

# Cytochrome oxidase: An alternative model

(O<sub>2</sub> reduction/metalloprotein structure/Fe(IV) heme/peroxidases)

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**ABSTRACT** Oxidative titration of reduced cytochrome oxidase (cytochrome *c* oxidase; ferrocyanide oxidoreductase, EC 1.9.3.1) in the presence of carbon monoxide and sulfide, at potentials greater than +500 mV (vs. the neutral hydrogen electrode), have failed to produce new copper signals in the electron paramagnetic resonance spectrum of this enzyme. This observation implies that one of the copper centers in cytochrome oxidase remains Cu(I) under strongly oxidizing conditions. The rationalization of this fact, and the possible explanation of a great accumulation of spectroscopic data, is that cytochrome *a*<sub>3</sub> may be a two-electron redox center, with stable Fe(IV), Fe(III), and Fe(II) states during its redox cycle. This oxidase model does not require an antiferromagnetic coupling scheme, in contrast to currently prevalent models.

Mitochondrial cytochrome oxidase (cytochrome *c* oxidase; ferrocyanide oxidoreductase, EC 1.9.3.1) is a large ( $M_r$  140,000) enzyme known to contain two copper atoms and two heme groups. The two heme iron moieties in the enzyme are prosthetic groups for cytochrome *a* and cytochrome *a*<sub>3</sub>, these two cytochromes being distinguished by the observation that one of them (*a*<sub>3</sub>) binds cyanide, carbon monoxide, and other ligands, whereas the other does not (1).

The principal spectroscopic anomaly associated with this enzyme is that the electron paramagnetic resonance (EPR) spectrum of oxidized oxidase shows only an oxidized low-spin ferric heme, presumably from cytochrome *a*, and a mildly unusual Cu<sup>2+</sup> signal, corresponding in intensity to one copper (Cu<sub>A</sub>). Thus one copper atom (Cu<sub>B</sub>) and cytochrome *a*<sub>3</sub> are "missing" from the EPR spectrum of the enzyme in its oxidized state. Upon partial reduction a high-spin heme iron EPR signal appears, and then disappears as reductive titration proceeds to completion (2, 3).

Because cytochrome oxidase has a four-electron redox cycle, early investigators thought it reasonable to assume a Cu<sup>2+</sup> → Cu<sup>1+</sup> reduction step for both copper atoms, and an Fe<sup>3+</sup> → Fe<sup>2+</sup> reduction step for both irons. Under this assumption, the "missing" EPR signals from Cu<sub>B</sub> and cytochrome *a*<sub>3</sub> were the result of an antiferromagnetic coupling between Cu<sub>B</sub><sup>2+</sup> and Fe<sup>3+</sup> (*a*<sub>3</sub>), giving an EPR-silent  $S = 2$  spin system. This picture of oxidase has caused Cu<sub>B</sub> frequently to be designated as Cu<sub>A</sub>, and different models for the possible metal-ligand arrangements that could produce such a coupling have been offered in the literature (3, 4).

There are two problems with these models as a class. The first problem is that no antiferromagnetic couplings of the type postulated have been found in nature (5); i.e., there are no antiferromagnetically coupled heme proteins, and there are no known couplings between dissimilar metals in natural systems, coupling having been proved only in Fe-Fe and Cu-Cu clusters. The second problem is that the Cu x-ray absorption edge spectrum of cytochrome oxidase should be sensitive to ligation but shows no change on addition of CN<sup>-</sup> (6) or CO (7), and when resolved into two components it shows virtually no oxidation-reduction edge shift for one of the two coppers (7, 8).

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We therefore have undertaken EPR experiments to test this model by attempting to define conditions under which EPR signals from both Cu<sub>A</sub> and Cu<sub>B</sub> would be observable. Previous reports of an EPR signal associated with Cu<sub>B</sub> are erroneous; in one case Seiter *et al.* (9) have found that a signal appearing during sulfide binding arises from transient free-radical formation, and in the other case (4), a signal ascribed to nitric oxide interaction with Cu<sub>B</sub> in the presence of azide is simply a feature of the EPR spectrum of partially reduced oxidase in the presence of azide alone (10).

The experiments described here monitor the oxidation of cytochrome oxidase in the presence of ligands (11, 12), in particular the oxidation of the reduced cytochrome oxidase-CO complex and the cytochrome oxidase-sulfide complex. Ferricenium was chosen as the oxidizing agent because of its high formal potential (+422 mV vs. the neutral hydrogen electrode) (13) and its complete "transparency" in EPR spectra (14) near  $g = 2$ , the region associated with copper signals. The reagent can be added in 100-fold molar excess in Triton X-100 solution with no damage to oxidase and with no spectral interference in the regions of interest; thus oxidizing potentials approaching +600 mV are readily attained.

## METHODS

Ferricenium was prepared by an antimony pentachloride oxidation of ferrocene (Aldrich) in methylene chloride solution. The isolated blue crystals [(SbCl<sub>6</sub>)<sup>-</sup> is the ferricenium counterion] were dissolved in 0.2 M potassium phosphate buffer (pH 7.2) at 5% Triton X-100 concentration.

Cytochrome oxidase was prepared according to Sun *et al.* (15) from beef heart mitochondria provided by Y. Hatefi. Oxidase concentration was calculated from  $\epsilon_{605}$  (reduced - oxidized) taken as 24 mM<sup>-1</sup>cm<sup>-1</sup>. Solutions were made anaerobic by repeated flushings with nitrogen or argon (Matheson, Newark, CA), and reduction of enzyme was accomplished with solid sodium dithionite (MCB, Norwood, OH).

EPR spectroscopy was performed on a Varian E-12 spectrometer equipped with an Air Products Heli-Tran cryostat (LTD-2-100) with temperature controller. Temperature was monitored with a gold/chromel thermocouple 2 mm below the sample. Microwave frequency was determined with a Hewlett-Packard frequency counter (5245L) and converter (2590B).

## RESULTS

Fig. 1 shows the spectrum of reduced oxidase reoxidized with ferricenium, and the spectrum of the carbon monoxide and sulfide complexes of oxidase in the presence of ferricenium at a high concentration. Neither the classical CO complex (1) nor Nicholls' (11) complex produce a Cu<sub>B</sub> signal, despite Soret and visible band shifts in the heme spectrum. Many and various alkyl sulfide (as opposed to H<sub>2</sub>S) complexes have been tested (12), and these also show only the original  $g = 2$  region signal attributed to Cu<sub>A</sub>.

Abbreviation: MCD, magnetic circular dichroism.

## DISCUSSION

Whether cytochrome  $a_3$  is held as  $\text{Fe}^{2+}$  (CO complex) or  $\text{Fe}^{3+}$  ( $-\text{SH}$  complex),  $\text{Cu}_B$  shows no  $\text{Cu}^{2+}$  signal, at oxidizing potentials up to +600 mV. In the case of the  $a_3$ -SH complex, there is an EPR signal that accounts for 94% of the expected intensity of the  $a_3$  species, so the absence of a  $\text{Cu}_B$  signal cannot be explained by antiferromagnetic coupling to  $a_3$ . If  $\text{Cu}_B$  fails to appear as  $\text{Cu}^{2+}$  at potentials approaching those of  $\text{O}_2$  itself (+810 mV), the possibility is strongly suggested that there is no  $\text{Cu}_B^{2+}$  state in the redox cycle of oxidase and that this species remains  $\text{Cu}_B^{1+}$  in the enzyme fully oxidized by  $\text{O}_2$ .

The observation that oxidized cytochrome  $a_3$  does not appear in the EPR spectrum of resting oxidized oxidase must then be explained without recourse to antiferromagnetic coupling. Such an explanation is not difficult to find. Oxygen-binding hemes [e.g., myoglobin and hemoglobin (16)] have a stable  $\text{Fe}^{4+}$  state at high oxidation potentials (+400 to +700 mV). Likewise, peroxidases and catalase have well-characterized  $\text{Fe}^{4+}$  states (17). Because cytochrome  $a_3$  must bind both oxygen and peroxide in its functional cycle, the possibility suggests itself that the resting oxidized state of iron in cytochrome  $a_3$  is  $\text{Fe}^{4+}$ , a  $d^4$  system that has spin state  $S = 2$  in its high-spin form and  $S = 1$  in its low-spin form. The species cytochrome  $a_3^{4+}$  has been mentioned as a possible short-lived low-temperature intermediate [compounds  $\text{B}_2$  and  $\text{C}_2$  of Chance *et al.* (18)]; we are suggesting that it is a stable species in fully oxidized oxidase. The resulting simple model for the oxidase  $\text{O}_2$  reaction site is contrasted with earlier models of oxidized oxidase in Fig. 2.

This picture of cytochrome  $a_3$  calls for a reinterpretation of existing data and suggestions for further experimentation. EPR, magnetic, and Raman results will be reviewed, and possible magnetic circular dichroism (MCD), magnetic, and Mössbauer experiments will be proposed.

(i) *EPR data.* The absence of an  $a_3$  signal in the oxidized oxidase spectrum has been attributed to an antiferromagnetic coupling between  $a_3^{3+}$  and  $\text{Cu}_B^{2+}$ ; in this model this absence is explained by postulating  $a_3^{4+}$  as the fully oxidized form. The high-spin heme signal from partially reduced oxidase in earlier models is said to result from disruption of antiferromagnetic coupling by reduction of  $\text{Cu}_B^{2+}$ ; here it is a simple consequence of the reduction of  $a_3^{4+}$  to  $a_3^{3+}$ . The heme ligands nitric oxide and sulfide (4, 19) should be readily oxidized by a heme of re-

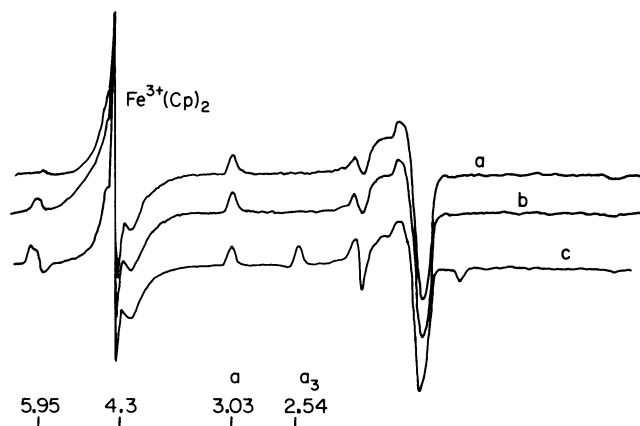


FIG. 1. EPR spectra of cytochrome oxidase: a, reduced, then reoxidized with ferricenium; b, reduced, treated with carbon monoxide, and reoxidized with ferricenium; c, treated with sodium sulfide and reoxidized with ferricenium. Oxidase concentration: 0.15 mM in 2% Triton X-100, phosphate buffer at pH 7.2. Sulfide 0.2 mM for c. EPR conditions: temperature 10 K; 10-mW power at 9.356 GHz; modulation amplitude 6 G. The high-spin signals near  $g = 6$  in b and c disappear completely at higher ferricenium [ $\text{Fe}^{3+}(\text{Cp})_2$ ,  $g = 4.3$ ] concentrations (Cp, cyclopentadiene).

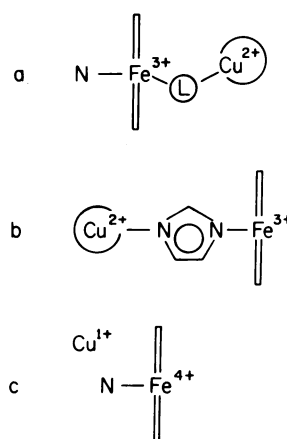


FIG. 2. Cytochrome  $a_3$  models. In models a (17) and b (1), antiferromagnetic coupling is mediated between  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  by ligands. In model c, the  $\text{Cu}^{1+}$  center may be anywhere in the protein.

duction potential higher than +300 mV, and in the present model oxidation of these ligands results in a high-spin rhombic  $a_3^{3+}$  signal in the nitric oxide case and a low-spin  $a_3^{3+}$  signal in the sulfide case, as observed. [Because  $\pi$ -acceptor ligands such as CO and  $\text{CN}^-$  have no measurable effect on the copper x-ray absorption edge spectrum (6, 7), the suggestion (4) that NO binds to and reduces  $\text{Cu}_B$  is difficult to credit.] An assignment of one-electron reduction potentials for the four species  $\text{Cu}_A^{2+}$ ,  $a_3^{3+}$ ,  $a_3^{4+}$ , and  $a_3^{3+}$ , including a heme-heme redox interaction (20) will be presented elsewhere as a computer fit to EPR reductive titration data.

(ii) *Magnetic data.* Susceptibility studies (21) have asserted that oxidized cytochrome oxidase contains an  $S = 2$  species in addition to the species  $a_3^{3+}$  ( $S = 1/2$ ) and  $\text{Cu}_A^{2+}$  ( $S = 1/2$ ). The conventional model ascribes the  $S = 2$  component to the coupled pair  $a_3^{3+}$  ( $S = 5/2$ ) and  $\text{Cu}_B^{2+}$  ( $S = 1/2$ ); here the  $S = 2$  component is the  $d^4$  system  $a_3^{4+}$  (see Fig. 3A). MCD data (22) have also been presented as evidence for an  $S = 2$  system. The temperature dependence of the MCD signal in myoglobin (23) should be compared to that of oxidase (24) over a wide temperature range and in a great variety of derivatives, because the details of this temperature dependence may yield information about possible spin-state transitions. It should perhaps be noted that proof of an antiferromagnetic coupling in magnetic studies requires the demonstration of a magnetic transition. The supposed coupling in oxidase ( $J \approx -200 \text{ cm}^{-1}$ ) corresponds to a transition above room temperature, so the enzyme cannot be made to yield positive evidence for this coupling.

(iii) *Resonance Raman.* The principal resonance Raman result on oxidase is that the enzyme is readily photoreduced by Soret-wavelength illumination (25, 26). No explanation of this

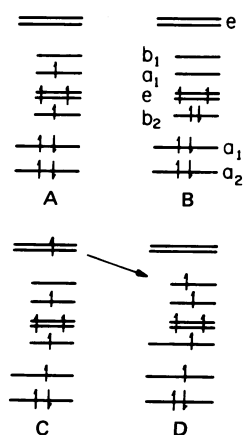


FIG. 3. Orbital scheme for model 2c. The Fe set [ $b_1, a_1, e, b_2$ ] and porphyrin set [ $a_1, a_2$ ] of orbitals under  $C_{4v}$  symmetry are shown for cytochrome  $a_3^{4+}$ . Case A represents resting oxidase; case B represents a low-spin complex such as  $a_3$ -CN, and cases C and D represent a scheme for explaining the photoreduction of iron in cytochrome  $a_3$  when Soret illumination excites a porphyrin electron.

effect is offered in the conventional model, and in fact no isolated heme  $a^{3+}$  model system is photoreducible (26). On the other hand,  $d^4$  porphyrins, most notably  $Mn^{3+}$  porphyrins, are readily (27) photoreducible in the Soret region. A simplified diagram of this effect is offered in Fig. 3 C and D. It is perhaps not surprising that resonance Raman has not yielded much new information about cytochrome oxidase, because the spectra obtained with illumination at 441 and 600 nm should both be dominated by the spectral contributions of cytochrome  $a$  (28–30) rather than  $a_3$ .

(iv) *Experiments.* Three lines of evidence to support the  $Fe^{4+}$  model of cytochrome  $a_3$  can be attempted. First, MCD studies of the species produced by one-electron oxidation of metmyoglobin and of catalase with ferricenium, and cyanide derivatives of these, should be compared to analogous studies on oxidase. Second, magnetic susceptibility studies of methyl isonitrile complexes and alkyl sulfide complexes of oxidase would provide spin-state data without the complication of reduction, a potential problem arising in the 24-hr incubation of oxidase with potassium cyanide as currently practiced (22). Third, Mössbauer experiments (31) on  $^{57}Fe$ -enriched oxidase, from yeast or other sources, could provide valuable data in comparison to existing results on peroxidases (32). These three magnetic experiments are indicated as the most reliable characterization of  $d^4$  systems.

### CONCLUSION

The model presented here postulates an oxidation scheme starting with reduced cytochrome oxidase as cytochrome  $a_3^{2+}$ , cytochrome  $a^{2+}$ ,  $Cu_A^{1+}$ . ( $Cu_B^{1+}$  does not participate in the oxidation–reduction cycle.) Oxygen binds to cytochrome  $a_3$  to produce the intermediate species cytochrome  $a_3^{4+}-O_2^{2-}$ . Cytochrome  $a$  and  $Cu_A$  pass an additional electron each through cytochrome  $a_3$  to the “bound peroxide” species. Because the rates of electron transfer from  $a$  and  $Cu_A$  to  $a_3$  are presumably both rapid but not necessarily identical, there may be a transient cytochrome  $a_3^{3+}-O_2^{2-}$  intermediate. As  $H^+$  ions are supplied from solvent water, the reaction proceeds to give cytochrome  $a_3^{4+}$  and two water molecules; the other redox components are now cytochrome  $a^{3+}$  and  $Cu_A^{2+}$ . Certain of the intermediate species postulated here are recognized as similar to those of peroxidase reactions.

The presence of a copper center in oxidase ( $Cu_B$ ) with a reduction potential higher than +800 mV has several precedents in copper biochemistry (5). Perhaps  $Cu^{1+}$  in oxidase is a structural component, like  $Zn^{2+}$  in zinc enzymes, or perhaps it has a functional role yet to be discovered.

The immediate result of  $O_2$  reduction, requiring uptake of four  $H^+$  ions on the cytochrome  $a_3$  side of the mitochondrial membrane, is an alkalization of the mitochondrial interior. This could appear to be a proton-pumping function in some experimental systems (33) and not in others (34). *In vivo*, only this alkalization may be important in oxidase energetics, because  $H^+$  ejection into strongly buffered cellular interiors is electrochemically futile. In summary, this oxidase model calls only for redox interactions that are already familiar in biochemistry, and it accounts for the principal spectroscopic and chemical observations on this important and interesting system. It further suggests a class of porphyrin model systems that should be efficient synthetic catalysts for oxygen reduction.

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