Structural Basis for the Function of the N-terminal Domain of the ATPase CopA from Bacillus subtilis

The solution structure of the N-terminal region (151 amino acids) of a copper ATPase, CopA, from Bacillus subtilis, is reported here. It consists of two domains, CopAa and CopAb, linked by two amino acids. It is found that the two domains, which had already been separately characterized, interact one to the other through a hydrogen bond network and a few hydrophobic interactions, forming a single rigid body. The two metal binding sites are far from one another, and the short link between the domains prevents them from interacting. This and the surface electrostatic potential suggest that each domain receives copper from the copper chaperone, CopZ, independently and transfers it to the membrane binding site of CopA. The affinity constants of silver(I) and copper(I) are similar for the two sites as monitored by NMR. Because the present construct “domain-short link-domain” is shared also by the last two domains of the eukaryotic copper ATPases and several residues at the interface between the two domains are conserved, the conclusions of the present study have general validity for the understanding of the function of copper ATPases.

The mechanisms regulating the homeostasis of essential metal ions in living organisms has been the subject of many investigations in the recent past (1–5). Attention was particularly directed toward the reactive and potentially toxic copper, and specific copper uptake and intracellular copper trafficking pathways have been identified (6–11). A critical element of this fascinating machinery for copper homeostasis is represented by the P-type copper-transporting ATPases (12–16). A peculiar feature of this kind of ATPases is that the cytoplasmic N terminus contains one or more domains with a M

Crystallographic structure, formed by two 

/ β-helices and four 

β-strands arranged in a classical “ferredoxin-like” βαββββ-fold. The two cysteines of the CXXC motif are exposed on the surface of the protein readily accessible for binding the metal ion. Moreover, cytoplasmic copper chaperones such as Hah1 in humans, Atx1 in Saccharomyces cerevisiae, and CopZ in Bacillus subtilis and Enterococcus hirae bacteria, which deliver copper(I) to ATPases, share the same MXCXXC metal binding motif and the same βαββββ-fold (21–24). In addition, interaction between the N-terminal region of the copper ATPase Ccc2 from S. cerevisiae and Atx1 as well as of the bacterial homologues, the copper ATPase CopA and the chaperone CopZ from B. subtilis, was demonstrated by yeast and bacterial two-hybrid assays, respectively (25–27). Complex formation of either Atx1 or CopZ with one N-terminal domain of either Ccc2 or CopA was also confirmed and characterized by in vitro NMR studies (28, 29).

The functional role of the multiple domains needs further investigation. Several studies are available on the human ATPases, called also Menkes and Wilson proteins (30–32) because their genetic alteration is responsible of these disease, but none of all these studies completely addressed the role and the inter-correlation of the various domains in the process of copper binding.

To gain further information on this machinery we have, therefore, determined the solution structure of the N-terminal region of the copper ATPase CopA from B. subtilis, which contains two soluble metal binding domains. The solution structures of the two isolated domains are available (19, 33). In the present study a protein segment of 151 amino acids, which contains the 2 metal binding domains and 4 other amino acids at the C terminus (CopAab hereafter), was expressed. A mutation in position 46 (S46V) was introduced to have a folded protein construct (33), and the solution structure was solved in its apo form. The interaction with copper(I) and silver(I) has been also investigated. The structural features found here for this system most likely hold for all two domain pumps and for the two domains closest to the membrane when the domains are more than two.

**EXPERIMENTAL PROCEDURES**

**Cloning and Purification**—The plasmid for the expression of the N-terminal region of CopA from B. subtilis was prepared as previously described (19). The single amino acid substitution was introduced using the QuikChange™ site-directed mutagenesis kit from Stratagene. Sequencing of the engineered DNA fragments was achieved using an automatic sequencer ABI 377. The expression and purification of the protein were performed as previously described (19). The 

15N-labeled proteins were obtained by growing the cells in the labeled Silantes media Escherichia coli OD2-CN and OD2-N (Silantes GmbH), respectively.

**Sample Preparation**—As a precaution against possible disulfide for-
Solution Structure of the N-terminal Region of CopA

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Table I

Acquisition parameters for NMR experiments performed on apoCopAa from B. subtilis

| Experiments | t₁ | t₂ | t₃ | F₁ | F₂ | F₃ | F        | n° | Reference |
|-------------|----|----|----|----|----|----|-----------|----|-----------|
| [1H, 1H] NOESY | 1024(1H) 2048(1H) | 15 15 | 64 57 |
| [1H, 1H] TOCSY | 1024(1H) 2048(1H) | 15 15 | 64 58 |
| 1H, 15N HSQC | 512(1H) 1024(15N) | 40 15 | 16 59 |
| 1H, 13C HSQC | 256(13C) 2048(1H) | 70 14 | 16 59 |
| CBCA(CO)NH | 136(13C) 56(15N) | 2048(1H) | 88 40 | 12 16 | 60 |
| CBCANH⁴ | 136(13C) 56(15N) | 2048(1H) | 88 40 | 12 16 | 60 |
| HNCO | 80(13C) 40(15N) | 1024(1H) | 16 40 | 12 8 | 60 |
| HN(CO)C⁴ | 80(13C) 40(15N) | 1024(1H) | 16 40 | 12 16 | 60 |
| (HCCH) TOCSY | 272(13C) 96(15N) | 1024(1H) | 88 88 | 12 4 | 61 |
| CC(CO)NH TOCSY⁵ | 128(13C) 48(15N) | 1024(1H) | 88 40 | 12 16 | 62 |
| 15N-Edited [1H, 1H] NOESY | 368(1H) 64(15N) | 1024(1H) | 15 40 | 15 16 | 63 |
| 13C-Edited [1H, 1H] NOESY | 320(1H) 80(15N) | 1024(1H) | 15 40 | 15 8 | 63 |
| HNHA⁶ | 256(1H) 40(15N) | 1024(1H) | 15 40 | 15 16 | 35 |
| HNHB⁶ | 128(1H) 32(15N) | 1024(1H) | 15 40 | 15 32 | 45 |

¹ Number of acquired scans.
² Data acquired on a 800- or 600-MHz spectrometers.
³ Data acquired on a 700-MHz spectrometer.
⁴ Data acquired on a 500-MHz spectrometer equipped with cryoprobe.

The structures were evaluated using the programs PROCHECK-NMR (40) and AQUA (40). The figures were generated with the program MOLMOL (41).

Relaxation Measurements and Analysis—Relaxation experiments were collected at 298 K on a 1.5 mM sample on a Bruker Avance 600 spectrometer operating at proton nominal frequencies of 600.13 MHz. ¹N R₁ and R₂ and steady-state heteronuclear NOEs were measured with the gradient-enhanced, sensitivity-enhanced pulse sequences as described by Farrow et al. (42). R₁ were measured using refocusing times (t₁) of 450 μs in the Carr-Purcell-Meiboom-Gill (CPMG) detection scheme. All experiments use the “water flipback” scheme to suppress the water signal without its saturation. A recycle delay of 3 s was used for R₁ and R₂. The steady state heteronuclear ¹H, ¹3N NOE was obtained by recording spectra with and without proton saturation. In the case of reference spectra without proton saturation, a relaxation delay of 6 s was employed, whereas a delay of 3 s before the proton saturation was employed for spectra with proton saturation. The latter was achieved with a train of 120 ¹H pulses at 20-ms intervals.

1024 × 256 data points were collected for each map using 8 scans for each experiment. A spectral window of 40 ppm in the F₁ (¹N frequency) dimension and of 16 ppm in the F₂ (¹H frequency) dimension were used. Quadrature detection in F₁ was obtained by using the TFFP method. Integration of cross-peaks for all spectra was performed by using the standard routine of the XWINNMR program.

Relaxation rates R₁ and R₂ were determined by fitting the cross-peak intensities measured as a function of the delay within the pulse sequence to a single exponential decay. Errors in the rates were estimated through a Monte Carlo approach. Heteronuclear ¹H, ¹5N NOE values were calculated as the ratio of peak volumes in spectra recorded with and without ¹H saturation. The heteronuclear ¹H, ¹5N NOE values and their errors were estimated by calculating the mean ratio and the S.E. from the available data sets.

The abbreviations used are: TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect (NOE) spectroscopy; HSQC, heteronuclear single quantum coherence; REM, restrained energy minimization; r.m.s.d., root mean square deviation.

The NMR samples of the apo protein were in 20 mM sodium phosphate buffer, pH 7, 90% H₂O, 10% D₂O. The final protein concentration ranges between 1 and 1.5 mM in the presence of 2 mM dithiothreitol.

The NMR samples were prepared in a Vac atmosphere nitrogen chamber at room temperature. Protein concentrations were determined using a calculated extinction coefficient of 5360 M⁻¹ cm⁻¹. Circular dichroic spectra were collected on a Jasco J-810 spectropolarimeter with a fused quartz cuvettes with a 1-cm path length (Merck).

The NMR experiments recorded on 13C,15N- and 15N-enriched and unlabeled samples are summarized in Table I. All three-dimensional and two-dimensional spectra were collected at 298 K, processed using the standard Bruker software (XWINNMR), and analyzed through the XEASY program (34).

The backbone assignment was performed using the three-dimensional spectra CBCANH, CBCA(CO)NH, HN(CA)CO, and HN(CA)CO (CA and CB represent carbon alpha and carbon beta, respectively). The assignment of the aliphatic side chain resonances was performed through the analysis of three-dimensional CC(CO)NH and (HCCH) TOCSY spectra together with 15N-edited NOESY-HSQC and 13C-edited NOESY-HSQC spectra. Distance constraints for structure determination were obtained from 15N-edited and 13C-edited three-dimensional NOESY-HSQC experiments and from two-dimensional NOESY (see Table I). 3JHNH coupling constants, determined through the HNHA experiment, were transformed into backbone dihedral φ angles through the Karplus equation (35). Backbone dihedral φ angles for residue (i-1) were also determined from the ratio of the intensities of the dₙᵢ₋₁(13C) and dₙᵢ₋₁(15N) NOEs obtained from the 15N-edited NOESY-HSQC spectrum. The elements of secondary structure were determined on the basis of the chemical shift index (36), of the 3JHNH coupling constants, and of the backbone NOEs.

An automated CANDID (37) approach combined with the fast DYANA torsion angle dynamics algorithm 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 (38) followed by restrained energy minimization (REM) with AMBER 5.0 (39) applied to each member of the family. The quality of
Contributions to the relaxation data of those NHs that have exchange by monitoring the 1H,15N HSQC spectral changes upon the addition of tubes. The starting 15N-labeled protein samples were 1.0 or 0.5 mM in delivering small amounts of metal solution to the labeled samples in NMR NOESY15N HSQC experiments were recorded on silver(I) samples with checked through atomic absorption measurements. Three-dimensional resonances are missing for residues Met-1, Leu-2, Ala-18, and producing low 1H,15N NOE values.

The metal-bound content was 15N-labeled apo protein with copper(I), added as [(CH3CN)4Cu]PF6, or silver(I), added as AgNO3, both solved in acetonitrile, were performed concentration. The additions range from 0.2 or 0.1 mM to 3.0 or 2.0 mM concentration. 147 of the expected 151 15N back-resonances (Table I).

CopAab construct is structurally characterized in the demetalized form. In vivo this construct is followed by eight transmembrane helices and by the ATP binding and the Actuator domains (30). CopAab construct contains 2 domains with 40% identity and Cys copper binding motifs separated by 68 amino acids. In vitro this construct is followed by eighth transmembrane helices and by the ATP binding and the Actuator domains (30). CopAab construct is structurally characterized in the demetalized form.

NMR Structure of ApoCopAab—The resonance assignment was accomplished by the analysis of triple and double resonance experiments (Table I). 147 of the expected 151 15N backbone amide resonances were observed and assigned. The amide resonances are missing for residues Met-1, Leu-2, Ala-18, and Ala-86, as they were not determined in the separated domains (19, 33). In total the resonances of 92% of carbon atoms, 96% of nitrogen atoms, and 95% of protons were assigned. These values compare well with those found for the separated domains (19, 33). The 1H, 13C, and 15N resonance assignments of apoCopAab from B. subtilis (19, 33). In total the resonances of 92% of carbon atoms, 96% of nitrogen atoms, and 95% of protons were assigned. These values compare well with those found for the separated domains (19, 33). The 1H, 13C, and 15N resonance assignments of apoCopAab from B. subtilis (19, 33). In total the resonances of 92% of carbon atoms, 96% of nitrogen atoms, and 95% of protons were assigned. These values compare well with those found for the separated domains (19, 33). The 1H, 13C, and 15N resonance assignments of apoCopAab from B. subtilis (19, 33). In total the resonances of 92% of carbon atoms, 96% of nitrogen atoms, and 95% of protons were assigned. These values compare well with those found for the separated domains (19, 33).

The atomic coordinates and structural constraints have been deposited in the Protein Data Bank (accession code 1P6T).

RESULTS

CopAab construct contains 2 domains with 40% identity and 2 MXYCXXC copper binding motifs separated by 68 amino acids. In vivo this construct is followed by eight transmembrane helices and by the ATP binding and the Actuator domains (30). CopAab construct is structurally characterized in the demetalized form.

NMR Titration of the Apo Protein with Cu(I) and Ag(I)—Titration of 15N-labeled apo protein with copper(I), added as [(CH3CN)4Cu]PF6, or silver(I), added as AgNO3, both solved in acetonitrile, were performed by monitoring the 1H, 15N HSQC spectral changes upon the addition of increasing amounts of metal ions. Aliquots were added in a Coya chamber under a nitrogen atmosphere at 298 K using a Hamilton syringe to deliver small amounts of metal solution to the labeled samples in NMR tubes. The starting 15N-labeled protein samples were 1.0 or 0.5 mM in concentration. The additions range from 0.2 or 0.1 mM to 3.0 or 2.0 mM in Cu(I), and from 0.2 to 1.6 mM in Ag(I). The metal-bound content was checked through atomic absorption measurements. Three-dimensional NOESY 15N HSQC experiments were recorded on silver(I) samples with protein/metal concentration ratios of 3:1.

Coordinates—The atomic coordinates and structural constraints have been deposited in the Protein Data Bank (accession code 1P6T).

Ala-86, as they were not determined in the separated domains (19, 33). In total the resonances of 92% of carbon atoms, 96% of nitrogen atoms, and 95% of protons were assigned. These values compare well with those found for the separated domains (19, 33). The 1H, 13C, and 15N resonance assignments of apoCopAab can be found as Supplementary Table 1.

Chemical shift index analysis (36) on H

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### Table I

| Parameter                          | REM (30 structures) | <REM> |
|------------------------------------|---------------------|-------|
| Statistical analysis**             |                     |       |
| % of residues in most favorable regions | 75.1                | 78.3  |
| % of residues in allowed regions   | 22.2                | 17.8  |
| % of residues in generously allowed regions | 2.5                | 3.1   |
| % of residues in disallowed regions | 1.1                | 0.8   |
| H-bond energy (kJ mol\(^{-1}\))    | 2.93 ± 0.05         | 2.92  |
| Overall G-factor                   | −0.25 ± 0.02        | −0.24 |
| Experimental restraints analysis†  |                     |       |
| % completeness of experimentally observed NOE up to 4 Å cut-off distance | 70.5% | 66.2% |
| % completeness of experimentally observed NOE up to 5 Å cut-off distance | 49.8% | 46.4% |

** The number of experimental constraints for each class is reported in parenthesis.

† Medium range distance constraints are those between residues (i,i + 2), (i,i + 3), (i,i + 4), and (i,i + 5).

‡ As it results from the Ramachandran plot analysis. For the PROCHECK statistics, an average hydrogen bond energy in the range of 2.5–4.0 kJ mol\(^{-1}\) and an overall G-factor larger than −0.5 are expected for a good quality structure.

‡‡ As it results from AQUA analysis.

An estimate of the overall tumbling correlation time and the local correlation times for the NH vector of each residue were derived from the measured R\(_2\)/R\(_1\) ratios. In this analysis care was taken to remove from the input the relaxation data of those NHs that have exchange contributions to the R\(_2\) value or which are exhibiting internal motions producing low 1H,15N NOE values.

NMR Structure of ApoCopAab—The resonance assignment was accomplished by the analysis of triple and double resonance experiments (Table I). 147 of the expected 151 15N backbone amide resonances were observed and assigned. The amide resonances are missing for residues Met-1, Leu-2, Ala-18, and Ala-86, as they were not determined in the separated domains (19, 33). In total the resonances of 92% of carbon atoms, 96% of nitrogen atoms, and 95% of protons were assigned. These values compare well with those found for the separated domains (19, 33). The 1H, 13C, and 15N resonance assignments of apoCopAab can be found as Supplementary Table 1.

Chemical shift index analysis (36) on Hα, CO, Cα, and Cβ resonances, the 3J\(_\text{HNH}\) coupling constants, the 3J\(_\text{HNHa}\) / 3J\(_\text{HNHb}\) ratios (43), and the NOEs patterns indicated the presence of eight β strands and four α helices, ordered in two β-strands and four α-strands in white.

![Fig. 2. 30 lowest energy structures of apoCopAab (residues 3-144) from B. subtilis, shown as a tube with a radius proportional to the backbone r.m.s.d. value of each residue. 3α-helix and α-helices are in black, and β-strands are in white.](image-url)
The interacting residues are shown in red. The cysteine ligands are shown in yellow. Backbone amide resonances experiencing chemical shift differences between the separated domains and the complete N-terminal region are mapped in green on the backbone of the structure. Hydrogen bonds at the domain-domain interface of apoCopAab are represented as black dashed lines.

The two domains are closely packed to form a single rigid molecule. There are two interacting regions between the two domains, one involving residues 11–12 and 72 with residues 119–122 and the other involving residues 9–10, 57, and 61 with residues 103–104 (Fig. 3). The interdomain interaction surface is experimentally well determined by 22 interdomain NOE values. The final structure indicates an H-bond network at the surface is experimentally determined by 22 interdomain NOE values. The final structure indicates an H-bond network at the solvent accessible surface (Fig. 3). The solvent accessibility of the residues at the domain interface shows a dramatic decrease when passing from the isolated domains to the entire construct.

Dynamic Properties of ApoCopAab—Backbone mobility in the subnanosecond time range is homogeneous along the entire polypeptide sequence as it results from 15N relaxation properties determined through 15N, R1, and 1H,15N NOE measurements (data are reported in Supplementary Fig. 1). Only eight residues (96–98 and 125–127) have R1 values larger than the average, thus suggesting conformational exchange processes on the ms–μs time scale. Moreover, the C- and N-terminal protein segments display negative NOEs values that suggest a flexibility in the subnanosecond time scale. The average values of R1, R2, and 1H,15N NOE, excluding the N- and C-terminal stretches and, as far as R2 is concerned, the above 8 residues, are 1.14 ± 0.19 s⁻¹, 13.04 ± 1.98 s⁻¹, and 0.80 ± 0.11, respectively. The small range of these values indicate a homogeneous dynamic behavior of the molecule in the subnanosecond time scale. From the R1/R2 ratio the overall tumbling correlation time (τc) was calculated to be 11.3 ± 1.1 ns. This value is consistent with the molecular mass of this protein according to the Stokes-Einstein relation (44), and it is almost twice that found for the two isolated domains (29, 33). This result indicates that the two domains are rigidly held one with respect to the other.

Interaction of ApoCopAab with Metal Ions—The interaction of apoCopAab with copper(I) and silver(I) ions was studied through NMR titrations by monitoring chemical shift variations of the amide resonances in 1H,15N HSQC experiments. Initial additions of copper(I) (up to a copper/protein ratio equal to 0.5) to a 1.0 mM solution of apoCopAab produce a significant line broadening, in some cases beyond detection, of some amide resonances close to each metal binding site (i.e. Thr-16, Thr-84, Ala-21, Ala-89, and the Cys ligands), whereas the majority of amide resonances remains unperturbed. Further additions of copper(I) up to copper/protein ratio equal to 1.0 (i.e. 0.5 eq/ metal binding site) cause the appearance of new NH peaks (Thr-16, Thr-84, Ala-21, and Ala-89) with chemical shift values
very close to those observed in the Cu(I) form of each isolated domain (Fig. 4). This might indicate that the two domains bind copper(I) with a similar affinity. Furthermore, it appears that Cu(I) binding to both metal binding sites is occurring with slow/intermediate exchange rates on the NMR time scale. Further additions of copper(I) (up to a copper/protein ratio equal to 3.0, i.e. 1.5 eq/per metal binding site) determine the disappearance/broadening of most of NH signals in the 1H,15N HSQC spectra (Fig. 4). These effects exclusively originate from copper binding and are reversible; when copper is removed with a very high affinity copper(I) ligand such as bathocuproine disulfonate, the spectra of the apo form are completely recovered. When the titration with copper(I) is performed using half-concentration of protein with respect to the first titration, the resonances in the 1H,15N HSQC spectrum of CopAab with a copper/protein ratio equal to 3.0 are largely conserved even if with weaker intensity with respect to the spectra of the apo form. Therefore, the line broadening of the backbone NH resonances is not only copper-dependent, but also protein concentration-dependent. Line broadening up to the complete disappearance of the

Fig. 4. 1H,15N HSQC spectra (700 MHz, 298 K) of apoCopAab in the presence of 0, 0.5, and 1.5 eq/metal binding site of Cu(I) and Ag(I). The initial protein concentrations are 1 and 0.5 mM for Cu(I) and Ag(I) titrations, respectively.
15NH signals can be due to molecular aggregation induced by the coordination properties of copper(I), which prefers a three/four-coordination state. A similar phenomenon was observed in the case of Cu(I)CopZ from *E. hirae* (24). Because aggregation does not occur within separated domains upon copper binding, the interaction probably occurs between different domains.

A further titration was performed using Ag(I), which adapts a similar coordination state and for this reason is sometimes used as a substitute of copper(I) (20). The 1H,15N HSQC spectra performed during the titration show that NH resonances close to both metal binding sites, with characteristic shifts of the apo form, disappear with the concomitant formation of new peaks, which are due to the silver(I)-bound form (Fig. 4). The latter form is characterized by chemical shifts very similar to those found for the copper(I)-bound form of the two isolated domains. No significant line broadening occurs (Fig. 4). The analysis of the 1H,15N HSQC together with a three-dimensional NOESY-HSQC experiment shows that the chemical shifts of the backbone NHs of all residues belonging to the interdomain interaction region are not strongly affected by the presence of silver(I) (Fig. 4), suggesting that the binding of the silver(I) does not determine significant relative structural rearrangements of the two domains.

During the titration of the apo protein with silver(I), the variation in the normalized intensities of the signal of Ala-21 of the first domain and of Ala-89 of the second domain were followed with respect to [Ag(I)]. At a 1:1 [Ag(I)]/[CopAab] ratio, the first domain is metalized for about 60%, whereas the second domain is metalized for about 40%. The metal binding site of the first domain results completely saturated at a 2:1 [Ag(I)]/[CopAab] ratio, whereas the other domain required a slight excess of silver(I) to obtain a fully metalized state. This behavior provides evidence that the first domain has an affinity constant \( K_{\text{A}} \approx 10^4 \text{ M}^{-1} \), whereas the second has a slightly lower affinity.

**DISCUSSION**

A comparison between the structure of apoCopAab with that of the two separated domains shows an essentially identical overall fold and length of secondary structure elements, with global backbone r.m.s.d. values with the first and second isolated domains of 1.2 and 1.5 Å, respectively. All the secondary structure elements and loops are well superimposed. However, the apoCopAab structure experiences meaningful differences with respect to the isolated apoCopAa and apoCopAb structures, specifically in the side chain conformation of some resi-
dyes at the interdomain interface, Met-10, Gln-11, Gln-61, Asn-119, and Glu-122 (Fig. 5). From the isolated domains to the double domain construct Met-10 moves from the interior of the protein core toward the interaction surface in the vicinity of Ala-103, and Gln-61 moves to approach the side chain of Asn-104 to form a H-bond with it. The side chains of the other residues display structural rearrangements to favor the domain-domain interactions (Fig. 5).

Gln-11 forms hydrogen bonds with Asn-119 and Glu-122, which connect the two domains. Sequence analysis of the fifth and sixth domains of all mammal copper transporting ATPases indicates that these two domains are always connected by a short stretch formed by only a few amino acids and that a Gln residue is always conserved in the sequence position corresponding to Gln-11 for the fifth domain, and Asp and Glu residues are always conserved in place of Asn-119 and Glu-122 for the sixth domain (17, 19). Therefore, it may be suggested that a hydrogen bond network, which connects the fifth and sixth domains, is conserved also in the superior organisms in humans and constitutes a key structural element for the domain interactions.

The solution structure of apoCopAab as well as its dynamic properties show that the two domains are closely packed to form a single rigid molecule, with both metal binding sites easily accessible. Therefore, considering that a functional relation between CopA and CopZ from B. subtilis has been recently detected in vitro (27) and protein-protein interaction has been characterized in vitro (29), it is reasonable to conclude that the protein partner CopZ can release copper(I) to both metal binding sites independently. The partner protein-protein interaction was shown to occur between the negatively charged surface of CopZ and the positive surface of the second domain of CopA (29). Both domains of apoCopAab have an accessible positively charged region (Fig. 6); thus, both have the possibility of interacting with the partner CopZ.

Interaction with Metal Ions and Related Biological Implications—The copper-protein stoichiometry of the Wilson and the Menkes copper binding N-terminal domains was extensively characterized (46–48). A recent paper (49) reports that each domain is capable to bind copper or silver but that the array of six tethered domains binds four equivalents of Cu(I) to produce a monomer containing four Cu(I) ions in a solvent-stabilized environment. According to the authors (49), this suggests that a tertiary structural reorganization of the domains makes interdomain copper(I) interactions possible. These conformational changes may act in vivo as a trigger for the translocation of the protein from the trans-Golgi network to the plasma membrane or to a vacuolar compartment (50, 51). Furthermore, the N-terminal region of the Wilson protein is shown to interact with the ATP binding domain (52). In the copper-bound form such interaction is weakened, determining an increase in affinity of ATP for the ATP binding domain (52), thus triggering the copper translocation process. A similar functional role for the N-terminal domains has been also proposed for other ATPases (53, 54).

The present studies and the interaction of CopAab with copper(I) and silver(I) together with the solution structure of the apo form and its dynamic properties suggest that the two domains, which both have positively charged, solvent-exposed patches, are organized to receive copper independently one from the other from its copper chaperone partner, CopZ, to start the metal transfer process. Our data fit within a model proposed for the function of the Wilson protein in vivo (51, 55), where the binding of copper to the domains closest to the transmembrane segments is the first step of the copper transport cycle, sufficient for the basic activity and for the delivery of copper to the copper oxidase target. When the copper concentration increases, other metal binding sites become occupied, thus inducing conformational changes that determine the translocation process of the Wilson protein from the trans-Golgi network to the plasma membrane. This model could also explain the reason why eukaryotic organisms have developed a higher number of domains that are needed to activate the protein translocation process, whereas the bacterial ATPases have only one or two domains since they do not have internal organelle, and therefore, translocation processes are not required. It has been also observed that, in copper(I) ATPases with multiple copper binding domains, those closest to the transmembrane region appear to be functionally more important than those closest to the N terminus for the copper transport function (50, 56). Our data show that the two domains close to the transmembrane region do not interact with each other and that there are no significant conformational changes upon metal binding. They presumably transfer copper to residues within the channel for subsequent translocation across the membrane without playing an important role in the protein translocation process.

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Solution Structure of the N-terminal Region of CopA

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