetic coupling between the iron of cytochrome a_3 and Cu_B is broken. In the absence of oxygen however, partial reduction gives none of these forms, but a state in which Cu_B is reduced and cytochrome a_3 oxidized, producing a g 6 EPR signal (Fig. 1A).

In a previous publication [15] a catalytic scheme for cytochrome c oxidase was suggested, based on observations of the reaction with O_2 at low temperature. The finding of the new Cu^{2+} EPR signal during reoxidation of reduced enzyme and during turnover conditions allows an extension of the earlier proposed model.

On the basis of the results in the present investigation we suggest the following scheme for the catalytic cycle of cytochrome oxidase (see Fig. 5), where for simplicity only the O_2-reducing unit, Cu_B and cytochrome a_3, has been considered: When the reaction between reduced cytochrome oxidase and O_2 is started by thawing of the frozen sample, intermediates I, II and III, previously identified at low temperature [8], are rapidly formed in sequence. The oxygen-binding unit in intermediates II and III in [8] were written as $[Cu_Ba_3O_2]^{2+}$ and $[Cu_Ba_3O_2]^{+}$, respectively. We have here suggested the configurations $Cu_B^{2+} - O^- - a_3^{3+}$ and $Cu_B^{2+} - O^{2-} - a_3^{3+}$ for these intermediates, i.e. we have allowed for the expulsion of one oxygen as water.

Intermediate III, which lacks the 655 nm absorption band, is suggested to be converted into an enzyme form with this band. The 'pulsed' [16] rather than

the resting enzyme, as suggested in Ref. 17, is preferred in the cycle, since the former has been shown to form rapidly on reoxidation of the fully reduced enzyme [18], and is the more reactive species [16].

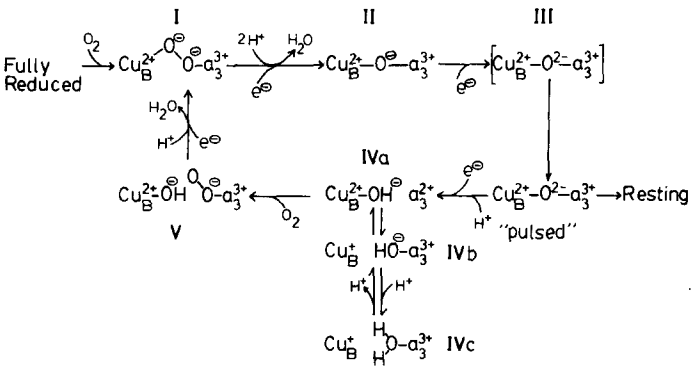
Further reaction leads to the formation of the partially reduced form IVa (in equilibrium with IVb and c), which on reaction with oxygen forms intermediate V, the species responsible for the new Cu^{2+} signal.

If during turnover, all oxygen is consumed before the reducing substrate, intermediates IVb and c accumulate, giving rise to low- and high-spin haem signals, respectively. If, on the other hand, the reducing substrate ceases, some enzyme molecules are ultimately trapped as intermediate V.

The lack of Cu_B^{2+} EPR signal in reductive titrations and reoxidation experiments in which oxygen is absent or exhausted (Fig. 1A) is in our model accounted for by the displacement of the equilibria involving IVa, b and c towards the latter two intermediates. At low pH, intermediate IVc, with H_2O as ligand in the free coordination position of cytochrome a_3^{3+}, gives rise to a large haem signal at g 6, which decreases at higher pH as the low-spin haem form IVb accumulates as a result of deprotonation [19]. A low-spin form of cytochrome a_3 has been suggested to be responsible for the EPR signal at g 2.6 [20].

Following the suggestion in [17], the free coordination position of Cu_B^{2+} in intermediate IVa and V contains an hydroxyl ion at neutral pH. Intermediate V represents the form of the enzyme which gives rise to the EPR signal from Cu_B. Fig. 6 visualizes intermediate V, including an imidazole as axial ligand to Fe^{3+} [21].

The slow decay of intermediate V to the resting state, is easily accounted for, since this reaction involves the release of a bound O_2^-, formed as a result of a one-electron transfer from reduced cytochrome a_3. With an oxidation-reduction potential for cytochrome a_3 of +350 mV [22], the minimum free energy of activation for the reaction is 66 kJ · mol^{-1}, which is a significant energy barrier to overcome.



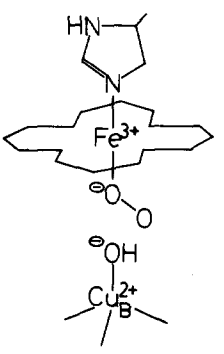


Fig. 5. Simplified hypothetical catalytic mechanism for cytochrome oxidase. The oxygen-binding unit (Cu_B, cytochrome a_3) is assumed to receive electrons via intramolecular transfer from cytochrome a and Cu_A. For details, see text.

Fig. 6. Hypothetical structure of the oxygen-binding unit in cytochrome oxidase with Cu_B in EPR detectable form.

On the basis of this study and a previous report from this laboratory [5], it should be evident that Cu_B in cytochrome oxidase can not always be classified as a tetrahedral type 1 [23] or an axial type 2 [6] copper. Although the optical absorption in the 600 nm region may be fairly high, the EPR parameters of signals from Cu_B^{2+}, obtained during reoxidation and turnover, place Cu_B in a category of its own, similar to the type 3 copper in tree laccase [5]. The apparently conflicting observations of the structure of Cu_B in cytochrome oxidase and the resulting confusion about how to label this copper may be the result of naturally occurring changes in the conformation of the enzyme. It would be surprising if the rigid classification nomenclature used so far for copper in proteins was able to cover the expected dynamic situation in an enzyme such as cytochrome *c* oxidase.

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