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MINI-REVIEW

Cytochrome *c* Oxidase Metal Centers: Location and Function

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Abstract

Cytochrome *c* oxidase of *Paracoccus denitrificans* is spectroscopically and functionally very similar to the mammalian enzyme. However, it has a very much simpler quaternary structure, consisting of only three subunits instead of the 13 of the bovine enzyme. The known primary structure of the *Paracoccus denitrificans* subunits, the knowledge of a large number of sequences from other species, and data on the controlled proteolytic digestion of the enzyme allow structural restrictions to be placed on the models describing the binding of the active metal centers to the polypeptide structure.

Key Words: *Paracoccus denitrificans*; Cytochrome *c* oxidase; heme *a*; copper A, B, and C; electron transfer; proton pump.

Introduction

The enzyme, which plays a fundamental role in cell energetics, has a remarkably complicated structure and function. The active centers of cytochrome *c* oxidase contain metal ions which undergo redox changes with resultant modifications in their absorption and EPR[4] spectra. These properties of cytochrome *c* oxidase, together with the ease of its preparation, have resulted in extensive spectroscopic and functional studies. The three main subunits of

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[4] Abbreviations: RR, resonance Raman spectroscopy; ENDOR, electron nuclear double resonance spectroscopy; EXAFS, extended X-ray absorption fine structure.

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bacterial cytochrome *c* oxidases have been shown to possess structural, spectroscopic, and functional properties very similar to eukaryotic oxidases. Moreover, their structural analogy to a functionally different bacterial oxidase (cytochrome *bo*) is highly significant (Chepuri *et al.*, 1990). Such a broad evolutionary conservation has been invoked in the attempt to identify the location of the enzyme active centers, not covalently bound to the protein backbone. The following discussion will extend to structural information obtained from cytochrome *c* oxidase of other organisms as these have provided useful insights. Spectroscopic and kinetic aspects of cytochrome *c* oxidase which are not strictly related to *Paracoccus denitrificans* will not be covered, since these aspects have been previously addressed in detail (Wikström *et al.*, 1981; Fee *et al.*, 1986; Hill *et al.*, 1986; Brunori *et al.*, 1987; Ludwig, 1987; Naqui and Chance, 1987; Müller and Azzi (1988); Azzi and Müller, 1990; Bisson, 1990; Chan and Li, 1990).

This review will focus on the contribution of *Paracoccus denitrificans* studies to the elucidation of structural aspects of cytochrome *c* oxidase.

General Properties of *Paracoccus denitrificans* Cytochrome *c* Oxidase

Paracoccus denitrificans cytochrome *c* oxidase was originally isolated as a two-subunit enzyme (Ludwig and Schatz, 1980). The discovery of three genes (Raitio *et al.*, 1987) led to the search for a third subunit and to the purification of a three-subunit enzyme. The third subunit, although probably always expressed, is not consistently present in the purified enzyme preparations (Haltia *et al.*, 1988; Müller *et al.*, 1988). More recently it was found that *Paracoccus denitrificans* has two genes for subunit 1(I) (Raitio *et al.*, 1990). The three subunits are structurally homologous to those of other bacterial and eukaryotic cytochrome oxidases. Subunits 1(I) and 3(II)[5] contain all the redox active centers, two heme *a* and two copper atoms, as inferred from their metal content analysis, absorption spectra, and catalytic properties (Ludwig and Schatz, 1980; Solioz *et al.*, 1982; Reichhardt and Gibson, 1983; Bolli *et al.*, 1986). An additional copper atom was recently reported to be associated with the two-subunit enzyme (Steffens *et al.*, 1987), but its redox properties have not yet been characterized.

[5]A new type of nomenclature of cytochrome *c* oxidase subunits will be used in this article, which is based on the subunit molecular weight, calculated from their sequence, as proposed by Azzi and Müller (1990). An Arabic number defines the subunit according to the new nomenclature; it is followed by a Roman number in parentheses corresponding to the old classification.

Heme *a*

Heme *a* is located in subunit 1(I) as inferred from experiments by which a 71-amino acid fragment of subunit 3(II) amino terminus was digested by chymotrypsin (Müller *et al.*, 1988a,b). This treatment eliminates the only possible heme ligands remaining after accommodating a copper in this subunit. The iron of this center, Fe_A, is coordinated by the four nitrogen atoms of the planar heme *a* moiety and by two axial nitrogen atoms from at least one histidyl residue (most probably two), leaving therefore no free valency for additional ligands, as is the case in hemoglobin. The above model is based on EPR and resonance Raman (RR) spectroscopy studies of the bovine heart enzyme and model compounds (Babcock and Callahan, 1983; Argade *et al.*, 1986), as well as electron nuclear double resonance (ENDOR) spectroscopy on the native and ^{15}N-histidine-substituted yeast enzyme (Martin *et al.*, 1985).

Cytochrome *a*, together with Cu_A, accept electrons from cytochrome *c* and are in a very fast kinetic equilibrium (Antalis and Palmer, 1982). The identity of the first center to be reduced is still not firmly established (Morgan *et al.*, 1989). The redox potential of cytochrome *a* has a pH dependence of 30 mV/pH unit (Artzatbanov *et al.*, 1978). This observation, together with evidence obtained by laser raman spectroscopy studies, suggested that the formyl group of cytochrome *a* could be part of the proton pump mechanism (Babcock and Callahan, 1983). It was recently reported that in the bovine heart cytochrome *c* oxidase a water molecule is associated with the formyl and the vinyl group of the heme (Sassaroli *et al.*, 1989). These molecules may have an important role in the proposed proton pump mechanism.

Heme a_3

Cytochrome a_3 is a binuclear metal center located in subunit 1(I) (Azzi and Müller, 1990) responsible, in cytochrome *c* oxidases, for the final reduction of oxygen to water. The isolation of a cytochrome aa_3 from *Halobacterium halobium* composed of only one polypeptide of apparent molecular weight 40,000 lacking Cu_A and Cu_B but containing two heme *a* (Fujiwara *et al.*, 1989) is consistent with the location of heme a_3 within subunit 1(I).

In addition to the four nitrogen atoms of the heme *a* plane, a nitrogen atom (also in this case from a histidyl residue) and a sixth ligand which would bridge between Fe_B and Cu_B (Stevens and Chan, 1981) have been suggested as possible ligands of Fe_B. The identity of the Cu_B ligands, intensively studied by ENDOR (Cline *et al.*, 1983) and by extended X-ray absorption fine structure (EXAFS), is still open to discussion (Powers *et al.*, 1981; Li *et al.*,

1987; Powers and Kincaid, 1989). There seems to be agreement on three imidazole nitrogens (or three oxygen atoms) but not on the sixth coordination ligand, a sulfur or a chloride atom bridging Cu_B with Fe_B. This model has to be taken with some reservation [cf. Azzi and Müller (1990) for a detailed discussion] since it is based on the assumption that only two copper ions are present per oxidase monomer. If there are three copper ions, as claimed by a number of laboratories, and the copper content (found to be two per monomer of enzyme) of the samples utilized for the EXAFS studies was underestimated, the assignment of the ligands discussed above should be revised. The catalytic mechanism by which the binuclear center (Fe_B/Cu_B) of the enzyme reduces molecular oxygen to water has been described by assuming six different intermediate states. An important intermediate of the reaction is the oxyferryl form of cytochrome a_3, which has been demonstrated using different spectroscopic techniques (EPR, RR, Mössbauer spectroscopy, EXAFS; Witt and Chan, 1987; Kumar *et al.*, 1988). Electron transfer from cytochrome a/Cu_A to the oxyferryl form of cytochrome a_3 has been proposed to be involved in proton translocation (Wikström, 1989). The complicated oxygen chemistry utilized to describe the catalytic function of the binuclear center of cytochrome *c* oxidases is reviewed by Hill *et al.* (1986) and by Chan and Li (1990).

The Coppers

Of the three coppers constantly found to be associated with good cytochrome *c* oxidase preparations, Cu_B has been discussed above as an integral part of the binuclear metal center present in subunit 1(I).

A second copper, Cu_A, is spectroscopically defined by its optical absorption in the near-infrared region with a maximum around 820–840 nm and by its characteristic EPR signal at $g = 2.00$. Of all of the three copper ions, Cu_A is the only one detected by EPR spectroscopy. When *Paracoccus denitrificans* cells were grown in salt-rich media the $g = 2.00$ signal was almost completely covered by the resonances of Mn ions present in such preparations (0.2 equivalents of Mn/enzyme monomer; Seelig *et al.*, 1981; Müller and Azzi, 1988). Growth in Mn-poor media permitted us, however, to isolate an active enzyme devoid of the Mn signal and still fully active, suggesting that Mn does not play a role in the enzyme function. The assignment of Cu_A to subunit 1(I) or to subunit 3(II) implies a very different coordination. Traditionally, Cu_A has been assigned to subunit 3(II), and a model has been proposed describing its coordination by two histidine nitrogen atoms and two cysteine sulfur atoms. This model proposed by Chan's group on the basis of ENDOR spectroscopy data obtained with the yeast enzyme (Stevens *et al.*, 1982;

Martin *et al.*, 1988) was supported by similar results obtained for the bovine heart and *Pseudomonas stutzeri* enzymes (Hall *et al.*, 1988; Li *et al.*, 1987; Scott *et al.*, 1989).

Criticism and limitations of the spectroscopic observations mentioned before are reviewed by Azzi and Müller (1990). The failure to detect a copper EPR spectrum in a purified fragment of subunit 3(II) produced by trypsin treatment of *Bacillus* PS3 cytochrome oxidase although it contained equimolar amounts of copper (Müller, Sone, and Azzi, unpublished) would speak against such a model. Further evidence supporting the location of Cu_A in subunit 1(I) comes from the observations of Zimmermann *et al.* (1988) on an isolated cytochrome ba_3 oxidase from the bacterium *Thermus thermophilus*. This enzyme, composed of a single subunit, belongs to the cytochrome aa_3 structural family, similar to subunit 1(I) of cytochrome *bo* (Saraste *et al.*, 1988; Chepuri *et al.*, submitted). It still possesses a typical EPR Cu_A spectrum. Nevertheless, until the amino acid sequence of cytochrome ba_3 is elucidated, the presence of CuA ligands similar to those of subunit 3(II) cannot be excluded. Finally the cytochrome aa_3 isolated from *Erythrobacter longus* (Fukumori *et al.*, 1987), composed of two identical polypeptides of M_r 43,000 linked together by a disulfide bridge, contains equimolar amounts of Cu and heme *a*. This is consistent with a model of *E. longus* cytochrome oxidase composed of two identical subunits 1(I), each of them associated with two hemes *a* and two copper atoms.

The major criticism of the classical location of Cu_A in subunit 3(II) was based on experiments showing that subunit 1(I) depleted of subunit 3(II) contained not only Fe_A, Fe_B, and Cu_B (Müller *et al.*, 1988a, b) but also CuA, detected by its absorption spectrum (Müller and Azzi, 1990) and EPR signal (Azzi and Müller, 1990). However, the conclusions drawn from these experiments must be withdrawn as the apparent removal of subunit 3(II) by digestion with *Staphylococcus aureus* V8 protease can be attributed to proteolytic activity in sodium dodecyl sulfate during preparation of the sample for polyacrylamide gel electrophoresis (Zabal and Azzi, unpublished results). In addition to its role as electron acceptor, Cu_A was proposed by Chan and coworkers to be part of the proton pump mechanism (Gelles *et al.*, 1986; Chan and Li, 1990). They postulate the involvement of a tyrosyl residue (or another amino acid with similar pK) in the catalytic cycle of the proton pump. The absence of Cu_A in the cytochrome oxiase of *Nitrosomonas europea* seems not to affect electron transfer from cytochrome *c* to oxygen (Numata *et al.*, 1989), indicating that Cu_A is not a prerequisite for at least one of the catalytic functions of the enzyme. Proton translocation was not studied in this bacterium. However, a proton pump (Puustinen *et al.*, 1989) was described in the cytochrome *bo* of *E. coli*, which does not contain Cu_A (Salerno *et al.*, 1990).

Recently, more sophisticated analytical techniques and a more accurate analysis of results have uncovered the existence of a third copper atom, Cu_C, not only in *Paracoccus denitrificans* (Steffens *et al.*, 1987) but also in heart (Einarsdöttir and Caughey, 1985; Bombelka *et al.*, 1986; Öblad *et al.*, 1989), liver (Bombelka *et al.*, 1986), and bacterial cytochrome *c* oxidases (Yoshikawa *et al.*, 1988; De Vrij *et al.*, 1989). The location of this third Cu has not been discussed until now, except for the model proposed by Azzi and Müller (1990).

In this model, Cu_C may be located in subunit 3(II) and cannot be detected by EPR under physiological conditions (but see Baker and Palmer, 1987) since it is constantly kept reduced by its two cysteine sulfur ligands, as suggested by Hemmerich (1966).

Possible Ligands for the Metal Centers of Subunit 1(I)

If indeed five metal centers are present in the two-subunit cytochrome *c* oxidase and one of them, Cu_C, is bound to subunit 3(II), subunit 1(I) must coordinate the other four metal centers, two Fe and two Cu atoms. However, the conserved possible ligands available in subunit 1(I) are very limited in both number and type. Analysis of almost 30 different cytochrome oxidase subunit 1(I) sequences revealed the presence of a total of about 50 evolutionarily conserved amino acids. The following residues may be taken into consideration as possible ligands for Fe and Cu: 6 histidines, 1 methionine, 2 lysines, 2 tyrosines, 3 aspartic acids, 1 glutamic acid, and 4 tryptophans (Fig. 1). An additional methionine (M_{324}) may be also conserved since there are some uncertainties about its real substitution by leucine in *S. pombe*.

The sequence present in subunit 1(I) H_{322}-H_{323}-M_{324} is identical with that responsible for copper binding in the active center of bovine dopamine β-hydroxylase (Robertson *et al.*, 1990; Farrington *et al.*, 1990), and responsible for copper binding. However, a *S. cerevisiae* auxotroph mutant grown on [^{2}H]-cysteine was used in ENDOR experiments to show that cysteine is involved in copper coordination (Stevens *et al.*, 1982), thus excluding subunit 1(I) as the Cu_A binding center, since this polypeptide does not contain any conserved cysteines. The possible enzymatic conversion of [^{2}H]-cysteine to [^{2}H]-serine by the enzyme serine-sulfhydrase (Meisch and Kappesser, 1987) present in yeast leads to the alternative interpretation that a serine and not a cysteine is responsible for the partial loss of the signal intensity observed in the ENDOR experiments. A serine is present in *s. cerevisiae* adjacent to the sequence histidine-histidine-methionine mentioned above as a possible copper-binding site.

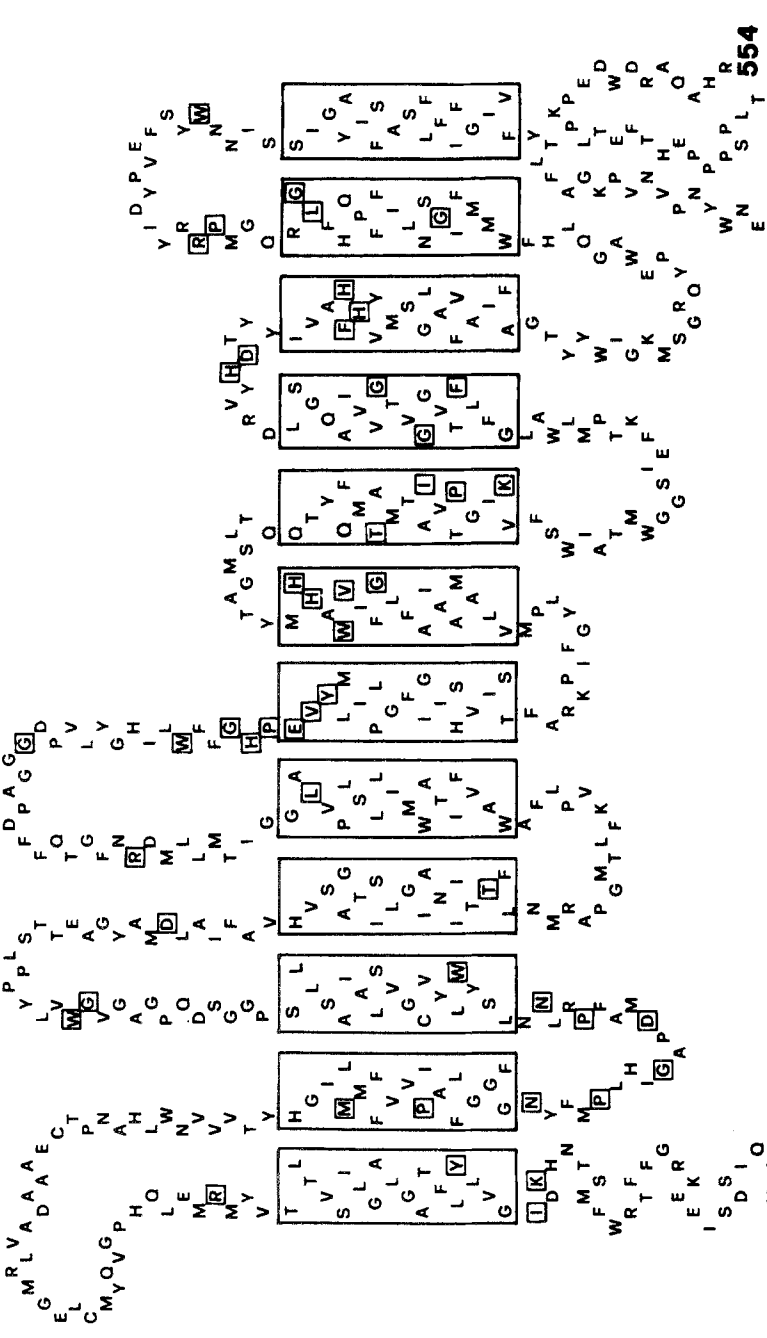

Fig. 1. The primary structure of *Paracoccus denitrificans* cytochrome *c* oxidase subunit I(I). Amino acids enclosed in a square are conserved in all known sequences. Putative α-helical segments are enclosed in frames.

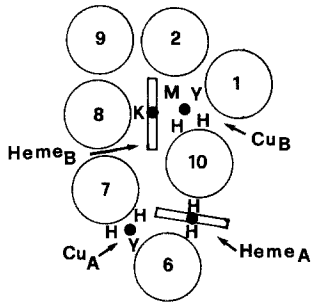

Fig. 2. Putative arrangement of α-helices, hemes, and copper atoms in subunit 1(I) as described in the text.

The sequence H_{406}-X-H_{408} was found in other copper oxidases, i.e., ceruloplasmin, laccase, and ascorbate oxidase (Huber, 1989; Messerschmidt and Huber, 1990) and, in addition, in the N_2O reductase of *P. stutzeri* (Viebrock and Zumft, 1988). These two conserved histidine clusters may represent the Cu_A and Cu_B nitrogen ligands. The two remaining histidines

Table I. Comparison of the Amino Acid Sequence of Cu-Binding Domains of Some Blue Copper Proteins, N_2O Reductase, and Cytochrome Oxidase Subunit 3(II)[a]

Protein	A	B	C	References[b]
N. crassa laccase				
Domain D3	H	70	CxxxxHxxxxx	1
Human ceruloplasmin				
Domain D2	H	41	CxxxxHxxxxx	2
Domain D4	H	41	CxxxxHxxxxM	
Domain D6	H	44	CxxxxHxxxxM	
Cucumber ascorbate oxidase				
Domain D3	H	52	CxxxPHxxxxM	3
Plant azurins	H	65	CxxPxHxx--M	4
Plastocyanins	H	40–46	Cx-P-HxxxxM	5
Pseudomonas AM1				
Amicyanin	H	38	Cx-P-Hxx--M	6
Pseudoazurin	H	37	Cx-P-Hxx--M	6
Stellacyanin	H	34	CxxPxHCxxxx	7
Cucumber blue protein	H	33	CxxPxHCxxxM	7
P. stutzeri N_2O reductase				
N-terminal	H(78)	78	CxxP-Hxx--M	8
C-terminal	H(583)	42	CxxxCHxx-HM	
Cytochrome oxidase				
Subunit 3(II)'s	H	34	CxExCGxx-Hx	9

[a] A: Distal ligand; B: number of amino acids intercalating between distal ligand and proximal ligand; C: proximal-ligand region. Amino acids are represented by one-letter code. x, spacing amino acid; -, space introduced to match the putative ligand positions.

[b] 1. Germann *et al.*, 1988; 2. Koschinsky *et al.*, 1986; 3. Ohkawa *et al.*, 1989; 4. Adman, 1985; 5. Yoshizaki *et al.*, 1989; 6. Ambler and Tobari, 1985; 7. Murata *et al.*, 1982; 8. Viebrock and Zumft, 1988; 9. Bisson, 1990.

Table II. Comparative Table of the Absorption Characteristics of Some Copper Proteins[a]

Protein	A	B	C	References[b]
Azurin				
P. aeruginosa	1	628 (5, 3)	2H, 1C, 1M	1
Azurin mutant				
P. aeruginosa	1	633 (6, 0)	2H, 1C	1
Plastocyanin				
E. proliferate	1	597 (4, 7) 780 (1, 8)	2H, 1C, 1M	2
Auracyanin				
C. aurantiacus	1	596 (2, 9) 725 (1, 4)	2H, 1C, 1M	3
Galactose oxidase				
P. circinatus	1	630 (1, 0) 775 (0, 9)	2H, 1Y, 1H2O	4
Nitrite reductase				
Alacaligenes sp.	1	596 (3, 7) 775 (1, 6)	2H, 1C	5
N_2O reductase				
A. cycloclastes	4			
N-terminal region	1	625 (3, 6) 780 (2, 9)	2H, 1C, 1M	6
C-terminal region	1	No spectrum?	2H, 2C	
Cytochrome. *c* oxidase 3				
Subunit 3(II)/Cu_C	1	No spectrum?	2H, 2C	
Subunit 1(I)/Cu_A	1	830 (2, 0)	2H, ?	7

[a] A: Total number of copper atoms or copper atoms involved in a specific site. B: Absorption maxima (nm) and in parentheses molar extinction coefficient ($mM^{-1}cm^{-1}$); only maxima > 550 nm are listed. C: Main copper ligands or putative ligands (i.e., in the case of nitrite and N_2O reductases, cyt. *c* oxidase).

[b] 1. Karlsson *et al.*, 1989; 2. Yoshizaki *et al.*, 1981; 3. Trost *et al.*, 1988; 4. Ettinger, 1974; 5. Suzuki *et al.*, 1989; 6. Hulse and Averill, 1990; 7. Wikström *et al.*, 1981.

(H_{273} and H_{398}) could coordinate heme *a*, and heme a_3 could be coordinated by a nitrogen ligand different from imidazole, for example by the conserved lysyl residue (K_{351}). Axial ligands different from histidine have recently been suggested to be present in cytochrome *d* from *E. coli* (Fang *et al.*, 1989) although they have not yet been identified. Finally the unique conserved methionine (M_{95}) could serve as bridging ligand between Fe_B and Cu_B, as indicated by EXAFS studies. Alternatively Cu_A could be coordinated as well by oxygens (still consistent with the EXAFS data) provided by the conserved glutamic acid 275 and tyrosine 277. Tyrosine as copper ligand was found to be present in galactose oxidase (Whittaker *et al.*, 1989; Whittaker and Whittaker, 1990). The data discussed above is summarized in Fig. 2 in which an attempt to resolve the following dilemma is made: three coppers seem to be associated with cytochrome *c* oxidase, while only one is visible by optical and EPR spectroscopy. This dilemma can be resolved by placing Cu_C in subunit 3(II), in a permanently reduced state, complexed by four ligands: C_{216}, C_{220}, H_{181}, and H_{224}. The remaining two copper ions are then bound to subunit 1(I), one in a paramagnetically coupled manner in the binuclear center and the other visible. The binding site of Cu_C would then be similar to that of blue copper proteins as originally proposed by Steffens and Buse

(1979). Table I illustrates this similarity, as well as the differences of this copper site from that of the copper sites in blue copper proteins.

P. stutzeri N_2O reductase (Scott *et al.*, 1989) has also been proposed to be analogous to cytochrome *c* oxidase subunit 3(II). *P. stutzeri* N_2O reductase contains a sequence in its C-terminal region where several possible ligands for a Cu_C type center are present as well for a blue-copper-type center (Table I). However, the proposed role of serine as copper ligand (Scott *et al.*, 1989) cannot be extended to cytochrome oxidase since some enzymes do not have it (in PS3 cytochrome oxidase it is substituted by an alanine). In the N-terminal region of *P. stutzeri* another possible region containing potential ligands for a blue type copper can be found, in agreement with the EPR results obtained with the N_2O reductase of *Achromobacter cycloclastes* which suggest the presence in the enzyme of one Cu(I), one Cu(II), and one mixed-valence Cu(I)–Cu(II) binuclear center (Hulse and Averill, 1990). The Cu(II) would correspond to Cu_C, while Cu(I) could be bound by the residues found in the N-terminal region. The EPR spectra and the optical properties of Cu_A are different (Li *et al.*, 1989) from those of N_2O reductases (Table II), which resemble that of a blue copper.

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