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Minireview

The nature of the Cu_Acenter in cytochrome *c* oxidase

Bo G. Malmström and Roland Aasa

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

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The merits of the suggestion that Cu_A in cytochrome oxidase is a mixed-valence binuclear site is reviewed on the basis of recent analytical and spectroscopic studies. First an alternative mononuclear model is presented. Metal analyses indicate that homogeneous oxidase preparations with high activity contain 3Cu/2Fe. Multifrequency EPR measurements demonstrate a close similarity with a copper site in nitrous oxide reductase, and this is also supported by optical and MCD spectra. Strong evidence for a binuclear site is provided by a 7-line hyperfine structure in the EPR spectra of both enzymes. A binuclear model consistent with amino acid sequence data can be formulated.

Cytochrome oxidase; Nitrous oxide reductase; Copper-A; Mixed-valence Cu center; Multifrequency EPR

1. INTRODUCTION

Keilin suggested in 1938 already that cytochrome oxidase is a copper protein, but with the discovery of cytochrome a_3 in the following year the possible functional involvement of copper was largely ignored (see [1] for documentation of these historic aspects). Interest in the copper component was renewed in the 1950s, even if it remained controversial for another 20 years. With the application of the EPR technique, starting in 1959, a picture gradually evolved in which the functional unit of the oxidase contains 3 redox centers, cytochrome *a* and Cu_A, the primary acceptors of electrons from cytochrome *c*, and the binuclear cytochrome a_3-Cu_B unit, the O_2-reducing site. This picture appeared to be established and generally accepted until 1988, when Kroneck et al. [2] suggested that Cu_A is really a mixed-valence binuclear site. This suggestion was immediately challenged [3], but during the last few years it has received considerable experimental support. It is the purpose of this contribution to review critically the evidence for and against the hypothesis of a binuclear site.

In 1960 it was demonstrated by EPR that Cu^{2+} in some blue copper proteins (type 1) have unusually small hyperfine coupling constants (<10 mT) compared to non-blue proteins (type 2) or synthetic copper complexes [4]. The EPR spectrum ascribed to Cu_A^{2+} was, however, later found to be quite unique, with a hyperfine coupling constant $A_z < 4$ mT and an anomalously low *g* value, more like that of a free radical [5]. Kroneck et al. [2] showed that nitrous oxide reductase (N_2OR) has EPR parameters very similar to those of Cu_A^{2+}, so that cytochrome oxidase is no longer unique. In addition, they found a seven-line hyperfine pattern, and this observation formed the basis for their suggestion that both enzymes contain a binuclear site. The presence of a mixed-valence copper pair had, in fact, been suggested by Beinert et al. in a long footnote to their 1962 paper [5], but they soon retracted this idea. In the following, recent studies pertaining to the hypothesis of a binuclear copper site in cytochrome oxidase will be briefly discussed. This discussion will be preceded by a short presentation of a mononuclear model.

2. A MONONUCLEAR MODEL

Cu_A is bound to subunit II of the oxidase. This was first suggested [6] on the basis of sequence homologies with the blue copper proteins, azurin and plastocyanin, and it has now been proven experimentally by genetic engineering of the homologous subunit II from a cytochrome *o* quinol oxidase [7]. This enzyme lacks Cu_A, but the site has been restored by the introduction of the presumed copper ligands. This also demonstrates that the copper-binding region of subunits II has the β structure of blue copper proteins, the so-called cupredoxin fold.

Correspondence address: B.G. Malmström, Department of Biochemistry and Biophysics, Chalmers University of Technology, S-412 96 Göteborg, Sweden. Fax: (46) (31) 772 2813.

Abbreviations: ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; N_2OR, nitrous oxide reductase; MCD, magnetic circular dichroism.

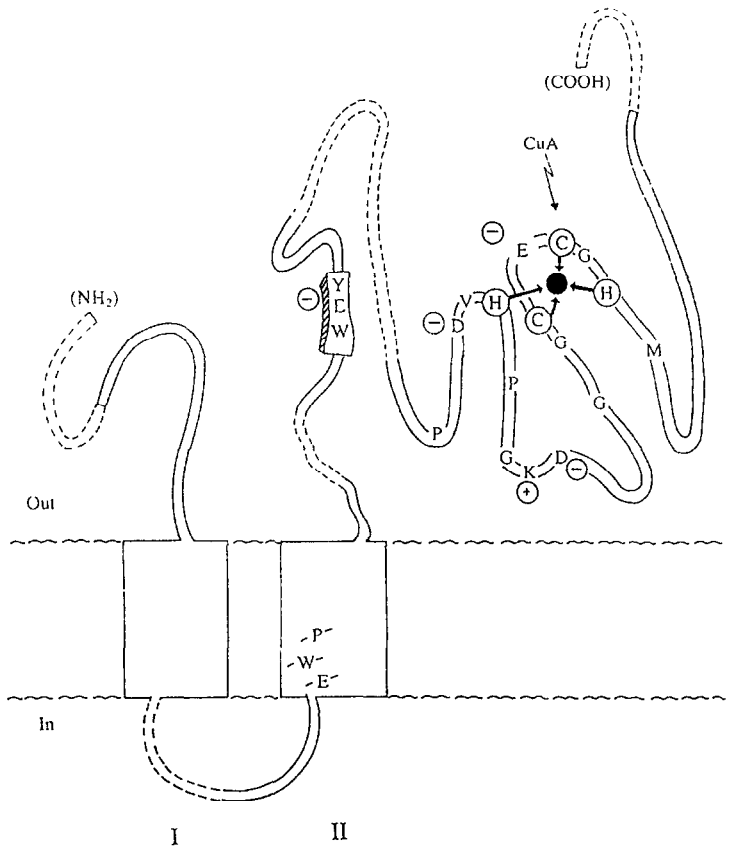

Fig. 1. Model of the membrane-anchored subunit II of cytochrome oxidase with a mononuclear Cu_A site. Invariant amino acid residues are shown. From [8].

Subunit II is thought to be anchored to the membrane by two transmembrane helices, with the Cu_A site located outside the membrane, as shown in Fig. 1 [8]. The structure of this region in the COOH-terminal part of the peptide chain has been modelled [9,10] on the basis of conserved cysteine and histidine residues and the known azurin structure. In these models the Cu_A site is assumed to be mononuclear with two cysteine and two histidine residues as ligands (Fig. 1). Such a coordination is supported by ENDOR spectra [11,12], and the presence of two cysteines may also result in electron delocalization away from sulfur onto copper, explaining the free-radical character of the EPR spectrum. Some other features are, however, better described in terms of a binuclear structure (section 3).

3. EVIDENCE FOR A BINUCLEAR SITE

3.1. *Analytical evidence*

Crucial to the question of whether or not the Cu_A site is binuclear is the Cu/heme ratio of well-defined oxidase preparations. Steffens et al. [13] have reported that bovine, as well *Paracoccus*, oxidase contains 3Cu/2Fe, but other groups [14,15] have found 2.5Cu/2Fe. A few years ago we analyzed a large number of oxidase preparations for metals by an X-ray fluorescence method [16]. The EPR characteristics, peptide compositions, protein and phospholipid contents, as well as the catalytic activities, of the samples were also determined. According to our results, preparations that were good by other criteria (high catalytic activity in particular) always had Cu/Fe ratios close to 1.5. It is also notable that the integrated intensity of the Cu_A EPR signal was 1.0, whereas the preparations we made in the 1970s showed an intensity of 0.7–0.8 and contained 2.7–2.8Cu/2Fe [17]. In other words, more pure preparations have both higher total Cu content and higher EPR intensity, which supports the binuclear model.

A blue Cu site has also been created in subunit II of cytochrome *o*, but the Cu_A mutant always contained more Cu/mg protein compared to the blue protein [7] (and data presented by M. Saraste at the 7th European Bioenergetics Conference). Again, this would agree with the binuclear hypothesis.

3.2. *Spectroscopic evidence*

3.2.1. EPR spectroscopy

Vänngård [18] showed in 1972 that, in a plot of $A_{\parallel}$ vs.

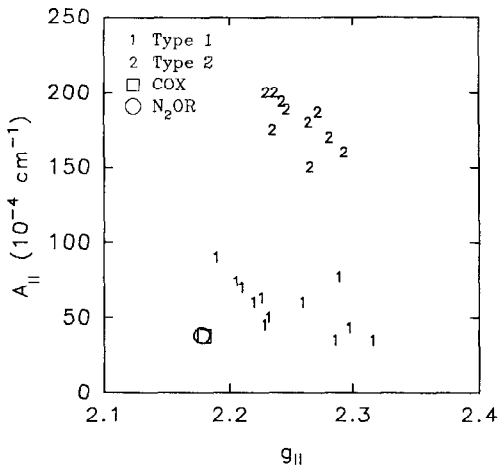

Fig. 2. A plot of $A_{\|}$ vs. $g_{\|}$ for a number of copper proteins. The upper points (2) represent type 2 centers, whereas the lower points (1) are for type 1 proteins; the double point in the lower left-hand corner shows the parameters for cytochrome oxidase and N_2OR. Modified from [18].

$g_{\|}$, the parameters for type 1 and type 2 Cu^{2+} centers fall in different regions, but Cu_A was found to be in a category by itself. As seen in Fig. 2, the parameters for N_2OR [2] are very close to those for the oxidase, and the strong relationship between the sites in the two proteins has been further documented by multifrequency EPR [19,20] and electron spin echo spectroscopy [21]. These data alone are sufficient to say that the sites must be nearly identical, and this has been demonstrated also by other types of spectroscopy (section 3.2.2) as well as by homologies in amino acid sequence [22].

Froncisz et al. [23] were the first to show that hyperfine structure can be resolved in the $g2$ signal of oxidized cytochrome oxidase at microwave frequencies < 9 GHz. They suggested that this could only in part be ascribed to an interaction with an $S = 3/2$ copper nucleus, but that an interaction with a proton or another paramagnetic site must be involved as well. As a possibility they considered interaction with cytochrome *a*, and this has also been invoked by other authors [3,24]. Such an interaction could also account for another unusual feature of the cytochrome oxidase $g2$ signal, namely that it disappears at temperatures above 150 K [25]. The EPR signal from the Cu_A mutant of cytochrome *o*, which lacks cytochrome *a*, also disappears at this temperature, however, but this protein has, in addition, another paramagnet, a type 2 Cu^{2+} ion [7]. It appears, on the other hand, rather unlikely that cytochrome *a* in another subunit and a type 2 ion in subunit II would have exactly the same effect.

With N_2OR a 7-line hyperfine structure can be resolved even at 9 GHz (X-band) [2], and this is most readily explained in terms of one unpaired electron interacting with two $S = 3/2$ nuclei. Thus, a mixed valence binuclear site, [Cu(1.5)...Cu(1.5)], $S = 1/2$, was suggested for this enzyme, and this hypothesis was strengthened by the multifrequency EPR [19,20] and spin echo [21] studies already mentioned. Kroneck and co-workers [19,20] have also investigated the EPR properties of cytochrome oxidase at several frequencies, and their results are best interpreted in terms of a Cu–Cu interaction in both enzymes. In particular, for a 4-line pattern from a mononuclear Cu center, no consistent g_x value could be obtained. The main difference between the two enzymes is that hyperfine structure cannot be resolved at X-band with cytochrome oxidase. It should be noted, however, that such structure is seen also at X-band in our Cu_A mutant (Fig. 3), so perhaps its absence in the intact oxidase is caused by a line broadening due to interactions with the other paramagnetic centers. Simulation on the basis of the binuclear model can nicely account for the N_2OR spectrum, as shown in Fig. 3.

Even if the EPR properties strongly support a binuclear site in both enzymes, such a model cannot explain the unusual temperature dependence of the $g2$ signal [25]. Thus, it may still be necessary to invoke an interaction with another paramagnetic site. Our mutant work

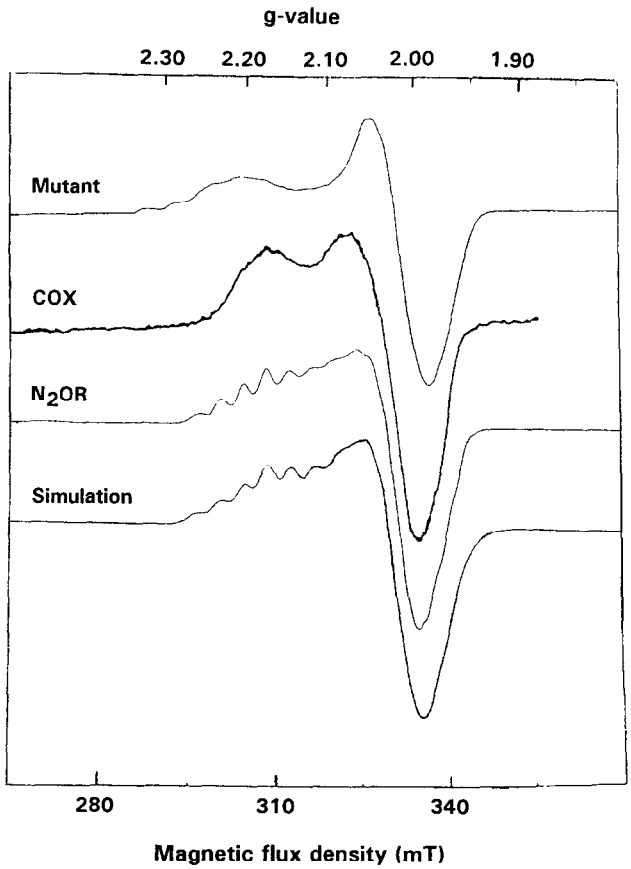

Fig. 3. EPR spectra at 9 GHz of the Cu_A mutant of cytochrome *o* [7], of cytochrome oxidase and of N_2OR [26] as well as a simulated spectrum for a binuclear site in N_2OR.

[7] speaks against this, however, and this property may be the result of a unique binding-site geometry [17].

3.2.2. Other spectroscopic properties

The optical spectrum of the Cu_A mutant of subunit II in cytochrome *o* [7] closely resembles that of N_2OR [26], both having a strong band around 550 nm and a weaker one close to 800 nm. This again emphasizes the close relationship between the copper sites in these two enzymes, which has also been demonstrated by MCD measurements [26–28]. Thus, if the site in N_2OR is binuclear, then the Cu_A site is so as well. Even if this is not definitely established, it certainly offers the best explanation for the 7-line hyperfine structure in the EPR spectrum (section 3.2.1).

The MCD results indicate that 2 cysteine residues are associated with the Cu_A site [28], and this has also been demonstrated by EXAFS data [29,30]. These findings would be consistent with a mononuclear model (section 2), but they do not exclude a binuclear center. In the latter case, the two cysteines would interact with two copper ions, as suggested in a model by Zumft et al. [22] (section 4).

4. CONCLUDING REMARKS

Unfortunately it does not appear possible to us to state with absolute certainty that Cu_A constitutes a mixed-valence binuclear site, but the weight of evidence is in favor of this hypothesis. Particularly strong support comes from the analytical data (section 3.1) and the multifrequency EPR studies (section 3.2.1).

For the binuclear model to be acceptable, it must be consistent with the amino acid sequence information [6,22]. It is possible to associate the conserved cysteine and histidine residues with two copper ions rather than with one, as in the mononuclear model [9,10]. This has, for example, been done in a model formulated by Antholine et al. [20], partly on the basis of suggestions by Farrar et al. [26]. They propose the following structure: N(His)-S(Cys)-[Cu(1.5)-S(Cys)-Cu(1.5)]-N(His)-S(Met), with a cysteine S serving as the bridging ligand. The 7-line hyperfine pattern requires a structure with a certain symmetry, but this does not have to be absolute [22], so the model is feasible. As an alternative bridging ligand, a carboxylate group was suggested, since there is a conserved aspartate residue separated by one amino acid from one of the ligand histidines.

In conclusion, we think there is strong support for a mixed-valence binuclear site in N_2OR as well as in cytochrome oxidase, and such a model is consistent with all spectroscopic and structural infomation.

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