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Restoration of a lost metal-binding site: construction of two different copper sites into a subunit of the *E.coli* cytochrome *o* quinol oxidase complex

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The cupredoxin fold, a Greek key β-barrel, is a common structural motif in a family of small blue copper proteins and a subdomain in many multicopper oxidases. Here we show that a cupredoxin domain is present in subunit II of cytochrome *c* and quinol oxidase complexes. In the former complex this subunit is thought to bind a copper centre called Cu_A which is missing from the latter complex. We have expressed the C-terminal fragment of the membrane-bound CyoA subunit of the *Escherichia coli* cytochrome *o* quinol oxidase as a water-soluble protein. Two mutants have been designed into the CyoA fragment. The optical spectrum shows that one mutant is similar to blue copper proteins. The second mutant has an optical spectrum and redox potential like the purple copper site in nitrous oxide reductase (N_2OR). This site is closely related to Cu_A, which is the copper centre typical of cytochrome *c* oxidase. The electron paramagnetic resonance (EPR) spectra of both this mutant and the entire cytochrome *o* complex, into which the Cu_A site has been introduced, are similar to the EPR spectra of the native Cu_A site in cytochrome oxidase. These results give the first experimental evidence that Cu_A is bound to the subunit II of cytochrome *c* oxidase and open a new way to study this peculiar copper site.

Key words: blue copper/Cu_A/cytochrome *c* oxidase/protein design/quinol oxidase

Introduction

The cytochrome oxidase family has two branches. One is formed by enzymes which reduce oxygen using cytochrome *c* as electron donor. This aa_3-type cytochrome *c* oxidase is a characteristic enzyme of the mitochondrial respiratory chain but is also present in a large number of aerobic bacteria (Ludwig, 1987). The other branch is formed by quinol oxidases and may be peculiar to bacteria (Chepuri *et al.*, 1990; Lübben *et al.*, 1992; Santana *et al.*, 1992). The enzyme complexes belonging to these branches are structurally homologous and share the same functional core (Lemieux *et al.*, 1992; Minagawa *et al.*, 1992; Shapleigh *et al.*, 1992). They also have a similar mechanism in energy conservation (Puustinen *et al.*, 1989, 1991; Babcock and Wikström, 1992). All members of the family are probably redox-linked proton pumps.

The main distinction between the branches is that they use different electron-donating substrates. In mitochondria oxidation of ubiquinol and reduction of oxygen to water are catalysed by two separate enzymes, ubiquinol:cytochrome *c* oxidoreductase and cytochrome *c* oxidase, respectively. Bacterial quinol oxidases, such as the *Escherichia coli* cytochrome *o* complex, catalyse quinol oxidation directly with dioxygen (Anraku and Gennis, 1987). The entry of an electron from either a water-soluble cytochrome *c* or a lipid-soluble quinol must correlate to structural differences between the two types of terminal oxidases.

Subunits I and II form the functional core of the enzymes within the family (reviewed by Bisson, 1990; Chan and Li, 1990; Malmström, 1990; Saraste, 1990; Haltia and Wikström, 1992). All family members possess three common metal centres. The O_2-binding site in subunit I is formed by a high-spin haem iron and a copper called Cu_B. These are located at 3–5 Å distance from each other. The third redox centre is a low-spin haem involved in electron transfer to the active site. Subunit II of cytochrome *c* oxidase (COII) is thought to bind another copper centre, which is likewise involved in the internal electron transfer from cytochrome *c* to the active site. This centre, Cu_A, has been shown to be absent from the *E.coli* (Puustinen *et al.*, 1991) and *Bacillus subtilis* (Lauraeus *et al.*, 1991) quinol oxidases. The presence or absence of Cu_A appears to be diagnostic for substrate specificity (Saraste *et al.*, 1991).

Subunit II of both quinol and cytochrome *c* oxidase complexes has the same membrane topology (Chepuri and Gennis, 1990; Figure 1). It contains an N-terminal membrane anchor consisting typically of two transmembrane segments and a C-terminal domain exposed to the outside of the membrane. This may form the cytochrome *c*-binding site in cytochrome *c* oxidase (Capaldi *et al.*, 1982). It is likely that electrons enter via this domain and its resident Cu_A on their way to the active site. The spectroscopic evidence for the nature of the ligands that bind Cu_A has been summarized by Martin *et al.* (1988). The consensus model predicts that two cysteines and probably two histidines bind a single copper. Indeed, the COII sequences contain two invariant cysteines and two invariant histidines, which are all absent from the homologous subunits of quinol oxidases (Chepuri *et al.*, 1990; Lübben *et al.*, 1992; Santana *et al.*, 1992).

Subunits II of cytochrome *c* and quinol oxidases have enough sequence similarity to allow an unambiguous alignment. The alignment suggests (Saraste, 1990) that at their C-termini both proteins contain a subdomain, which is a Greek-key β-barrel characteristic of cupredoxins, a family of blue copper proteins of plants and bacteria (Adman,

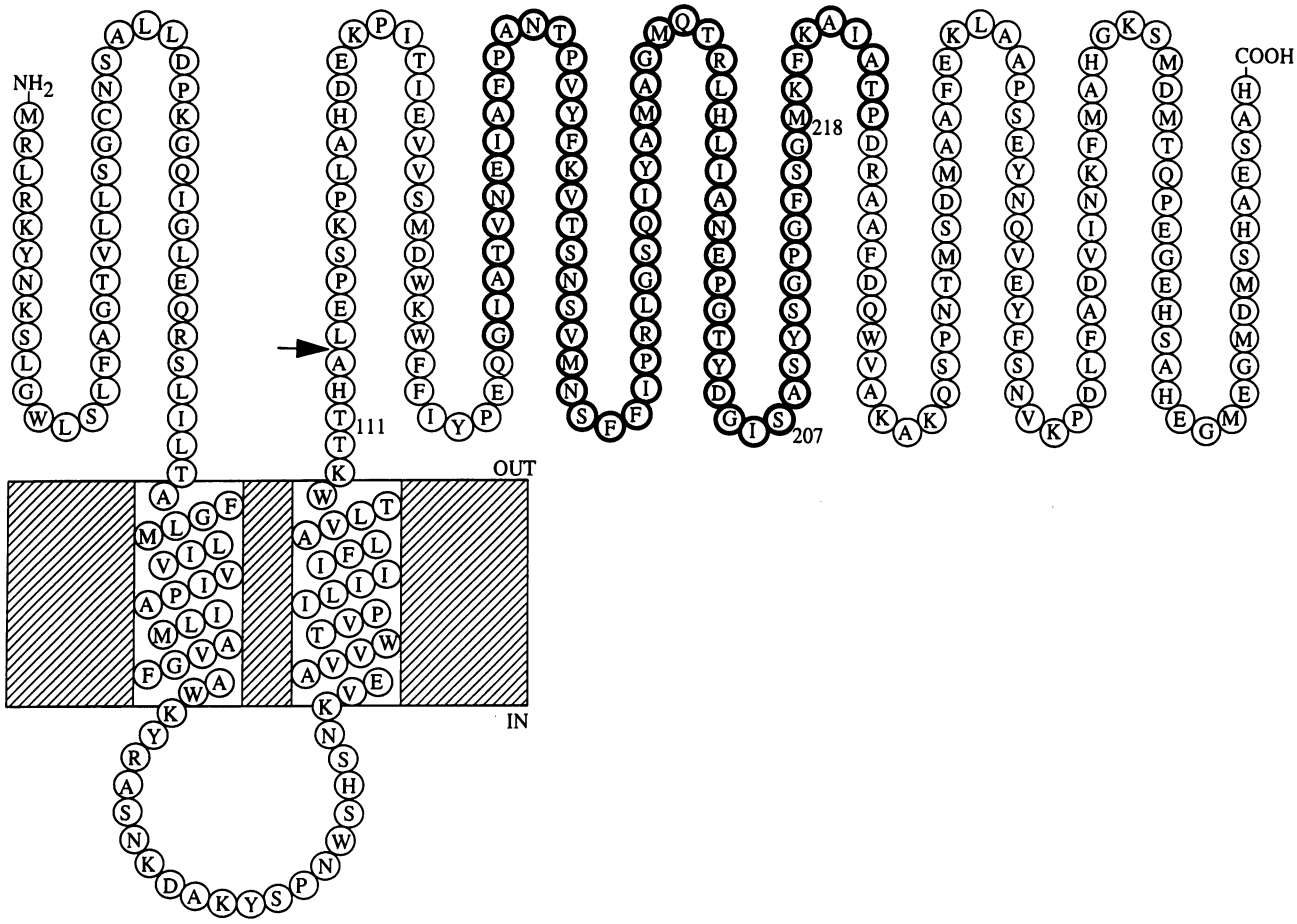

Fig. 1. Topology of the CyoA subunit in the membrane. The CyoA sequence is taken from Chepuri *et al.* (1990). Both N- and C-termini face the periplasm. The arrow indicates the site for proteolytic cleavage which liberates the water-soluble C-terminal fragment (Figure 2). The sequence shown in bold may correspond to a cupredoxin fold (Saraste, 1990).

1991a,b). Here we use this model to design two different copper-binding sites into the copperless CyoA subunit of the *E.coli* cytochrome *o* quinol oxidase complex. We have been able to express the C-terminal part of CyoA in a soluble form and to construct a Cu_A-like centre as well as a blue copper centre into it. The Cu_A-like site has also been introduced into the entire cytochrome *o* complex.

Results

Expression and purification of the C-terminal fragment from CyoA

Several different attempts were made to prepare the C-terminal subunit II fragment of bacterial cytochrome *c* and quinol oxidases expressing different variants of this domain individually or as fusion proteins. The results in these experiments varied from the formation of large amounts of inclusion bodies to the total lack of expression. Figure 2 shows how we finally found the way to carry out a successful experiment.

The CyoA subunit of the *E.coli* cytochrome *o* is mostly membrane-bound when it is overexpressed in *E.coli* after the induction with isopropyl-thio-β-D-galactoside (IPTG) (see Materials and methods). However, prolonged incubation produces a 23 kDa fragment which crossreacts with antibodies raised against cytochrome *o*. It is soluble and

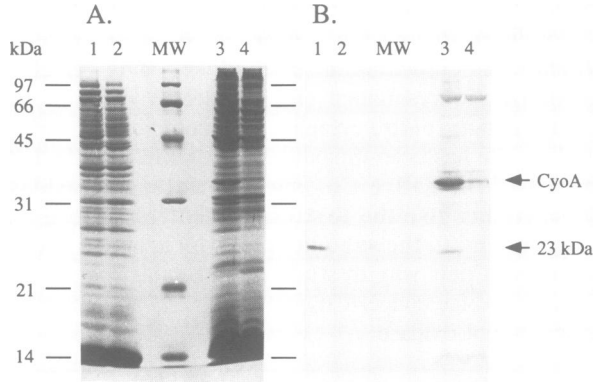

Fig. 2. Overexpression of CyoA in *E.coli*. Expression of CyoA was carried out as described in Materials and methods using the plasmid pCYOA1. The vector pUC18 was used in the control experiments. Soluble and membrane fractions were separated, and proteins were analysed by electrophoresis in the presence of SDS and by immunoblotting. (**A**) Lanes 1 and 3 show proteins in the soluble and membrane fractions, respectively, from cells expressing CyoA; lanes 2 and 4 are the corresponding controls. (**B**) Immunoblots of the same samples. Native CyoA protein and its 23 kDa soluble fragment are labelled on the right, and molecular weight markers (MW) are shown in the middle.

present in the periplasm (not shown). We purified this fragment with the method described below, and found that its N-terminal amino acid sequence is LEPSK. The cleavage

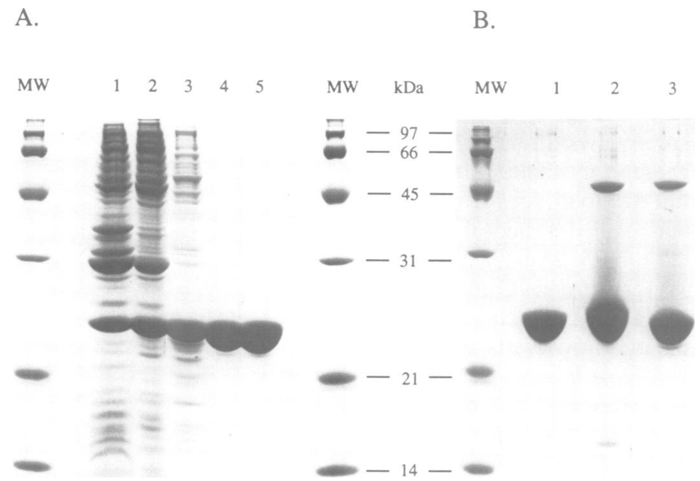

Fig. 3. Purification of the CyoA fragment and its mutants. (**A**) Protein composition of the preparations after different purification steps. Lane 1: soluble fraction of *E.coli* cells expressing the CyoA fragment; lane 2: proteins precipitated with ammonium sulphate (30−60% saturation) and desalted by gel filtration on PD-10 columns; lanes 3−5: preparation after chromatography on Q-Sepharose, 'copper column' and Superdex 75, respectively; see Materials and methods for details. (**B**) Purified wild-type CyoA fragment (lane 1), Cu_A-like mutant (lane 2) and blue copper mutant (lane 3). Note that a 50 kDa protein band is present in the mutants.

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                                  A     B     B'     C                  D.
Amicyanin      1  AGALEAVQEAPAGSTEVKIAKMKFQTPEVRIKAGSAVTWTNT-----EALPHNVHFK
Blue Cu                                                               H
CyoA         147                     ..GIATVNEIAFPANTPVYFKVTS----NSVMNSFFIP
CuA                                                                   H
COII         155                     ..LLATDNPVVVPVGKKVLVQVTA----TDVIHAWTIP
N2OR         552                     ..PAFGVQEFTVKQGDEVTVTITNIDQIEDVSHGFVVV

                                    E         F                 G     .
Amicyanin     53  SGPGVEKDVEGPMLRSNQTYSVKFNA--PGTYDYIC-----TPHPFMKGKVVVE*
Blue Cu                                              C-----TPHPF
CyoA         179  -----RLGSQIYAMAGMQTRLHLIANE-PGTYDGISASYSGPGFSGMKFKAIATP..
CuA                                                  C EIC   H
COII         187  -----AFAVKQDAVPG-RIAQLWFSVDQEGVYFGQCSELCGINHAYMPIVVKAVS..
N2OR         588  -----NHGVSMEISPQ-QTSSITFVADKGPLHWYYCSWFCHALHMEMVGRMMVEPA*
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Fig. 4. Design of copper mutants. Amino acid sequences of *Methylococcus extorquens* amicyanin (Ambler and Tobari, 1985), *Escherichia coli* CyoA (Chepuri *et al.*, 1990), *Paracoccus denitrificans* COII (Raitio *et al.*, 1987) and *Pseudomonas stuzeri* N_2OR (Viebrock and Zumft, 1988) are shown. Location of β-strands in amicyanin have been assigned by NMR (Lommen *et al.*, 1991). These are overlined and labelled A−G. It is difficult to decide where the N-terminal end of cupredoxin-like subdomain (β-strands A and B) would be located in CyoA and COII. In a multiple sequence alignment some COIIs and CyoA have a long gap prior to the sequence position shown in the figure; this may indicate location of a domain boundary (Saraste, 1990). The histidine and cysteine copper ligands and the corresponding residues in CyoA as well as the conserved methionine (residue 218 in CyoA) are shown in bold. The mutations designed to create a blue copper site and a Cu_A site are indicated. Gaps are shown with (−), (*) mark the C-termini of amicyanin and N_2OR. Residue numbers are on the left.

site of an endogenous protease is marked with an arrow in Figure 1. It is located close to the position where the CyoA protein is predicted to emerge from the membrane.

Relatively small amounts of the 23 kDa fragment could be produced by spontaneous proteolysis in the periplasm. Therefore we constructed a new expression plasmid using a pET vector, and expressed the protein in the cytoplasm. The construct was designed to code for a protein which begins with the N-terminus MTHALEPS and ends with the natural C-terminus (Figure 1, Table III). The cytoplasmic expression and subsequent purification of the fragment are shown in Figure 3A. Protein sequencing showed that threonine is the N-terminal residue.

The CyoA fragment is expressed at a very high level in the cytoplasm (Figure 3A, lane 1). It was purified after ammonium sulphate precipitation (30−60% saturation) using three chromatographic steps: (i) anion exchange on Q-Sepharose, (ii) affinity chromatography on immobilized copper and (iii) gel filtration on Superdex 75 (see Materials and methods for details). The preparations made with this procedure contain a single 23 kDa band (Figure 3A, lane 5); virtually no contaminating proteins can be detected after silver staining (not shown). About 40−50 mg of protein can be purified from a 10 l cell culture.

Design of Cu-binding sites into CyoA

A proper alignment of CyoA and COII sequences is the prerequisite for the mutagenesis experiments described in this paper. This alignment suggests that both proteins contain a basic structural motif characteristic to the cupredoxin family of copper-binding proteins and identifies the residues which replace the putative copper ligands of COII in CyoA

(Saraste, 1990). Figure 4 shows the design for two mutants. The first was designed to have a blue copper site, the second to have a Cu_A site.

The blue copper proteins (type 1 copper proteins) have four amino acid residues that are involved in metal binding (Adman, 1991a,b). Three of these, a cysteine, a histidine and a methionine, reside within the loop between the β-strands closest to the C-terminus (called strands F and G by Van Beeumen *et al.*, 1991). The fourth ligand, a histidine, is located in a different loop (between strands C and D, see Figure 4). The methionine residue is not obligatory for construction of a blue copper site in azurin (Karlsson *et al.*, 1991).

The FG loop appears to be longer in Cu_A-containing proteins. This sequence contains two cysteines and one histidine and ends at a methionine. The latter residue is conserved in CyoA whereas all others have been substituted. However, the length of the CXXXCGXXHXXM loop is the same in CyoA as in COII, and it contains the glycine that is strictly conserved in COII. Figure 4 also shows the homologous sequence in nitrous oxide reductase (N_2OR), the only protein other than COII that is known to have a Cu_A-like site (Viebrock and Zumft, 1988; Scott *et al.*, 1989; Kroneck *et al.*, 1990b).

Amicyanins are cupredoxins found in methylotrophic bacteria. Their sequences show the best local match to the sequences in CyoA which flank the FG loop. The closest similarity seems to be between the *Methylobacterium extorquens* amicyanin (Ambler and Tobari, 1985) and the CyoA sequence. This alignment gave us the rationale for changing the FG loop to construct a blue copper centre (Figure 4). First, the N172H mutation in the CD loop was made in order to recreate the histidine ligand. Then the SASYSGPGFSGM sequence of CyoA (residues 207–218) was replaced with the CTPHPFM sequence, which is the invariant FG loop in amicyanins (Van Spanning *et al.*, 1990; Van Beeumen *et al.*, 1991; Lommen *et al.*, 1991).

The FG loop for a Cu_A-like site was constructed in a similar way. The SASYSGPGF sequence was substituted with CAEICGPGH. In addition to the potential Cu ligands, two cysteines and a histidine, we created the glutamate and isoleucine/leucine positions, which are conserved in COII between the cysteines [see Saraste (1990) and Figure 4]. The details of mutagenesis are described in Materials and methods.

Properties of the copper-binding mutants

The mutants were expressed and purified similarly to the wild-type CyoA fragment. The apoproteins were routinely loaded with copper during metal affinity chromatography, which was carried out in the presence of free $CuCl_2$ (see Materials and methods). 2% dimethyl-sulfoxide was included in buffers since we found that it protects effectively against the loss of bound copper during storage. Such losses easily occur in the absence of DMSO and particularly in the presence of imidazole during freezing and thawing. Introduction of cysteines into the CyoA fragment results in the appearance of a cross-linked product in SDS–polyacrylamide gels (Figure 3B). The nature of this product is not known; it cannot be removed by the addition of reducing agents such as β-mercaptoethanol to the electrophoresis gel.

Figure 5 shows electronic spectra of the blue copper and Cu_A mutants of the CyoA fragment. The oxidized spectrum

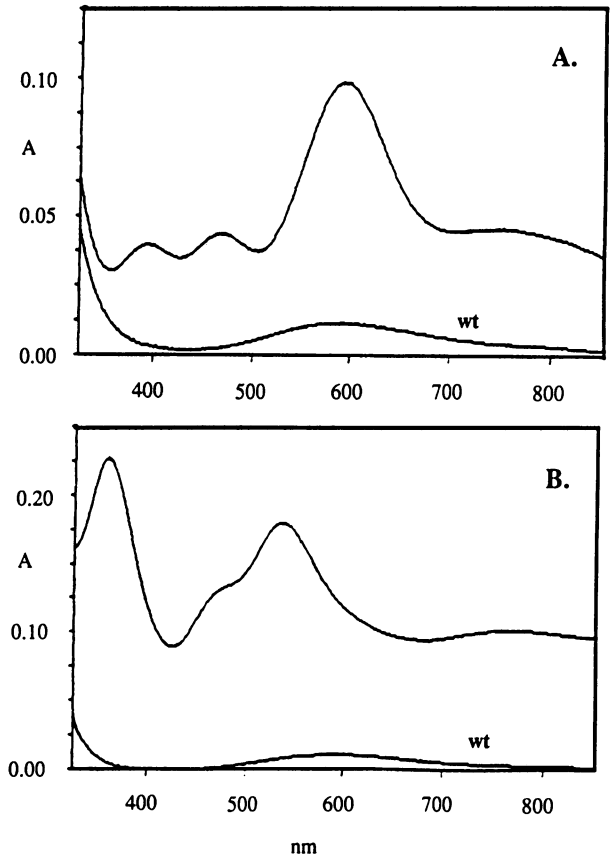

Fig. 5. Optical spectra of copper-containing mutants. Spectra were recorded at room temperature. The samples (115 μM protein) were prepared as described in Materials and methods by gel filtration in the presence of 0.1 mM $CuCl_2$ and 2% DMSO in standard Tris buffer; this solution was used as a reference. Under these conditions copper binding to the Cu_A-like site is almost saturated. The absorbance of the blue copper site can be increased by 10–20% by addition of $CuCl_2$ up to 1.0 mM concentration. (**A**) Blue copper mutant. (**B**) Cu_A-like mutant. The spectra of the wild-type CyoA fragment are labelled wt.

Table I. Spectral properties of the copper-binding mutants

Blue copper		Cu_A	
nm	mM^{-1} cm^{-1}	nm	mM^{-1} cm^{-1}
		765	0.9
594	1.2	536	1.6
		358	2.0
(A_{278}/A_{594} = 22)		A_{278}/A_{536} = 16)	

Absorption maxima and extinction coefficients of the mutants constructed into the soluble CyoA fragment were measured in the presence of 0.1 mM Cu^{2+}.

of the blue copper mutant has the largest absorption maximum at 594 nm. The location of this maximum is similar to those found in amicyanin (595 nm; Husain *et al.*, 1986) and plastocyanin (597 nm; Ramshaw *et al.*, 1973). The apparent extinction coefficient is, however, lower (1.2 mM^{-1} cm^{-1}; Table I) than the coefficients of the latter natural copper proteins (4–5 mM^{-1} cm^{-1}). Two smaller maxima at 390 and 466 nm are also present in the blue copper mutant (Figure 5A).

The Cu_A-like mutant is purple. Its absorption spectrum has two strong peaks at 358 and 536 nm, a pronounced

Table II. Copper/protein ratios

	Cu/protein (mol/mol)	Extra Cu (mol/mol)	EPR-detectable Cu[a]	
			Total (%)	Extra (%)
CyoA fragment	1.7 ± 0.2	–	100	–
Blue copper mutant	2.4 ± 0.3	0.7 ± 0.1	–	low
Cu_A mutant	2.8 ± 0.2	1.1 ± 0.1	–	30

Copper binding to the wild-type and mutant CyoA fragments was measured in the presence of free 0.1 mM Cu^{2+} (this background concentration is subtracted from the values).
[a]For EPR measurements free copper was removed from the samples by passage through either a PD-10 column or a chelating Sepharose resin (Materials and methods); these treatments also remove the same amount of bound copper from all three proteins (the Cu/protein values shown on the left go down by 0.4 and 0.8, respectively).

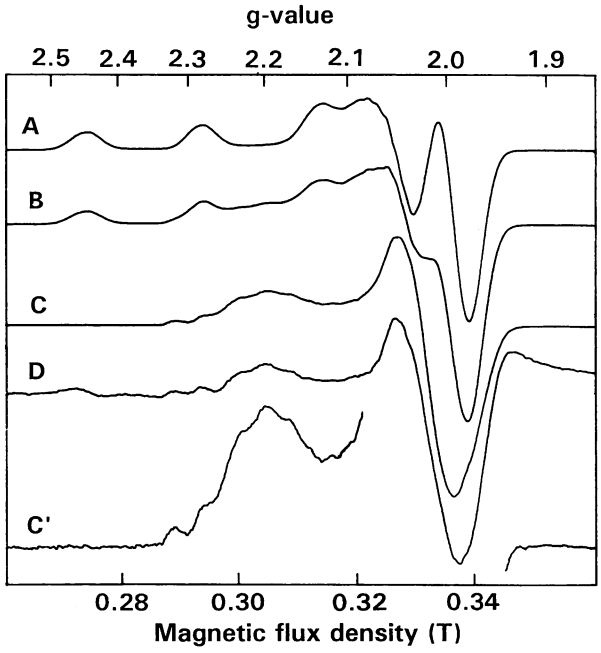

Fig. 6. EPR spectra of CyoA and its Cu_A mutant. Spectrum (**A**) is the wild-type CyoA fragment and (**B**) the Cu_A mutant. The difference spectrum (**C**) was obtained by subtracting (A) from (B) and amplified twice. (**C'**) is amplified four times from (C). (**D**) is the difference spectrum of the Cu_A mutant as 20 K and 160 K. Spectrometer conditions: frequency 9.367 GHz, modulation amplitude 2 mT, microwave power 10 mW and temperature 20 K (except in D).

shoulder at 475 nm and a flat peak between 750 and 780 nm. The spectrum shown in Figure 5B closely resembles that of the purple Cu_A-like site in N_2OR (Coyle *et al.*, 1985; Riester *et al.*, 1989; Farrar *et al.*, 1991). The major differences are the absorption peak at 358 nm, which is much weaker in the N_2OR spectrum, and the absence of a shoulder at 620 nm, which may arise from a different copper centre in N_2OR (Farrar *et al.*, 1991). The extinction coefficient of the Cu_A-like mutant at 536 nm (1.6 mM^{-1} cm^{-1}, Table I) is again 5-fold less than the coefficient of the purple site in N_2OR at 540 nm (7–8 mM^{-1} cm^{-1}, Coyle *et al.*, 1985; Riester *et al.*, 1989).

The entire optical spectrum of Cu_A has never been recorded in cytochrome *c* oxidase as most of it is masked by the absorbance of haems. However, magnetic circular

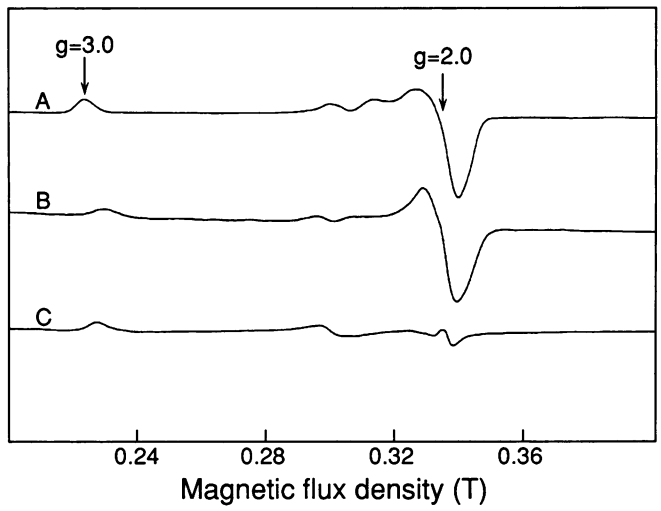

Fig. 7. EPR spectrum of the Cu_A mutant of cytochrome *o*. EPR spectra of the purified enzymes were recorded using a Bruker ESP300 spectrometer under the following conditions: modulation amplitude 2 mT, frequency 9,45 GHz, temperature 7 K, and power 10 mW. The protein concentration of (**A**), beef-heart cytochrome *c* oxidase is 8 mg/ml, (**B**), the Cu_A-containing cytochrome *o* complex is 3 mg/ml and (**C**), the wild-type cytochrome *o* complex is 4 mg/ml. The spectrum in (B) was multiplied by a factor of three.

dichroism (MCD) has been used to determine selectively the spectrum of Cu_A in the native enzyme (Greenwood *et al.*, 1983, 1988; Farrar *et al.*, 1991). The optical spectrum of the Cu_A-like mutant (Figure 5B) fits well with these MCD spectra. For instance, the MCD spectrum predicts a strong absortion maximum above 500 nm. This is clearly present in the electronic spectrum of the Cu_A mutant. Only the far-red part of the Cu_A spectrum is visible in cytochrome *c* oxidase, where the flat absorption maximum is ~820–840 nm (see Wikström *et al.*, 1981). This peak has shifted to 750–780 nm in the Cu_A mutant. A similar shift is found in the N_2OR spectrum (Coyle *et al.*, 1985; Riester *et al.*, 1989).

The wild-type CyoA fragment has rather specific copper-binding sites (Table II). The electron paramagnetic resonance (EPR) spectrum of this fragment is shown in Figure 6A. At higher resolution, using a lower modulation amplitude, clear extra hyperfine splitting due to ^{14}N can be observed (not shown), indicating that this copper is probably bound to histidine residues. The same type of 'extraneous' copper is often observed in other proteins and is, for instance, present in many bovine cytochrome oxidase preparations (Hartzell and Beinert, 1974; Aasa *et al.*, 1976).

The Cu_A-like mutant contains this extraneous copper as well as an additional and different copper site (Figure 6B). The difference spectrum, shown in Figure 6C, was obtained by subtracting spectrum A from spectrum B. This seems to eliminate the contribution of extra copper completely, and the resulting spectrum arises solely from the copper bound to the mutated site. Another way of obtaining a difference spectrum between the mutant and the wild-type is to change the recording temperature. The Cu_A spectrum in cytochrome *c* oxidase broadens beyond detection above 150 K, whereas the EPR spectrum of extraneous copper is nearly unaffected (Hartzell and Beinert, 1974). Figure 6D shows the difference between the spectra recorded at 20 K and 160 K. It is very similar to spectrum C. The noise in

the spectrum is probably due to a slight temperature dependence of the extraneous copper spectrum.

The spectrum shown in Figure 6C is strikingly similar to the EPR spectrum of Cu_A in cytochrome *c* oxidase (Aasa *et al.*, 1976; see Figure 7). The main difference is the slightly higher g_z value in the mutant spectrum (2.20 instead of 2.18). The spectrum shown in Figure 6D is rather sensitive to the numerical factors used in making the difference. There is, however, no doubt that the main part of this spectrum closely resembles the spectrum shown in Figure 6C. This means that the Cu_A-like mutant site has the same unusual temperature behaviour as native Cu_A. This is further strong evidence that the mutant is closely related to the Cu_A site in cytochrome *c* oxidase.

We have not been able to record the EPR difference spectrum of the blue copper mutant. This is possibly due to sensitivity of the site to freezing, upon which copper may dissociate from the apoprotein.

The redox potential of Cu_A-like mutant is 260 ± 10 mV. This is the same as the midpoint potential of the purple copper site in N_2OR (260 ± 5 mV; Riester *et al.*, 1989) and very close to the potential of the native Cu_A site (245 mV; see Wikström *et al.*, 1981).

We have tried to quantify the copper/protein ratios in the wild-type and mutant CyoA fragments. The results are shown in Table II. In the samples which were equilibrated with 0.1 mM free copper, the CyoA fragment contains 1.7 mol Cu/mol protein. Both mutants contain additional copper. In the blue copper mutant this additional copper amounts to 0.7 mol/mol and in the Cu_A-like mutant to 1.1 mol/mol.

Cu_A mutation in the cytochrome o complex

The Cu_A mutation described above was also incorporated into the *cyo* operon coding for the quinol oxidase complex (Chepuri *et al.*, 1990; see Materials and methods). The enzyme was expressed from a plasmid under the control of the native promoter. The mutant strain was able to complement a *cyo* deletion, but its growth was severely inhibited in comparison with the wild-type strain. Attempts were made to purify the mutant cytochrome *o* complex both from a *cyo*$^-$ and a *cyo*$^+$, *recA* strain. However, the mutant oxidase was unstable when purified from the former strain.

The instability of the mutant cytochrome *o* complex presented many complications that hindered its characterization. However, the EPR spectrum of the mutant cytochrome *o* could be measured (Figure 7). The signal of the Cu_A-like site around $g = 2$ in the mutant (middle) is similar to the Cu_A signal of cytochrome *c* oxidase (top). As expected (Puustinen *et al.*, 1991), the wild-type cytochrome *o* has no Cu_A signal (bottom). In contrast to the Cu_A-containing soluble fragment, the Cu_A-like site in the complex was not reconstituted during purification, but could be generated *in vivo* when the cells were grown in the presence of 0.02−1.0 mM $CuSO_4$. It is interesting to note that the EPR signal of the low-spin haem in cytochrome *o* at $g = 3$ is perturbed in the Cu_A-containing mutant (Figure 7).

The Cu_A mutant of cytochrome *o* was tested for cytochrome *c* oxidase activity. While the control cytochrome oxidase reduced cytochrome *c* rapidly, neither the wild-type cytochrome *o* nor the Cu_A mutant showed any significant activity (data not shown). The binding of cytochrome *c* and its subsequent oxidation must require more structural changes than introduction of copper into subunit II.

Discussion

Cupredoxin fold within subunit II of the oxidase family

The success of constructing copper-binding sites in this study suggests that our working hypothesis is correct. That is, the subunit II in cytochrome *c* and quinol oxidase complexes does contain a domain with a cupredoxin fold. The domain in CyoA and in the other related subunits of quinol oxidases is copperless. The ligands that are needed for binding of copper have been lost—an evolutionary adaptation that may reflect the change in enzymatic activity. As relatively small mutational changes (Figure 4) can recreate a binding site for copper, the domain framework must be preserved in the quinol oxidase subunits. Cupredoxin domains constitute parts of many multidomain copper-containing enzymes, such as ascorbate oxidase and nitrite reductase. Some of these domains have lost their copper (Messerschmidt and Huber, 1990; Adman, 1991a,b; Fenderson *et al.*, 1991).

A circular dichroic spectrum suggests that the CyoA fragment contains very little α-helix but has 70% β-sheet (data not shown). The domain with the cupredoxin fold is only a part of the CyoA fragment which may have several subdomains (Figure 1). In other cases (e.g. Lübben *et al.*, 1992), subunit II of oxidase complexes is smaller and lacks the sequence that extends from the cupredoxin domain to the C-terminus of CyoA. Our results also imply that the C-terminus of N_2OR has a cupredoxin domain (Figure 4).

This study demonstrates that blue copper and Cu_A sites are structurally related and that the properties of copper binding sites in the cupredoxin family (into which COII and N_2OR should become members) are probably mostly determined by the FG loop region.

The properties of Cu_A-like mutants

The optical spectrum of the Cu_A-like site constructed into the soluble CyoA fragment (Figure 5B) is very similar to the spectrum of the purple site in the multicopper enzyme nitrous oxide reductase. The close similarity between the Cu_A of cytochrome oxidase and the purple N_2OR copper site has been shown before using EPR and MCD spectroscopy as well as extended X-ray absorption fine structure (EXAFS) measurements (Greenwood *et al.*, 1988; Kroneck *et al.*, 1988, 1990a; Scott *et al.*, 1989; Farrar *et al.*, 1991).

The EPR spectra of Cu_A-like mutants closely resemble that of the native Cu_A in cytochrome *c* oxidase (Figures 6 and 7). It has a well resolved hyperfine splitting around g_z (Figure 6C′). A clear seven-line hyperfine splitting is present in the EPR spectrum of N_2OR and it has been suggested that it arises from a mixed valence [Cu(II),Cu(I)] spin = ½ site (Kroneck *et al.*, 1988, 1990b; Riester *et al.*, 1989; Farrar *et al.*, 1991). Equal spin coupling to both nuclei would give a seven-line spectrum with 1:2:3:4:3:2:1 intensity ratios. It has been claimed that such a seven-line splitting around $g = 2.2$ is also present in the Cu_A spectrum (Kroneck *et al.*, 1990a).

The binuclear Cu_A model is supported by the presence of 2.5−3.0 moles of copper per two haems to cytochrome oxidase preparations (e.g. Steffens *et al.*, 1987; Öblad *et al.*, 1989; Pan *et al.*, 1991). On the other hand it is not supported by EXAFS studies on cytochrome oxidase and N_2OR, in which the spectra can be simulated using a mononuclear model (see Martin *et al.*, 1988; Scott *et al.*, 1989; Malmström, 1990). This controversy might be solved with

quantitative copper analysis of the soluble mutant (Table II). The analysis is not informative, however, if the mutated copper-binding sites are not fully occupied as may be true in our case. This could be due to the presence of a modified form of protein that is not capable of binding copper. Low molar extinction coefficients (Table I) suggest that this is probably the case. A modified form of protein, which cannot be reconstituted with copper, contaminates azurin expressed in *E.coli* (Van de Kamp *et al.*, 1990).

The binuclear model would predict that about half of the extra copper in the Cu_A mutant is not detectable by EPR, because it is reduced Cu[I]. We found that only ~30% of the extra copper gives an EPR signal (Table II). This observation and the presence of 1.1 Cu/Cu_A site do not disprove the binuclear model, but more work is clearly needed for its support. In any case, the Cu_A and purple N_2OR sites must be almost identical; if the latter is binuclear, the Cu_A site is also.

Cu_A is bound to COII

Steffens and Buse (1979) noticed that the bovine COII sequence contains a set of putative copper ligands in the region we call the FG loop, and pointed out the analogy to blue copper proteins. This same idea has been used in modelling exercises (Holm *et al.*, 1987). Sequence comparisons and spectroscopic identification of the Cu_A ligands (Martin *et al.*, 1988; Chan and Li, 1990; Malmström, 1990; Saraste, 1990) have given circumstantial evidence that the Cu_A site of cytochrome *c* oxidase resides in COII. Thus far, no direct experimental evidence has supported this view.

The similarities in spectroscopic properties and in redox potentials of the Cu_A-like mutant, the native Cu_A and the purple copper site in N_2OR suggest that we have been able to create a genuine Cu_A site by protein engineering. This site has been constructed both into the intact CyoA, which is bound to the other subunits of the complex, and into the soluble CyoA fragment. Characterization of the C-terminal fragment from a cytochrome *c* oxidase subunit II—so far an unsuccessful experiment—may finally confirm the localization of the Cu_A site. However, the present study very strongly suggests that most, or all, of the Cu_A ligands are in COII.

In summary, we have been able to insert two different copper-binding sites into a protein that is homologous to a Cu_A-binding protein but has lost its copper during evolution. The properties of the novel copper site can be manipulated by protein engineering. Work is in progress to discover the roles of individual amino acid ligands for binding of copper in these engineered sites.

Materials and methods

Standard methods of molecular biology are described by Ausubel *et al.* (1991). Restriction enzymes as well as most of the other chemicals were obtained from Boehringer Mannheim. A Geneclean kit (BIO 101 Inc., La Jolla, CA) was used to purify DNA from agarose gels. AmpliTaq polymerase (Perkin–Elmer) was used in polymerase chain reactions (PCR). The *E.coli* strains were routinely grown in Luria Broth (LB) at 37°C. Ampicillin was added to 100 mg/l when required.

Expression of CyoA and its C-terminal fragment

The *cyoA* gene was multiplied as a 1.2 kb *Eco*RI–*Sal*I fragment from the plasmid pMC31 encoding the *E.coli cyo* operon (Lemieux *et al.*, 1992) using PCR. The *Eco*RI site was constructed with a PCR primer (Table III) 90 bp upstream from the 5′-end of *cyoA*; the *Sal*I site is present in the operon ~0.2 kb downstream from the 3′-end of *cyoA*. This restriction fragment was cloned with pUC18 in *E.coli* JM109. This construct, called pCYOA1, was used to express the native CyoA protein in *E.coli*. The cells were grown for 3–4 h to an optical density of 0.5 at 600 nm, and the expression was induced with isopropyl-thio-β-D-galactoside (IPTG, 0.2 mM). The cells were harvested 4 h after induction, washed with 20 mM Tris–HCl (pH 8.0) and stored at −20°C.

The expression of the C-terminal CyoA fragment in the cytoplasm of *E.coli* BL21 (DE3) (F^-, *ompT*, r_B m_B, λ lysogen containing T7 RNA polymerase gene under the *lac*UV5 promoter) was carried out with a modified pET vector, which was a derivative of pET3d (Studier *et al.*, 1990), made by deleting the 0.5 kb *Nco*I–*Hin*dIII fragment; this removes the downstream *Bam*HI cloning site and the Tφ transcription terminator of pET3d. The region of *cyoA* coding for residues 111–315 of the protein (Figure 1) was amplified with PCR. The PCR primers introduced the upstream *Bsp*HI and downstream *Hin*dIII restriction sites (Table III) which enabled ligation into the *Nco*I and *Hin*dIII sites of the vector. The resulting plasmid is called pET.E2.

We noticed that some of the plasmid constructs are unstable. Therefore freshly transformed colonies from LB plates containing ampicillin were used to inoculate 25 ml cultures in 100 ml Erlenmeyer bottles. After shaking (200 r.p.m.) for 2–3 h at 37°C these cultures were used to inoculate ten 1 litre cultures in 2 l Erlenmeyer bottles. The cells were harvested 2 h after induction with IPTG (see above).

Mutagenesis

The primers used in mutagenesis are shown in Table III. Mutations were introduced with PCR following the overlap extension method described by Higuchi *et al.* (1988). All PCR constructs were sequenced to rule out undesired mutations. The mutation that substitutes N172 with a histidine was carried out first using primer pairs #112/#84 and #83/#113. The mutated fragment was isolated and used in a second round of PCR for introduction of the FG loop mutations to create the Cu_A-like site (#112/#A1-2 and #A1-1/#113) or the blue copper site (#112/#123 and #122/#113). The mutated DNA fragments were ligated into pET vector. The resulting plasmids pET.E3 and pET.E4 were used for the expression of Cu_A and blue copper mutant, respectively, as described above.

Purification of the C-terminal fragment of CyoA

About 20 g (the yield of a 10 litre culture) of frozen and thawed *E.coli* cells were suspended with 100 ml of 'standard Tris buffer': 20 mM Tris–HCl (pH 8.0) containing proteolytic inhibitors phenylmethyl sulphonylfluoride (0.15 mM) and benzamidine (0.5 mM). Cells were broken by three passages through a French press at 4°C. The viscosity of the suspension was reduced by adding DNase (2 mg) and $MgSO_4$ (1 mM). The suspension was centrifuged for 1 h at 40 000 r.p.m. in a Beckmann Ti45 rotor, and the clear supernatant was recovered.

The CyoA fragment was precipitated from the extract with ammonium sulphate (AS) between 30 and 60% saturation. The precipitated proteins were dissolved into 20 ml of the standard Tris buffer, desalted with Sephadex G25 columns (Pharmacia PD-10) and applied to a Q-Sepharose Fast Flow anion-exchange column (XK-26, 50 ml bed volume) equilibrated with the standard Tris buffer. The flow rate was 4 ml/min. A linear NaCl gradient from 0 to 0.4 M was developed for 100 min. β-lactamase, which is coinduced with the CyoA fragment, elutes at 0.15 M NaCl. The peak fractions containing the CyoA fragment, which elutes at 0.25 M NaCl, were pooled, and protein was precipitated with 70% saturated AS. The pellet was dissolved into a small amount of the standard Tris buffer for the next purification step. The protein can be stored as a precipitated suspension for several days at 4°C.

A Chelating Superose column (Pharmacia HR 10/2) was equilibrated with the standard Tris buffer containing 0.5 M NaCl and 0.1 M NH_4Cl. 4 ml of the same buffer plus 0.2 M $CuCl_2$ was added to load the column with Cu[II]. One-third of the concentrated sample from the Q-Sepharose was applied to the 'copper column', and the bound proteins eluted with a linear imidazole gradient from 0 to 20 mM in the equilibration buffer. The flow rate was 1.0 ml/min; the gradient was developed for 60 min. The CyoA fragment elutes between 8 and 15 mM imidazole. The peak fractions from the runs were concentrated with centrifugation in Microsep concentrators (Filtron Co, MA, USA).

A Superdex 75 HiLoad gel filtration column (120 ml, Pharmacia) was equilibrated with the standard Tris buffer. Up to 2.0 ml of the sample could be loaded to this column. The flow rate was 1.0 ml/min. The CyoA fragment elutes at 65–70 ml.

All columns were run with a Pharmacia FPLC instrument at room temperature. Elution buffers of the 'copper column' contained 2% (v/v)

Table III. Primers used in plasmid construction and mutagenesis

1. PCR primers for construction of pCYOA1 and pET.E2

(A) pCYOA1: expression of CyoA

#79 5′- CGCGCGAATTCCCGTAAAATGCCCACACAC- 3′
3′- TTTCTCACCGACTGGAGGCAGCTGCGCGC- 5′

The *Eco*RI and *Sal*I sites are underlined.

(B) pET.E2: expression of the CyoA fragment

(111)
M T H A L E P S K. . .
#112 5′- GCGCGTCATGACTCACGCTCTTGAGCCTAGCAAG- 3′

#113 3′- GGTGCGCCTTAGGCGGGTAATTATTTTCGAAGCGCG- 5′
. . . H A E S A H *
(315)

*Bsp*HI and *Hin*dIII sites are underlined and the beginning and the end of the reading frame are shown.

2. PCR primers for mutangenesis

(A) NI72H

(172)
. . S V M **H** S F F. . .
#83 5′- CTCCGTGATG**C**ACTCCTTCTTCAT- 3′
#84 3′- GAGGCACTAC**G**TGAGGAAGAAGTA- 5′

(B) The FG loop of the blue copper mutant

(207) (218)
. . **C T P H P F** M K F K A I A T P.
#122 5′- **TGCACTCCCCACCCCTTTA**TGAAGTTCAAAGCTATTGCAACACCG
#123 5′- GGGCCGTGAATACTGCCATAG**ACGTGAGGGGTGGGGAAA**TACTTC-5′
. . P G T Y D G I **C T P H P F** M K. .

(C) The FG loop of the Cu_A mutant

(207) (218)
. . . D G I **C** A **E I C** G P G **H** S G M. .
#A1-1 5′- ATGACGGTATCT**GCGCCGAAATCTGCGGCCCGGGC**C**A**CTCAGGCATGA- 3′
#A1-2 3′- TACTGCCATAGA**C**GCGG**CTTTAGA**CGCCGGGCCCG**GT**GAGTCCGTACT- 5′

The nucleotide sequence of the template DNA has been reported by Chepuri *et al.* (1990). The protein sequence of the wild-type CyoA is shown in Figure 1. Mutations are shown bold. The residue numbers refer to the wild-type sequence.

dimethylsulphoxide and 1.0 mM $CuCl_2$, and the buffer used in Superdex column 2% DMSO and 0.1 mM $CuCl_2$, when the copper-binding mutants were purified.

Spectroscopy

Optical spectra were recorded with a Perkin–Elmer spectrophotometer λ2 at room temperature. EPR spectra in Figure 6 were recorded with a Bruker ER 200D-SRC X-band spectrometer equipped with a standard TE_{102} rectangular cavity and an Oxford Instruments ESR-9 helium flow cryostat. Quantifications of the EPR spectra were performed under non-saturating conditions as described earlier (Aasa and Vänngård, 1975) with Cu[II] in 2 M $NaClO_4$, pH 2, as a reference. For EPR the samples containing free copper were passed through PD-10 columns or small columns containing metal-free Chelating Sepharose. Copper was fully oxidized by addition of a small amount of laccase into the samples.

Protein and copper analysis

The protein concentration was obtained by quantitative amino acid analysis for determination of the UV extinction coefficient. The wild-type and mutated CyoA fragments at 1 mg/ml have the absorbance of 1.03 ± 0.11 at 278 nm; the millimolar extinction coefficient is 23.6 mM^{-1} cm^{-1}. The copper content of the samples was determined using the biquinone method (Broman *et al.*, 1962). Redox titrations were done as described earlier (Karlsson *et al.*, 1991).

The antibodies against CyoA were raised in rabbits using the *E.coli* cytochrome *o* complex as the antigen. IgG was partially purified following the method of Watt *et al.* (1980).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out using the buffer system of Laemmli (1970). Gels were routinely stained with Coomassie Blue. The N-terminal protein sequences were determined by Edman degradation using an Applied Biosystems Model 477A Sequencer.

Construction of a Cu_A site into the cytochrome o complex

In order to introduce the Cu_A mutation into the cytochrome *o* complex, the plasmid pL4 was constructed. The 1.7 kb *Eco*RI–*Sph*I fragment from pRCO6 (J.Ma and R.B.Gennis, unpublished), which lacks the *Kpn*I site at 5.5 kb in the *cyo* operon, was subcloned into *Eco*RI- and *Sph*I-digested pMC39 (Lemieux *et al.*, 1992), and the mutations described above were introduced into it. The 840 bp *Kpn*I–*Sal*I fragment containing the Cu_A mutations was then cloned into pL4 resulting in pL4CuA. The plasmid was able to produce a functional quinol oxidase in a genetic complementation test (see Lemieux *et al.*, 1992). The mutant oxidase was over-produced and purified from the *recA* strain, HB101. 15 litres of cells were grown as previously described (Lemieux *et al.*, 1992) with the addition of 1 mM $CuSO_4$ to the medium. The purification procedure for the wild-type and mutant enzymes was adapted from Minghetti *et al.* (1992). Type II copper, which is present in the soluble domain (Figure 6), was also found in the

wild-type cytochrome *o* complex, but not in the Cu_A-containing complex. This adventitious copper had to be removed by including 2 mM EDTA in the initial purification buffer and finally dialysing against 5 mM EDTA prior to EPR measurements. This EDTA treatment, however, did not affect the EPR spectrum of the Cu_A-containing complex.

Cytochrome *c* oxidase activity was measured spectrophotometrically following oxidation of reduced horse heart cytochrome *c* at 550 nm.

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