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Commentary

The nature of Cu_A in cytochrome *c* oxidase

Peter Mark Li, Bo G. Malmström* and Sunney I. Chan

Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, CA 91125, USA

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Kroneck et al. [(1988) FEBS Lett. 242, 70–74] have recently suggested, on the basis of a comparison with the EPR properties of nitrous oxide reductase, that cytochrome *c* oxidase contains a mixed-valence binuclear copper site, and that this is responsible for the EPR spectrum generally ascribed to Cu_A. Here we question this hypothesis in view of a multitude of analytical and spectroscopic data available. We maintain that a mononuclear Cu site with two cysteine sulfur and two imidazole nitrogen atoms as ligands is consistent with the current experimental information on the Cu_A site.

Cytochrome-*c* oxidase; Copper A; Nitrous oxide reductase; Mixed-valence binuclear center; EPR

Kroneck et al. [1] have recently reported multifrequency EPR spectra for nitrous oxide reductase. They observed a seven-line hyperfine pattern in both the X- and S-band EPR spectra and attributed this signal to a binuclear Cu site with the unpaired electron delocalized between two equivalent Cu nuclei. They furthermore suggested that a similar site is present in cytochrome *c* oxidase, and that this gives rise to the EPR spectrum generally assigned to Cu_A [2–4]. Here, we shall summarize a variety of experimental observations which would seem to contradict this hypothesis.

Steffens et al. [5] have suggested that cytochrome oxidase is a three-copper, two-heme A protein. A recent X-ray absorption investigation [6] has, however, shown that many preparations of cytochrome oxidase contain closer to two copper atoms per functional unit. Moreover, the extra copper present in some oxidase samples can be removed by various dialysis procedures [6,7]. These experiments indicate that two coppers are tightly bound by the enzyme, and that the third may be an adventitiously bound non-functional copper ion.

Extended X-ray absorption fine structure (EXAFS) studies [8,9] also constitute evidence strongly against a binuclear Cu_A site. There is no evidence for Cu-Cu scattering for either Cu_A or Cu_B. Recently, it has been shown [9–11] that Cu_A can be converted to a mononuclear type 2 copper site. A clear picture of the ligand structure for both Cu_A and Cu_B has emerged from EXAFS measurements on native and Cu_A-depleted oxidases [9], indicating that Cu_A has two N(O) and two S ligands.

In addition to the lack of spectroscopic evidence for a binuclear copper site, there is no functional evidence to support a binuclear Cu_A model. Redox titrations [12] show that Cu_A is a one-electron acceptor, displaying weak anticooperative redox interactions with cytochrome *a*, and possibly with the cytochrome a_3-Cu_B site. If Cu_A were a copper dimer, one would expect an anomalous redox behavior. Specifically, it should be possible for the proposed mixed-valence Cu-Cu dimer to be fully

Correspondence address: S.I. Chan, Arthur Amos Noyes Laboratory of Chemical Physics, 127-72 Caltech, Pasadena, CA 91125, USA

* *Permanent address:* Department of Biochemistry and Biophysics, University of Göteborg and Chalmers University of Technology, S-412 96 Göteborg, Sweden

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oxidized to a type 3 superexchange coupled site during turnover conditions [13].

Kroneck et al. [1] partially justify a two-copper model for Cu_A by stating that less than 40% of the copper in the oxidase is detectable by EPR in the native enzyme. This is a little misleading, however, because the hypothesized mixed-valence binuclear copper site is a Kramer's doublet with $S = 1/2$, without cause for an intensity anomaly. More likely, the somewhat less than 15% EPR intensity unaccounted for arises from a magnetic interaction of Cu_A with a nearby magnetic center, possibly cytochrome *a* [4,14] or the residual adventitious copper that is still bound to the enzyme.

Perhaps the strongest evidence against any similarity between cytochrome oxidase and nitrous oxide reductase is the lack of a seven-line hyperfine pattern in the cytochrome oxidase EPR spectrum in the X-band [2,3]. In fact, the X-band spectrum of cytochrome oxidase does not resemble the spectra of any known Cu-Cu dimers [15]. The *g* values extracted from the Q-band spectra of cytochrome oxidase [2] and nitrous oxidase reductase [1] are similar, but the actual spectra are quite dissimilar. That of reductase resembles spectra dominated by rapid passage, whereas the oxidase spectrum can be well simulated on the basis of a mononuclear $S = 1/2$ system [2]. It is true that cytochrome oxidase does give a seven-line hyperfine structure in the S-band [4]. This can, however, be accounted for in terms of hyperfine coupling to copper with some additional magnetic interaction, possibly with cytochrome *a* [4]. An electron spin relaxation study [14] has further shown that the Cu_A is coupled to a paramagnetic center 13–26 Å away, most likely cytochrome *a*. In addition to EPR spectroscopy, electron nuclear double resonance (ENDOR) has been used extensively to study the Cu_A site [3,16]. These studies confirm that the Cu_A site may be accounted for by a monomeric Cu(II) site with two histidine N ligands and two cysteine S ligands.

We feel the suggestion that the EPR spectrum generally ascribed to Cu_A actually originates from a mixed-valence Cu-Cu dimer can be excluded on the basis of the results summarized here. Not all oxidase preparations contain three coppers in their functional unit [6], but all contain one Cu_A per functional unit. In addition, the narrow, well-resolved EPR spectrum in the Cu_A modified oxidase [9–11] cannot arise from a copper pair. We have also pointed out that the EPR characteristics of nitrous oxide reductase and cytochrome *c* oxidase are quite distinct. Accordingly, we see no reason to abandon the model of Cu_A as a mononuclear site with the copper ion coordinated to two cysteine sulfurs and two imidazole nitrogens, particularly since this model has received strong support from such varied spectroscopic studies.

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