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Hypothesis

RNA sequence and the nature of the Cu_A-binding site in cytochrome *c* oxidase

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For cytochrome *c* oxidase subunit II (COXII), DNA and protein sequences suggest that Met-207 (bovine numbering) is conserved in all species except plants. Sequencing of plant mitochondrial COXII mRNAs now indicates that Met-207 is also conserved among plants as a result of a C-to-U type of RNA editing. Considering the strict evolutionary conservation of Met-207 and the homology of COXII to type I (blue) copper proteins and nitrous oxide reductase, we propose a model in which Met-207 is associated with the Cu_A-binding site (along with Cys-196, Cys-200 and His-204) and plays a role in determining its reduction potential and stability.

Cytochrome *c* oxidase; Copper binding site; RNA editing

1. INTRODUCTION

Cytochrome *c* oxidase is a complex metalloprotein of crucial importance in cellular respiration. It contains at least two distinct copper centers including Cu_A, which is thought to be involved in electron flow from cytochrome *c* to the dioxygen reduction site, and possibly in proton pumping [1–3]. While many aspects of the structure and function of cytochrome oxidase have yet to be firmly established [1,4], a large body of biochemical, biophysical and sequence data support the consensus that Cu_A is bound at least in part to subunit II of the enzyme (COXII) by at least one histidine and at least one (but probably two) cysteine(s) [1,2,5,6].

From the available data, Chan and coworkers have proposed a model for the Cu_A site which includes two histidine and two cysteine ligands [7] (see also [8,9]). There is good evidence for the involvement of two cysteines and one histidine as ligands in the Cu_A-binding site of COXII. However, there is relatively little support for a second histidine ligand. Extended X-ray absorption fine structure (EXAFS) data have been considered to support the involvement of a second nitrogen-containing ligand for Cu_A [10]; however, the analysis of such data is a matter of current debate [10,11] (see below).

The evolutionary conservation of two histidines at homologous positions (161 and 204 in the bovine sequence; all subsequent amino acid numbers refer to this sequence) has also supported the view that both may be copper ligands [8]. It is notable, however, that while His-204 is strictly conserved among thirty species, His-161 is apparently substituted by leucine in *Paramecium primaurelia* [12]. Furthermore, comparison of amino acid sequences of COXII polypeptides with those of related proteins suggests alternative ligand identities for Cu_A. The sequence similarity between blue copper proteins (such as plastocyanin and azurin) and COXII has been noted previously [13]. A number of these proteins have a C-X_m-H-X_n-M ($2 \leqslant m,n \leqslant 4$; CHM) motif which has been shown by X-ray diffraction to form part of the type I copper binding site [14] (Fig. 1a). The copper is bound by the cysteine and histidine of this motif and by an additional histidine residue. The methionine in this motif is associated with the type I copper at a relatively long Cu–S distance of 2.7–3.1 Å. Replacement of Met with Leu by site-directed mutagenesis of *Pseudomonas* azurin indicates that while the methionine of the CHM motif is not strictly necessary to form the copper binding site, it affects the stability and reduction potential of the site [15]. It is notable that the CHM motif common to blue copper proteins appears to be shared by COXII for all species investigated except plants [9], as well as by N_2O reductase [16] (the only other native protein known to contain a Cu_A type center).

Recent biophysical data lend support to the apparent homology between the Cu_A and type I copper proteins. While unambiguous interpretation of EXAFS data for

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Abbreviation: COXII, subunit II of cytochrome oxydase

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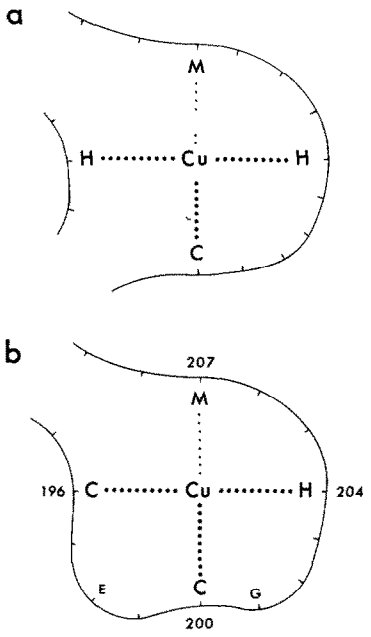

Fig. 1. (a) Type I copper binding site (azurin) based on X-ray diffraction data. (b) Proposed model of cytochrome *c* oxidase Cu_A-binding site showing conserved amino acid residues. Smaller dots connecting Cu and M denote a weaker interaction than in the case of the other three ligands.

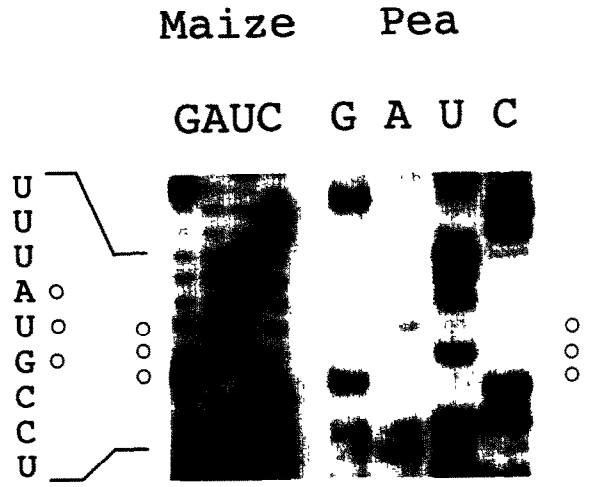

Fig. 2. Sequence autoradiograms for maize and pea COXII mRNA demonstrating the presence of a Met-207 codon (AUG). Mitochondrial RNA was extracted from etiolated seedlings of maize and pea [17] and sequenced [33] using oligodeoxyribonucleotides CCGGTTTAGTTGGTTTGGAG (maize) and CTTATCCCTCACCCTACTCT (pea) (Regional DNA Synthesis Laboratory, University of Calgary) as primers for reverse transcriptase (see [18] for additional details).

cytochrome oxidase is difficult [10,11], it is intriguing that evidence for a Cu-(S,Cl) interaction at 2.7 Å has been found for both cytochrome oxidase (oxidized and reduced forms) and N_2O reductase [16]. This suggests the possibility that the structure of the Cu_A site may be more similar to a type I site than is suggested by current models [7,8,9], in the sense that it may involve a Met ligand with a long Cu–S distance.

We report here plant COXII mRNA sequence data that address the question of the nature of the Cu_A-binding site in cytochrome *c* oxidase. Comparisons of plant COXII amino acid sequences (inferred from the corresponding gene sequences) have suggested a lack of conservation in wheat [17] of the cysteine of the CHM motif (position 200 in the bovine sequence; see [18] for an alignment). Furthermore, while evening primrose [19] and petunia [20] DNA sequences predict the presence of the methionine residue of the CHM motif, maize [21], wheat [17], rice [22], soybean [23] and *Zea diploperennis* [24] COXII gene sequences predict a threonine residue (codon ACG) at the homologous position. However, recent RNA sequence data have revealed a lack of correspondence between plant mitochondrial gene and mRNA sequences as a result of a C-to-U type of post-transcriptional editing [18,25,26]. Consequently, the COXII amino acid sequence of wheat is now predicted to contain the Cys and Met codons of the CHM motif at positions corresponding to residues 200 and 207 in the bovine COX-II sequence [18].

We have extended the RNA sequencing work to include maize and pea COXII (detailed results of this work will be reported elsewhere; P.S. Covello and M.W. Gray, submitted for publication). Fig.2 shows mRNA sequence data that demonstrate the presence of a Met-207 in both maize and pea, despite indications to the contrary from the published DNA sequences [21,27]. It is highly probable that the homologous threonine (ACG) codons in rice [22], soybean [23] and *Zea diploperennis* [24] are also edited to AUG (Met). Consequently, the RNA sequence data indicate a very high degree of conservation, not apparent from DNA sequence, of Cys-200 and Met-207. This is consistent with the hypothesis that Cys-200, as well as Cys-196, is involved in Cu_A binding [28].

We have verified the strict conservation of Met-207 by examination of thirty COXII sequences from nucleic acid and protein databases (GenBank, Release 62; EMBL, Release 22; NBRF Protein Information Resource, December 1989; Swiss-Prot Protein Sequence Databank, Release 13). No non-plant sequences were found that lacked the $C\text{-}X_3\text{-}C\text{-}X_3\text{-}H\text{-}X_2\text{-}M$ motif. (Of two independently determined sequences for the COXII gene in *Tetrahymena pyriformis* mitochondrial DNA, one [29] predicts a Met-207 equivalent (see [12]), whereas the other [30] does not. A rereading of the relevant autoradiograms has re-affirmed the sequence data in [29] (M.N. Schnare, personal communication).

The evolutionary conservation of Met-207 in COXII, as well as the occurrence of an apparently equivalent Met in N_2O reductase, leads us to propose an alternate model for the Cu_A-binding site of COXII which includes Met-207 as a ligand. A similar proposal had been made earlier by Buse and coworkers [13] but its accep-

tance appears to have been discouraged by the publication of the maize and other plant COXII DNA sequences [5]. We suggest that Cu_A is bound by the four closely spaced, highly conserved residues Cys-196, Cys-200, His-204 and Met-207, as shown in Fig. 1b. We further suggest the possibility that, as in many type I copper proteins [31,32], the methionine sulfur is at a relatively long distance from the copper atom. The major difference between this model and a type I copper site is the replacement of one of the histidine ligands in the latter with a cysteine (Fig. 1). This may explain the different spectroscopic properties of the two types of copper binding sites.

The scheme in Fig. 1b may not be entirely inconsistent with the Chan model in that we cannot rule out the possible additional involvement of His-161. However, it would appear that a Cu-N(His)S_2(Cys)S(Met) model is consistent with the EXAFS data. Firstly, the model explains the apparent Cu–S interaction at 2.7 Å in both cytochrome oxidase and N_2O reductase [16]. It is also consistent with the consensus view arrived at by Powers and Kincaid [11] (albeit somewhat reluctantly) regarding ligand numbers of 2 per copper atom in the case of (N,O) type ligands and 1.5 for (S,Cl) type ligands. Assuming that Cu_B has 3 (N,O) ligands and 1 (S,Cl) ligand [6], the reported EXAFS results could be taken to imply the presence of two cysteines and a histidine in the inner coordination shell of Cu_A, and in addition a more distant methionine.

If the Cu–S(Met) distance is 2.7 Å, this suggests that the methionine is very weakly bonded to the copper. However, by analogy to type I copper proteins [15], it seems likely that methionine-207 plays an important role in determining the reduction potential and stability of the Cu_A site. Site-directed mutagenesis of COXII polypeptides may provide a direct test of this hypothesis.

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