Solution Structure of Cox11, a Novel Type of β-Immunoglobulin-like Fold Involved in CuB Site Formation of Cytochrome c Oxidase* [S]

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Cytochrome c oxidase assembly process involves many accessory proteins including Cox11, which is a copper-binding protein required for Cu incorporation into the CuB site of cytochrome c oxidase. In a genome wide search, a number of Cox11 homologs are found in all of the eukaryotes with complete genomes and in several Gram-negative bacteria. All of them possess a highly homologous soluble domain and contain an N-terminal fragment that anchors the protein to the membrane. An anchor-free construct of 164 amino acids was obtained from Sinorhizobium meliloti, and the first structure of this class of proteins is reported here. The apoform has an immunoglobulin-like fold with a novel type of β-strand organization. The copper binding motif composed of two highly conserved cysteines is located on one side of the β-barrel structure. The apoprotein is monomeric in the presence of dithiothreitol, whereas it dimersizes in the absence of the reductant. When copper(I) binds, NMR and extended x-ray absorption fine structure (EXAFS) data indicate a dimeric protein state with two thiolates bridging two copper(I) ions. The present results advance the knowledge on the poorly understood molecular aspects of cytochrome c oxidase assembly.

Cytochrome c oxidase (CcO) is the terminal enzyme in the electron transport system, reducing oxygen to water and generating the proton gradient that drives ATP synthesis. Multiple subunits and several cofactors are necessary for catalytic activity including two hemes α, a magnesium ion, a zinc ion, and three copper ions. In particular, the copper ions are located in subunits COX I and COX II, which contain the CuB and CuA centers, respectively. The insertion of these cofactors and assembly of the CcO complex in the inner mitochondrial membrane requires accessory proteins (for a general review, see Ref. 1). It has recently become clear that the required metal ions cannot simply diffuse to the requisite compartment of the cell for insertion into the desired protein, but a complex machinery of metal importers and chaperones is required (2). In eukaryotes, two metallochaperones, Cox17 and Cox19, were proposed to shuttle copper in the mitochondrion (3, 4). Other nuclear genes also are required for the proper insertion of copper into CcO (5–8). In particular, it is well established that a mitochondrial inner membrane protein, Sco1, interacting with COX II subunit (9), is important for copper insertion into the binuclear CuB site (10, 11). On the contrary, the process through which copper is provided to COX I subunit, which contains a copper ion buried 13 Å below the membrane surface, still remains essentially obscure, even if it is known that it requires another mitochondrial inner membrane protein, Cox11. Cox11 was first shown to be implicated in the CcO maturation process from the observation that Δcox11 yeast lacked CcO activity and was deficient in heme α (5). It was also observed that, for Δcox11 mutants lacking CcO activity, RNA and protein synthesis of the core subunits I and II are normal, suggesting that Cox11 functions post-translationally to generate active CcO (5). However, it is now evident that other two proteins rather than Cox11 are the key enzymes involved in heme α formation (12, 13). More recently, it was indeed demonstrated that the CuB site is absent in CcO purified from Rhodobacter sphaeroides lacking cox11 (14). These results suggest that Cox11 functions primarily in CuB site formation, possibly delivering copper directly to that site. Alternatively, Cox11 could act as a co-chaperone, facilitating Cu(I) donation through another molecule. In particular, Cox11 may function in copper insertion with specificity for CuB site in a fashion analogous to that hypothesized for the metallation of the CuA site, i.e. Cox17 donates Cu(I) to Sco1, which inserts copper into the CuA site (1).

In yeast Cox11, a 34-kDa protein anchored by a single transmembrane segment to the inner mitochondrial membrane, the soluble C-terminal domain in a dimeric state both in the apoform and Cu(I) bound form (15). The protein binds one copper atom per monomer, and from the EXAFS data, it was observed that the two copper ions are in close proximity (15). It was suggested that copper is coordinated by three conserved cysteine residues, even if it was not possible to determine whether they all belong to the same subunit of the dimer or if one of them belongs to the other subunit (15). Mutation of any of these Cys residues reduces Cu(I) binding and confers respiratory incompetence and reduced CcO activity (15).

In this study, we browsed GenBank™ to search for homologs of yeast Cox11 in prokaryotic organisms and we selected five sequences, cloned them, and expressed them in a high throughput approach. We then have solved the three-dimensional so-
Solution Structure of ApoCox11 from *S. meliloti*

**EXPERIMENTAL PROCEDURES**

**Sequence Search and Analysis**—Cox11 sequences were searched in all of the non-redundant GenBank™ databases (coding sequences translations + Protein Data Bank + SwissProt + Protein Information Resource + Protein Research Foundation) using sequence similarity criteria. This was accomplished by starting from the sequence of Cox11 from yeast *Sacharomyces cerevisiae* and performing a protein-protein BLAST (16) search (www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were done with ClustalW (17). The prediction of trans-membrane helices and membrane topology of Cox11 sequences is obtained using HMMTOP and TMpred programs (18, 19).

**Protein and NMR Sample Preparation**—The genes coding Cox11 proteins from *Agrobacterium tumefaciens*, *Caolobacter crescentus*, *Pseudomonas aeruginosa*, *R. sphaeroides*, and *S. meliloti* were selected for protein expression and amplified by polymerase chain reaction (PCR) for protein expression and amplified by polymerase chain reaction (PCR) and were cloned into the pET21a vector for expression. Amplified genes were cloned in pET21a expression vectors producing both native and C-terminal His$_6$-tagged proteins. All of the constructs were screened for expression in *Luria-Bertani-ampicillin medium using BL21(DE3) Escherichia coli strains*. The cells were grown at 37 °C and induction was performed by isopropyl-β-D-thiogalactopyranoside.

Cox11 protein from *S. meliloti* was selected because it is mainly present in the supernatant at variance with all of the other protein constructs and showed the highest expression level. Purification of Cox11 from *S. meliloti* is performed by anion exchange chromatography followed by size-exclusion chromatography at 4 °C. The molecular weight of the native Cox11 protein was determined by SDS-PAGE and the protein concentration was measured by the method of Lowry (20).

**NMR Sample Preparation**—NMR samples were prepared in 20 mM potassium phosphate buffer at pH 7.2. Each sample was prepared under nitrogen atmosphere, and all of the subsequent handling was always conducted under inert atmosphere. The protein sample was loaded into a plastic vial with Kapton windows, which was completely washed with an 100 mM EDTA solution, rinsed with pure water and absolute ethanol, and dried. The sample cell was then mounted in a two-stage Displex cryostat (modified Oxford instruments) and kept at 20 K during data collection.

**NMR Spectroscopy**—NMR data were collected on a Varian spectrometer at 30 °C and 600.13 MHz, respectively. The NMR experiments used for the backbone and the aliphatic side chain resonances assignment and for obtaining structural restraints, recorded on 15N/13C- and 15N-enriched and unlabelled apoCox11 samples, are summarized in Supplemental Table 1. The 1H, 13C, and 15N resonance assignments of apoCox11 are reported in Supplemental Table 2. Distance constraints for structure determination were obtained from 15N-edited and 13C-edited three-dimensional NOESY-HSQC experiments and from two-dimensional NOESY. Backbone dihedral $\phi$ and $\psi$ angles were determined through HNHA and three-dimensional 13C-edited NOESY-HSQC experiments, respectively, as reported previously (20, 21). The elements of secondary structure were determined on the basis of the chemical shift index (22) of the $\delta_{	ext{H},	ext{C}}$, using constants of the backbone NOE restraints. The restrained Cα-CD approach combined with the fast DYANA torsion angle dynamics algorithm (23) was used to assign the ambiguous NOE cross-peaks and to have a preliminary protein structure. Structure calculations were then performed through iterative cycles of DYANA (24) implemented with the use of Ramachandran potentials (25) followed by restrained energy minimization with AMBER 5.0 (26) applied to each member of the final DYANA family. The assessment of the structures was evaluated using the programs PROCHECK-NMR (27) and AQUA (27, 28). Fold similarities in the Protein Data Bank were searched using the DALI (29), SCOP (30), and CATH (31) programs.

**X-ray Absorption Spectroscopy (XAS) Data Collection and Analysis**—Two Cu(I)Cox11 samples from different protein batches have been prepared for XAS measurements to check the reproducibility of the spectrum. Each sample consisted of a 1 mM protein solution in 50 mM Tris/MES buffer at pH 7.2. Each sample was prepared under nitrogen atmosphere, and all of the subsequent handling was always conducted under inert atmosphere. The protein sample was loaded into a plastic vial, which was completely washed with an 100 mM EDTA solution, rinsed with pure water and absolute ethanol, and dried. The sample cell was then mounted in a two-stage Displex cryostat (modified Oxford instruments) and kept at 20 K during data collection.

**XAS data were collected in fluorescence mode at the EMBL bending magnet beamline D2 (DESY, Hamburg, Germany) equipped with a Si(111) double crystal monochromator, a focusing mirror and a 12-element Ge solid-state detector. The XAS scans covered the energy range from 8725 to 9875 eV using variable energy step widths. In the X-ray absorption near-edge structure and EXAFS regions, the steps of 0.3 and 0.4–1.2 eV were used, respectively. The DORIS-III storage ring was operating at 4.5 GeV with 90–140 mA of ring current. Ionization chambers in front and behind the sample were used to monitor the beam intensity. The Bragg reflections of a static Si(220) crystal in the monochromator beam window, which was not flushed with gas, was used to monitor the energy calibration of the copper spectra (37). Harmonic rejection was ensured by an energy cut-off of the focusing mirror and a monochromator detuning to 60% of the peak intensity. Dead time correction was applied.

For the first Cu(I)-Cox11 sample, only nine scans could be collected, whereas for the second sample, 19 scans were collected. The individual scans were summed and averaged before data reduction, which was performed with the EXPLORE software package (designed by H.-F. Nolting and C. Hermes, H. F. EMBL, Hamburg, Germany). The XAS spectra from the two samples are identical within the experimental noise, and their analysis provides the same results. The reported spectrum and data analysis refer to the second (19 scans) higher quality dataset, which shows a better signal/noise ratio. The full k$^2$-weighted EXAFS spectrum (30–850 eV above $E_0 = 8983.6$ eV) and its Fourier transform calculated over the range 3.0–15.0 Å$^{-1}$ were compared with theoretical simulations with program EXCURVE9.20 (38). The Fermi energy offset was refined at the beginning of the simulation and then kept fixed at ~8.6 eV. A fixed amplitude reduction factor of 0.9 was used to compensate for the signal amplitude reduction due to multiple excitations. Ligand types and coordination numbers (kept as integers) were varied manually while distances and Debye-Waller factors were refined by the software. The quality of the fit was assessed by the parameter $e^2$ (39) and by the R-factor as defined within EXCURVE9.20 (38).

**Data Deposition**—Assignment restraints and the derived atomic coordinates for a family of acceptable structures and for the average minimized structure with all of the restraints used in structure determination are available at the Protein Data Bank (codes 1S09 and 1SP0).
RESULTS

Genome Browsing and Expression of Cox11 from *S. meliloti*—A BLAST search over all of the non-redundant GenBank™ genomes for proteins sharing >10% sequence identity with the amino acid sequence of Cox11 from yeast *S. cerevisiae* located 49 protein sequences. All of the searched sequences belong only to Gram-negative bacterial or to eukaryotic organisms. The Cox11 sequences have quite a variable length, but for a common region, a high average residue identity of 36 ± 8% was found, indicating a highly conserved protein family. A possible metal binding motif CFCF (not used in the search) is conserved in all of the sequences (Supplemental Fig. 1). A third cysteine at the N terminus at position 43 proposed to be involved in copper(I) binding in yeast Cox11 (15) is conserved in all of the sequences with the exception of three (Supplemental Fig. 1). A Lys residue at position 131 close to the metal binding motif CFCF is also fully conserved (Supplemental Fig. 1). A hydrophathy analysis indicates that this class of proteins contains a single N-terminal trans-membrane-spanning helix, suggesting that they are membrane-bound proteins and that a water-soluble segment can be produced if the N-terminal trans-membrane segment is taken out. This soluble segment entirely comprises the highly conserved amino acids region previously mentioned.

Sequences from five different bacterial organisms were selected to express the protein. The organisms are as follows: *S. meliloti; R. sphaeroides; A. tumefaciens; C. crescentus; and P. aeruginosa*. Protein cloning was successfully performed on all of the selected protein sequences, yielding both C-terminal His tag fusion proteins and native untagged proteins. Expression of all of the His tag constructs yielded non-soluble His tag fusion proteins and native untagged proteins. Ex-

![Solution Structure of ApoCox11 from *S. meliloti*](http://www.jbc.org/)
One sheet is formed by strands $\beta a$, $\beta b$, $\beta e$, $\beta d$, and the other sheet is formed by strands $\beta a'$, $\beta a0$, $\beta f$, and $\beta e'$ (Fig. 1). In addition, strands $\beta c$ and $\beta d'$ form a short antiparallel $\beta$-sheet. The most disordered regions are those involving residues 96–105 and the N and C termini, which show random coil conformation as the chemical shift index values indicate. $^{13}$N $R_1$, $R_2$, and $^1$H$-^{15}$N NOE values can provide information on internal mobility as well as on the overall protein tumbling rate. From the analysis of 112 backbone NH signals, which are well resolved in the $^{1}$H$-^{15}$N apoCox11 spectra, average $R_1$, $R_2$, and $^1$H$-^{15}$N NOE values of 1.72 ± 0.06, 12.7 ± 0.5, and 0.60 ± 0.05 s$^{-1}$ are found, respectively, at 600 MHz. The experimental relaxation data (Supplemental Fig. 3) are essentially homogeneous along the entire polypeptide sequence with the exception of residues located at the C and N termini. The correlation time for the molecule tumbling ($\tau_m$) as estimated from the $R_2/R_1$ ratio is $8.8 \pm 0.6$ ns as expected for a protein of this size in a monomeric state (40, 41), thus confirming the results from the gel-filtration analysis. From the spectral density function analysis (Supplemental Fig. 4), it appears that the residues at the N and C termini experience local motions in the sub-ns timescale, i.e. faster than the overall protein tumbling rate. The occurrence of sub-ns motions for residues belonging to the N- and C-terminal regions can explain the paucity of NOEs in these regions, thus determining their high root mean square deviation values.

Interaction with Copper: XAS and NMR Characterization—To characterize copper(I) binding to S. meliloti Cox11, the purified apoprotein was treated with an excess of copper(I) chloride, and then the copZ sample was analyzed. The EXAFS spectrum of apoCox11 and Cu(I)-Cox11 show sizable chemical shift variations for residues 83–86, 96, and 106 (Supplemental Fig. 5), whereas the CCF metal-binding region still eludes detection, possibly because of line-broadening due to conformational exchange processes. A similar phenomenon was already observed for CopZ from Enterococcus hirae (42). It is important to notice that the observed chemical shift changes are localized in the $\beta$-strands spatially closest to the CCF copper-binding region. In addition, even in the presence of copper(I) ion, the N and C terminal are not rigidified as NH signals of residues 12–20 and 154–164 are not observed and no significant chemical shifts are observed for the closer residues. The $^{13}$N relaxation rates, measured on a Cu(I)-Cox11 sample containing 1 mM DTT at 500 MHz, provide average values for $R_1$ and $R_2$ of 1.47 ± 0.05 and 19.6 ± 0.8 s$^{-1}$, respectively. The $R_2$ values overall show a sizable increase with respect to apoCox11, whereas the $R_1$ values show a global decrease. The correlation time $\tau_m$ of Cu(I)-Cox11 is 14.7 ± 1.0 ns, consistent with a predominantly dimeric state of the copper(I)-loaded form. A similar correlation time was also obtained when DTT was removed from the buffer. The same behavior is observed in the gel-filtration analysis where the Cu(I)-Cox11 complex is predominantly found in a dimeric state as it also occurs for yeast Cox11 (15).

X-ray absorption spectroscopy on S. meliloti Cu(I)-Cox11 shows, in the K-edge region, the presence of an absorption at 8983.9 eV (Supplemental Fig. 6A). This band is assigned to a 1s → 4p transition in the copper(I) compounds (43), thus clearly confirming the copper oxidation state as Cu(I). The height of the 1s → 4p transition suggests a three- or four-coordination for Cu(I) (44, 45). The EXAFS and the Fourier transform spectra of S. meliloti Cu(I)-Cox11 (Supplemental Fig. 6, B and C) are well reproduced by the presence of three sulfur atoms at 2.23 Å in the first coordination shell of the Cu(I) ion (Supplemental Table IV, Fit 2). This distance is typical of 3-coordinated Cu(I) complexes (43, 44). Attempts to fit the copper first coordination shell with two or four sulfur atoms lead to unrealistic fits (Supplemental Table IV, Fits 1 and 3). The presence of a higher frequency component of the EXAFS spectrum is revealed by the distinct second shell peak present at −2.7 Å in the spectrum Fourier transform (Supplemental Fig. 6C). The peak height is defined above noise and can be well simulated by a second copper backscatterer located at 2.70–2.71 Å (Supplemental Table IV, Fit 4). On the contrary, a shell of 1–5 light atoms (C/N/O/S) was unable to reproduce that feature (for example see Supplemental Table IV, Fit 5). The position and the height of this peak do not change if the Fourier transform is performed over a different $k$-range, suggesting that this peak is a real component of the spectrum. The high Debye-Waller factor ($2\sigma^2 = 0.021$ Å$^2$) suggests the presence of static disorder in the Cu environment. Indeed, splitting the 3-S shell in two different distances resulted in a significant improvement of the fit when 1 S at a shorter distance and 2 S at a longer distance were used for the spectrum simulation, providing an excellent fit with 1 S at 2.19 Å, 2 S at 2.24 Å, and 1 Cu at 2.70 Å (Supplemental Table IV, Fits 6 and 7). The same Cu(I) coordination (i.e. 3 S at 2.24 Å and 1 Cu at 2.70 Å) has been observed in yeast Cox11 for which Cys residues are involved in copper binding (15). The distance of 2.7 Å is in accordance with Cu-Cu distances observed in doubly bridging sulfur complexes, which range from 2.7 to 3.0 Å, whereas the Cu-Cu distances in singly bridged sulfur clusters are around 3.3 Å (45, 46). Similar EXAFS data were also found for Cu(I)-CopZ samples, indicating a common copper binding motif (47).

All of the experimental data on Cu(I)-Cox11 from S. meliloti indicate that copper(I) is bound to three sulfur atoms of Cys ligands organized in a binuclear cluster at the interface of the dimerization side.

DISCUSSION

Genome browsing for Cox11 homologues individualizes a highly conserved protein family present exclusively in eukaryotes and in Gram-negative bacteria. The available functional data indicate that Cox11 is restricted to a specific function in the CcO complex assembly mechanism (14). Its structure determination represents an essential step to unravel its functional role. The overall structure of the soluble domain of apoCox11 reveals a compact well defined domain composed entirely of $\beta$ strands, either parallel or antiparallel. The 10 $\beta$-strands are connected by an extensive backbone hydrogen bond network, thus forming an almost cylindrical $\beta$-barrel. Some of the $\beta$-strands are separated by short loops containing in some cases highly conserved residues of “special” conformational properties such as Gly or Pro, which can impart a shift to the strand position. This property makes strands $\beta a$ and $\beta a'$, which are separated by a highly conserved proline (Pro-51), form hydrogen bonds with strands $\beta b$ and $\beta a0$, respectively. The highly disordered copper-binding region containing Cys-100 and Cys-102 as ligands is located between two short $\beta$ strands ($\beta d$ and $\beta d'$), which form two different antiparallel $\beta$-sheets with strands $\beta e$ and $\beta e'$, respectively.

The core of apoCox11 structure is characterized by several hydrophobic interactions, which involve buried aromatic and aliphatic residues located in 9 of the 10 $\beta$-strands. This hydrophobic core is highly conserved in all of the bacterial and eukaryotic Cox11 homologous proteins (Supplemental Fig. 1), whereas the N- and C-terminal segments are characterized by a large variability in length as well as the nature of the amino acid sequence.
acids with the exception of few residues close to the transmembrane helix. In accordance, the structural characterization shows that the N- and C-terminal regions are not able to make hydrophobic contacts to get structured. Consequently, the possible copper ligand Cys-9 is located in a completely unstructured part of apoCox11, whereas the other potential copper ligands located in the conserved motif CFCF are inserted in the β-barrel structure.

The side of S. meliloti Cox11 structure, which contains the putative copper binding motif CFCF is highly hydrophobic with the exception of the adjacent positive charge of the fully conserved Lys-97 (Fig. 2A). The proximity of Lys-97 can counter-balance the overall negative charge produced by cysteine ionization when binding of Cu(I) occurs. Indeed, in Cu(I)Atx1 from S. cerevisiae, the approach of a conserved positively charged residue, Lys-65, to the copper site on copper binding represents a structural rearrangement that optimizes electrostatic interactions (48). The surface of the human Cox11 model displays a negative patch close to the copper binding motif CFCF, which is produced by two Gh2 residues (Fig. 2B), highly conserved in only eukaryotic organisms (Supplemental Fig. 1). This different charged distribution between bacterial and eukaryotic organisms could suggest that the negative patch close to the copper-binding region is important in eukaryotes for the interaction of Cox11 with the proposed copper chaperone partner, Cox17, which is indeed not present in any bacteria. To support the latter hypothesis, it should be noticed that even the other proposed protein partner of Cox17, Sco1, contains two negative charged residues close to the copper(I) binding motif CXXCP, which are analogous to Cox11 sequences highly conserved in eukaryotes but not in bacteria.

**Structural homologies and Mechanistic Implications**—Within proteins adopting a fold similar to that of apoCox11, the closest matches are with immunoglobulin-like domains. In particular, the search identified two structurally related proteins, i.e. a linker domain of a bacterial sialidase (Protein Data Bank code 1eut) and a motile major sperm protein of Ascaris suum (Protein Data Bank code 1msp) (Fig. 3). Cox11 from S. meliloti shares with the two above-mentioned proteins only a 10 and 13% residue identity, respectively.

Ig-like domains are classified into five subtypes (C, V, I, S, and H) according to the number of strands and the H-bond interactions between them (49). Backbone hydrogen-bonding patterns in the Ig-like domains define two sheets organized in a β-sandwich-fold, rather than a closed barrel. All of the Ig-like domains have a common core of six β-strands: βa, βb, and βe in one sheet and βc, βf, and βg in the other (colored gray in Fig. 3).

However, apoCox11 protein, despite a closely related fold, shows different structural features, which lead us to classify it as the prototype for a new class of Ig-related domain. Indeed, by comparing apoCox11 with the two closest structurally Ig proteins (Fig. 3), it appears that apoCox11 at the N terminus has an additional β-strand, βa0, which forms a parallel β-sheet with βf. Strand βa0 replaces, but in an opposite direction, the “Ig” strand βg, which is present at the C terminus in all of the Ig-related domains, and always forms antiparallel β-sheet with strand βf. On its turn, strand βf is interacting with strand βc in all of the Ig structures, thus forming a completely conserved three-stranded β-sheet. On the contrary, in the apoCox11 structure, the Ig strand βc is divided by a short loop into strands βc and βc’ (Fig. 3). Therefore, in this new subtype of Ig-like domains, the Ig-conserved βc, βf, and βc’ sheet is now formed by strands βa0, βf, and βc’ (Fig. 3).

It is known that Ig-like fold occurs in functionally diverse proteins, but in most cases, the common denominator is a recognition role at the cell surface (50, 51). This family of molecules is of fundamental importance in the immune system and in the intracellular recognition (50). The Ig-related molecules commonly form dimers to carry out their function, forming either a homodimer or heterodimers (50). A specific class of IgS called CD8 are dimers linked by an intermolecular disulfide bond present after a C-terminal trans-membrane helix, which is then followed by the one or more Ig-soluble domains (52–54). Apparently, any accessible part of the Ig domain surface can be involved in interactions with other molecules (50, 51). In few examples, the Ig-fold family is involved in metal binding and the metal binding sites are essentially located in the loops at the edges of the β-sandwich (55). In the Cox11 structure, the copper binding motif is inserted differently in the β-sheet region. This peculiar structural feature might be the factor determining the different functions of Cox11 with respect to those of Ig-like proteins. Indeed, the function of Cox11 is related to Cu₉ site formation, possibly delivering copper directly to that site. In vitro, Cox11 is capable of binding copper(I) forming at the interface of the dimerization domain, a binuclear copper(I) cluster, as indicated by the EXAFS data of Cu(I)Cox11. The formation of the copper(I) cluster could occur transiently in the cell and could be a response to the need for copper to reach a more energetically stable coordination. Indeed, copper(I) prefers three-coordination geometry, and when endogenous ligands are not available, it saturates its coordination sphere by binding an exogenous ligand as glutathione or sharing a ligand with another protein molecule, thus producing a dimer. A reasonable model for the copper site of the dimeric state of
Cu(I)-Cox11 contains one cysteine residue (Cys-100 or Cys-102) from each monomer acting as a monodentate ligand to one copper and one cysteine (Cys-100 or Cys-102) acting as a bridging bidentate ligand to both copper ions (Fig. 4). Indeed, the observed Cu-Cu distance of 2.7 Å is typical of doubly bridging sulfur complexes (56, 57) and was found in other copper(I) chaperones with similar rearrangements (45, 58). The N-terminal Cys-9, which is located far from the CFCF binding site and in an unstructured region only five/four residues of the inner membrane, would not be involved in copper binding but rather could be involved in an intermolecular disulfide bond with the corresponding cysteine of the other monomeric unit (Fig. 4). Indeed, the same occurs in the structurally related Ig CD8-dimeric protein, which dimerizes through the S–S bond (53, 54). This intermolecular disulfide bond can be essential for the in vivo function, thus explaining the respiratory incompetence observed in the yeast Cys-111 → Ala variant (15).

It is relevant to mention that also the apoform in the absence of reductant is predominantly in a dimeric state. The similar tendency to form a dimer, observed for both eukaryotes (yeast) (15) and for bacteria (S. meliloti), suggests that this self-aggregation process is mainly determined by the fold properties and/or by the possibility of intermolecular S–S bond formation, more than from electrostatic interaction, which should be minimal for eukaryotic proteins characterized by extensive negative regions close to the metal binding motif. The tendency to dimerize apoCox11, typical of most Ig-fold proteins, might play

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**Fig. 3.** Comparison between the structures and the two-dimensional topology of apoCox11 (A), a linker domain of a bacterial sialidase (Protein Data Bank code 1eut) (B), and a motile major sperm protein of A. suum (Protein Data Bank code 1msp) (C). The side chains of the Cys-100 and Cys-102 are indicated. The dashed lines indicate sheet formation between two β-strands. β-Strands a, b, c, e, f, and g (colored gray) are common to all of the Ig-like domains.

**Fig. 4.** Model for the Cu(I)Cox11-dimeric form. The binuclear copper(I) cluster involving the CFCF copper binding motif and the proposed disulfide bond involving Cys-9 are shown. IMS, intermembrane space.
a role in the process of copper binding, i.e. taking two protein molecules close each other to receive copper ion.

The flexibility of copper(I) coordination is known to be relevant for the copper transfer process (59–61). Assuming that Cox11 directly transfers the metal to COX I subunit, the copper transfer could occur in a heterodimeric complex where two different metal environment are present: 1) all-sulfur ligands in Cox11 and 2) all-nitrogen ligands in COX I. Analogously, this occurs in the copper transfer between the dimeric copper chaperone copper chaperone for superoxide dismutase containing a copper(I) binuclear cluster similar to Cu(I)Cox11 (45) and its protein target, the dimeric superoxide dismutase, where four His residues coordinate the copper ion (62). Therefore, it might be suggested that the dimeric state of Cox11 may stabilize copper binding, protecting the copper until transfer to COX I occurs. It was proposed that mitochondrial ribosomal proteins could be involved in signaling to Cox11 the incoming translation of Cox1(I), and concomitantly to the latter process, Cox11 may disrupt the dimer to form a heterodimer with Cox1, thus inserting the copper ion in the Ub site.

All of these considerations indicate a reasonable functional role of a dimeric Cu(I)Cox11 intermediate in this intriguing mechanism of copper delivery to the membrane protein CeCo, which is generally assumed to be a dimeric complex in vivo (63, 64).

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