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# The binuclear $Cu_A$ centre of cytochrome oxidase

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## 1. Introduction

Cytochrome oxidase is the terminal catalyst in the mitochondrial as well as in many bacterial respiratory chains. Cytochrome *c* oxidase contains three redox centres, $Cu_A$, cytochrome *a*, and the binuclear cytochrome $a_3$-$Cu_B$ centre where oxygen is reduced to water (Fig. 1). The latter are located in subunit I, wheras $Cu_A$ resides in subunit II (for reviews, see Refs. [1–5]). $Cu_A$ has been shown to be the primary electron acceptor in cytochrome *c* oxidases [6].

Aerobic bacteria possess respiratory enzymes that are homologous to mitochondrial cytochrome *c* oxidase. Some aerobic bacteria have also homologous enzymes that use quinol instead of cytochrome *c* as the electron donor. Quinol oxidases are structurally and functionally related to cytochrome *c* oxidases, but they have lost the $Cu_A$ during evolution [7]. We have been able to reconstruct a $Cu_A$-like centre to the initially copperless subunit II (CyoA) of the *E. coli* cytochrome *bo* quinol oxidase complex. This shows that the three-dimensional fold of this copper-binding domain is conserved between cytochrome *c* oxidases and quinol oxidases [8].

The spectroscopical characterization of $Cu_A$ has showed that it cannot be described within the classical scheme of three types of copper centre [9]. As shown in Fig. 2, the EPR spectrum of $Cu_A$ is striking similar to the spectrum of a copper centre in nitrous oxide reductase ($N_2OR$), an enzyme involved in the reduction of nitrous oxide to dinitrogen [10,11]. $N_2OR$ is a homodimer, in which each monomer contains two copper centres (Fig. 1A). Centre A that is similar to $Cu_A$, is believed to be an electron transfer site, wheras the EPR-silent centre Z may be the catalytically active site [11]. It has recently been proposed that the $Cu_A$ centre of cytochrome *c* oxidase has evolved from the centre A of $N_2OR$ [12].

## 2. Spectroscopical properties of $Cu_A$

It has not been possible to see the full optical spectrum of $Cu_A$ in the intact cytochrome *c* oxidase, because of the strong absorption of haems. Only a broad absorption peak around 800–850 nm has been assigned to $Cu_A$. However, magnetic circular dichroic spectra have shown that $Cu_A$ has transitions throughout the spectral region from 450 to 950 nm [13]. We have been able to express the $Cu_A$-binding soluble domain from the *Paracoccus denitrificans* cytochrome $aa_3$. This domain is free of other metal centres and therefore a suitable sample for spectroscopical work. The optical spectrum of this domain, as well as the spectrum of corresponding quinol oxidase domain with the engineered purple copper site, show absorption maxima at 480 and 530 nm and a broad peak around 800 nm [8,14]. Similar results have been obtained with the soluble $Cu_A$-binding domain from cytochrome oxidase of *Bacillus subtilis* [15]. These spectra are very similar to the optical spectra from $N_2OR$. The main difference is the absence of a 'shoulder' around 650 nm, a feature assigned to the centre Z of $N_2OR$ [11]. At alkaline pH, the copper centre in the isolated *Paracoccus* domain acquires a type-II-like character as seen in its EPR spectrum. This feature is seen neither in the intact oxidase nor in $N_2OR$, indicating that $Cu_A$ is more exposed to solvent in the soluble domain than in the native enzyme [14].

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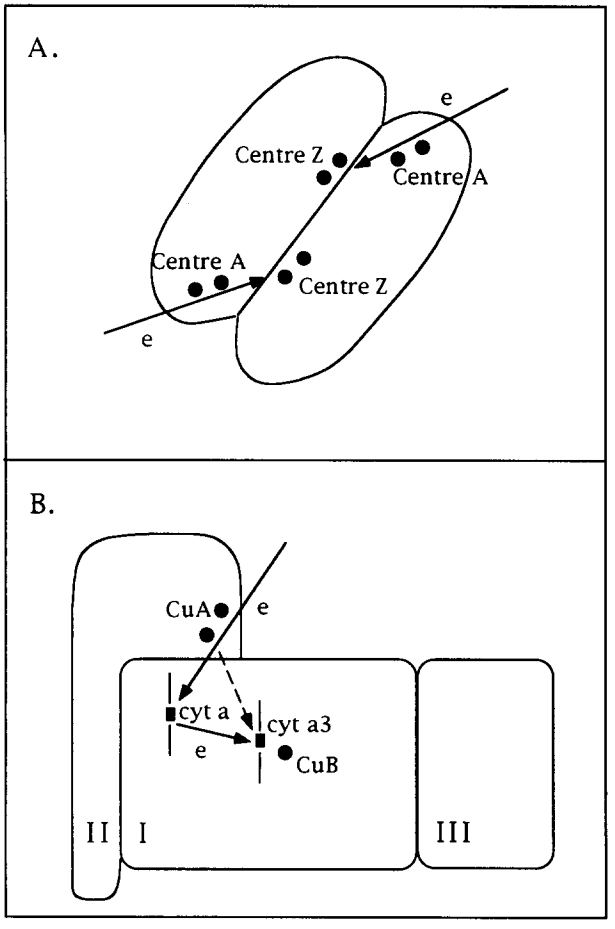


Fig. 1. Schematical models for the arrangement of the metal centres in nitrous oxide reductase ($N_2OR$) and cytochrome *c* oxidase. The proposed electron transfer routes are indicated by arrowheads. (A) $N_2OR$ homodimer is formed from two monomers. Each monomer contains two binuclear copper centres. Centre A is the putative electron transfer centre, and centre Z in believed to be the catalytic site [11]. (B) In cytochrome *c* oxidase, the $Cu_A$ is located in subunit II. The other catalytically active metal centres including the active site for oxygen reduction reside in subunit I.

The EPR spectra of $N_2OR$ and cytochrome *c* oxidase are similar and have anomalously low *g* values [5,16] (Fig. 2). One unusual feature common to both spectra is the broadening of the signal at temperatures above 150 K [17,18]. This has been proposed to arise from the interactions between $Cu_A$ and other metal centres. However, this feature is also seen in the isolated $Cu_A$-domain, and it must, therefore, be due to the unusual structure of this copper centre [14].

## 3. Binuclear $Cu_A$

The EPR spectrum of $N_2OR$ contains a well resolved seven-line hyperfine structure at 9 GHz (X-band). Kroneck et al. [19] have suggested that this feature arises from a mixed valence [Cu(1.5)...Cu(1.5)] $S = 1/2$ species with the unpaired electron delocalized between two equivalent copper nuclei. In the intact oxidase, this feature is more difficult to resolve in the X-band spectrum. However, the spectra of the isolated *Bacillus* and *Paracoccus* $Cu_A$-domains have a weakly resolved seven-line hyperfine structure at the X-band frequency [14,15], as shown in Fig. 2. Spectroscopic signatures of the copper centre in the soluble *Paracoccus* domain are almost identical to those seen in the intact cytochrome oxidase (P.L., M.S., J. Farrar and A. Thomson, unpublished data).

The binuclear $Cu_A$ model has recently been supported by many biochemical experiments. Many authors (see Ref. [5]) find that different cytochrome *c* oxidase preparations contain 3 Cu/2 Fe. We have measured the Cu/protein ratios of the isolated *Paracoccus* $Cu_A$-domain as well as of the engineered $Cu_A$-domain of CyoA. Electrospray mass spectrometry has showed that in both cases the mass of the copper-containing purple protein is 126–128 Da higher than the mass of the apoprotein [14,15,20]. This is approximately twice the mass of a copper atom (63.5 Da), indicating that two coppers are bound to the protein monomer. Chemical copper measurements give a value of 1.9 Cu/monomer of the isolated *Paracoccus* domain. Approximately one half of this copper is EPR-

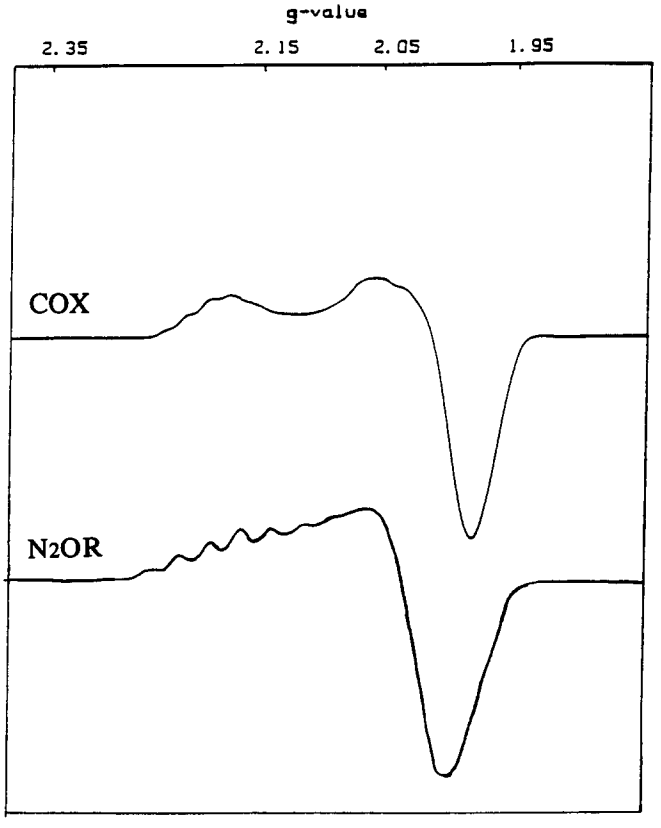


Fig. 2. X-band EPR spectra of the isolated *Paracoccus* $Cu_A$ domain (COX) and of $N_2OR$. The spectra were recorded at 10 and 15 K, respectively. The $N_2OR$ spectrum is taken from Ref. [11].

visible in good agreement with the mixed-valence binuclear $Cu_A$ model [14].

## 4. $Cu_A$ ligands

The alignment of cytochrome oxidase sequences have suggested that the copper-binding domain may have a topology analogous to the cupredoxin fold of blue copper proteins [1]. The copper atom is normally ligated by two histidines, a cysteine and weakly to a methionine in cupredoxins [21]. However, methionine seems not to be an essential ligand, because in azurin, it can be replaced by other amino acids without dramatical changes of the blue copper centre [22]. The topology of the protein fold and the positions of the copper ligands are well maintained in small blue copper proteins. One histidine is located in a loop between the $\beta$-strands C and D, where as cysteine, methionine and the other histidine are located in a loop between the $\beta$-strands F and G, using the nomenclature for amicyanin [23]. Subunit II of cytochrome *c* oxidase has a very similar pattern of plausible copper ligands. Cytochrome *c* oxidases have a second conserved cysteine in the region corresponding to the amicyanin FG-loop (see [8]), and three highly conserved carboxyl acids [1]. The histidine/cysteine ligation of $Cu_A$ has been experimentally supported by ENDOR and MCD studies [13,24]. Steffens et al. [25] have recently suggested that also a conserved aspartate that is also present in all known $N_2OR$ sequences, could serve as a ligand to $Cu_A$.

In order to identify the $Cu_A$ ligands, we have mutated all putative and conserved copper-binding residues of the isolated *Paracoccus* $Cu_A$ domain. The results of optical spectroscopy and quantitative Cu/protein measurements have showed that only five residues are important for the $Cu_A$ (P.L. and M.S., unpublished data). One of them is a histidine that is located in the region corresponding the CD loop of amicyanin, whereas the others, two cysteines, a histidine and a methionine, are located in the region corresponding to the amicyanin FG loop.

Replacement of the methionine either with a threonine or a leucine seems to cause problems in protein folding. This indicates that this methionine, which is also conserved in copperless quinol oxidases, may have an important role for the folding and geometry of the $Cu_A$ centre. We have no direct evidence that it is a ligand of $Cu_A$. Fig. 3 shows our current model for the binuclear $Cu_A$ centre. Two coppers are in the chemically identical environments which satisfies the prediction of the EPR spectra, and two histidines and two cysteines are the major ligands.

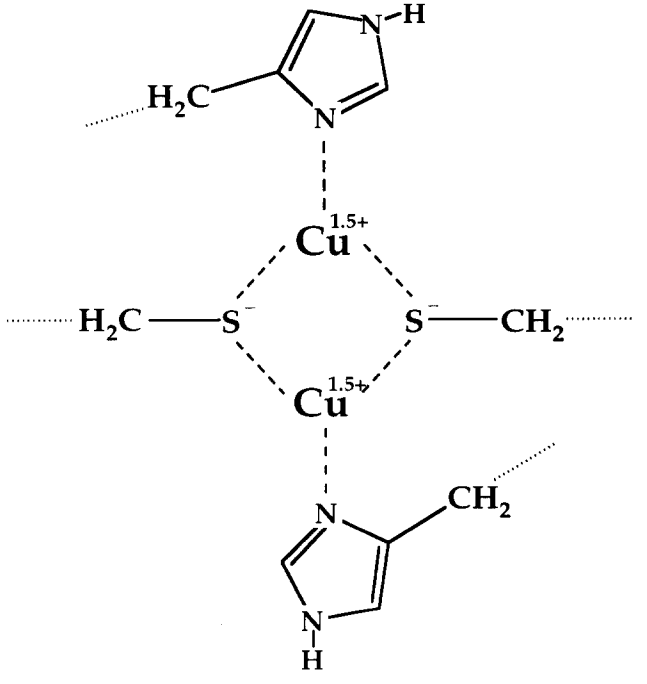


Fig. 3. Proposed model for the $Cu_A$ centre.

## 5. The possible role of the binuclear $Cu_A$

The functional reason behind the binuclear structure of $Cu_A$ is unclear. Chan and Li [26] have proposed that the $Cu_A$ centre could play a role in proton pumping. However, the absence of $Cu_A$ in quinol oxidases that are capable in proton pumping, makes this hypothesis unlikely. At the moment, it is widely agreed that this copper centre is involved in electron transfer to the catalytically active centres in cytochrome *c* oxidase and $N_2OR$. In the intact oxidase, $Cu_A$ does not have any water in the immediate coordination sphere of the metals [27]. The pH sensitivity of $Cu_A$ in the isolated domain suggests that it has become more exposed to the aqueous milieu [14]. This may indicate that $Cu_A$ is located close to the interface between subunits I and II. The distance between $Cu_A$ and haem *a* has been estimated to be 14–16 Å (see [28]). One possibility is that the binuclear CuA functions as a short electron conductor between cytochrome *c* and haem *a*, decreasing the effective distance between these redox centres. Even a short decrease in the distance between redox centres should have a significant effect on the electron transfer rate, because it decays exponentially with distance [29]. This hypothesis is also in agreement with the mixed valence nature of $Cu_A$.

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