

Interaction of the two soluble metal-binding domains of yeast Ccc2 with copper(I)–Atx1

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Abstract

Yeast Ccc2 is a P-type ATPase responsible for transport of copper(I) from the cytosol to the trans-Golgi network. It possesses a soluble cytosolic N-terminal region containing two copper(I)-binding domains. Homologous eukaryotic copper-transporting ATPases have from one to six domains. We have expressed a fragment encompassing residues 1–150 of Ccc2, which corresponds to the two domains, and found that the second domain was substantially less structured than the first. The first domain could bind copper(I) and interact with the partner protein Atx1 at variance with the second. Similar results are found in ATPases from other organisms and may represent a general feature, whose biochemical implications are not yet fully appreciated.

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Copper, an essential trace metal, is utilized as a cofactor in a variety of proteins, which, in eukaryotes, are found in various cellular locations. However, copper can be potentially toxic *in vivo* and thus its intracellular concentration is presumably strictly controlled [1,2]. In humans, the impairment of the ability to control copper levels leads to illness, such as in the Menkes or Wilson diseases [3]. The latter are caused by mutations, respectively, in ATP7A [4] and ATP7B [5], two P-type ATPases which remove copper(I) from the cytosol and pump it in the trans-Golgi network (TGN) (when copper levels are low) or, when copper levels are high, in vesicles or across the plasma membrane, following a relocalization of the polypeptide [6]. Ccc2 is a yeast homologue of the above-mentioned human copper(I)-transporting ATPases [7,8]. Yeast has been intensely exploited as a model organism for the understanding of

copper(I) homeostasis in eukaryotes. At variance with ATP7A and ATP7B, Ccc2 does not traffic from the TGN to the plasma membrane, but is exclusively localized at the TGN membrane and thus only serves to transport copper from the cytosol into this organelle where the metal is incorporated in cuproenzymes. Ccc2 can receive the copper(I) ions to be pumped across the membrane from a soluble metallochaperone, Atx1 (whose human counterpart is called HAH1 or Atox1) [1,9]. Related copper homeostasis mechanisms have been identified also in several prokaryotic organisms [10].

More in detail, Ccc2 is a multi-domain membrane protein, comprising a cytoplasmic region containing two distinct soluble metal-binding domains, which are quite similar in sequence to one another and are predicted to have the same fold ($\beta\alpha\beta\beta\alpha\beta$ ferredoxin fold) as the partner protein Atx1. In both domains of Ccc2, as well as in Atx1, copper(I) is bound by two cysteines in a well conserved consensus sequence (MTCXXC) located in a loop region [11,12]. Various biochemical assays indicate that each of the two soluble metal-binding domains can interact with

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Atx1 [13,14]. The structure of a copper-bridged adduct between the first metal-binding domain of Ccc2 (Ccc2a hereafter) and Atx1 has been solved using a combination of advanced NMR techniques and site-directed mutagenesis [15].

A long-standing open question is the role of the multiple cytosolic metal-binding domains in copper-transporting ATPases. Homologous ATPases even from phylogenetically close organisms display a variable number of such domains, with different fold stability and different capability of binding copper [10]. The simplest systems (found in prokaryotes) contain only one such domain. Yeast, as mentioned, contains two domains. The honey bee, the fruit fly and the *Caenorhabditis elegans* worm have four domains. Humans and several other mammals instead contain as many as six domains, whereas the rhesus macaque (*Macaca mulatta*) only has five. Notably, other metal-transporting P-type ATPases such as the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) or the Na⁺/K⁺ ATPase do not possess any cytoplasmic metal-binding domain [16]. Various experiments based on the rescue of yeast variants where the *ccc2* gene had been suppressed have shown that truncated variants of human ATP7A or ATP7B containing only two or even one soluble domain allow $\Delta ccc2$ yeast to grow on an iron- and copper-limited medium at levels close to that of wild-type yeast [17–19]. Another noteworthy feature is that in the ATPases containing multiple soluble metal-binding domains, the two closest in sequence to the transmembrane segments are most often closely spaced in sequence (4 to less than 10 amino acids), whereas the other domains, if any, are widely spaced in sequence, with the linker stretches spanning up to several dozen amino acids. The two domains closest to the C-terminus of the cytosolic copper(I)-binding region thus form a relatively compact unit, which is only loosely coupled, from a structural point of view, to the remainder of the tail. The organization of these two domains in the ATP7B protein prevents transfer of copper(I) from one domain to the other [20]. Based on these observations, it has been proposed that in the proteins containing more than two domains, the cellular role of the additional domains is that of regulating protein trafficking between the TGN and the plasma membrane [18,21–23]. To obtain further insight into these complex systems, we have undertaken an NMR investigation of the entire two-domain cytoplasmic region of Ccc2 and of its interaction with copper(I) and with copper(I)–Atx1.

Materials and methods

Protein preparation. A DNA segment corresponding to residues 1–150 of Ccc2 (Ccc2ab hereafter) was amplified by PCR and cloned into pET 20b(+) between the NcoI and XhoI restriction sites. Ccc2ab has been expressed in *Escherichia coli* BL21(DE 3) in minimal medium cultures. The expressed protein additionally contains a His₆-tag at C-terminal end. The protein localized to inclusion bodies, from which it was solubilized in 8 M urea, and purified by affinity chromatography based on the use of the His₆-tag, eluting the protein with buffer D (20 mM imidazole, 20 mM

phosphate, pH 8). Protein refolding was carried out by gradual buffer exchange in a dialysis cylinder using buffers with decreasing concentration of urea. For isotope enrichment, (1⁵NH₄)₂SO₄ and ¹³C-glucose were used. Samples of yeast Atx1 were prepared as previously reported [24].

All protein samples contained between 0.2 and 0.5 mM protein in 100 mM phosphate at pH 7.0. These were the same conditions used in previous studies [23,25], thereby enabling direct comparison of the chemical shifts and relaxation rates. Protein metallation was carried by adding copper(I) to samples containing 1.0 mM di-thiothreitol (DTT) in an inert atmosphere chamber (Coy Lab); the copper source was an acetonitrile solution of tetrakis(acetonitrile)copper(I) hexafluorophosphate. In titration experiments, Atx1 was added to Ccc2ab directly in the NMR tube under N₂ atmosphere.

NMR spectroscopy. NMR experiments were acquired using Bruker Avance spectrometers operating at proton frequencies of 500, 700, 800, and 900 MHz, all equipped with cryogenically cooled probes. Resonance assignments of Ccc2ab (both apo and copper-loaded) were performed through conventional multi-dimensional NMR techniques based on triple resonance experiments [26]. Chemical shift assignments have been deposited at the BMRB (Accession No. 15461). The backbone dynamics of the protein was investigated through the analysis of ¹⁵N R₁, R₂ relaxation rates and heteronuclear ¹H–¹⁵N NOEs [27] measured at 500 and 600 MHz.

Combined chemical shift variations between two different protein forms (e.g. apo and metallated) are calculated from the experimental ¹H and ¹⁵N chemical shift variations ($\Delta\delta(^1\text{H})$ and $\Delta\delta(^{15}\text{N})$, respectively) measured between corresponding peaks, through the following equation [28]:

$$\Delta\delta^{\text{combined}} = \sqrt{\frac{(\Delta\delta(^1\text{H}))^2 + \frac{1}{25}(\Delta\delta(^{15}\text{N}))^2}{2}}$$

Results

Assignment of NMR spectra

The present Ccc2ab construct contains two predicted domains (Fig. S1). The secondary structure of the first domain of Ccc2ab as determined from the chemical shift index is essentially the same as that observed in isolated domain Ccc2a [29]. The NMR spectra of apo–Ccc2ab are characterized by the presence of two sets of signals, some well spread indicating that the corresponding residues are located in a well folded domain, and other with reduced chemical shift dispersion, similarly to loosely folded proteins. From the assignment of the backbone atoms, which was obtained for 128 out of 149 non-proline residues, it appears that the first domain is well folded with the same secondary structure elements as in the isolated Ccc2a domain. For a more detailed view, a comparison of the backbone amide chemical shifts of apo–Ccc2a (the isolated first copper(I)-binding domain) and of apo–Ccc2ab is shown in Fig. 1. Indeed, significant variations are localized only at the N and C termini of the domain. On the contrary, in the second domain where most of the unassigned residues are located, only reduced secondary structure elements (as judged from the chemical shift index) are present.

The domains experience also quite different dynamical properties. Indeed, the average ¹⁵N R₁ and R₂ relaxation rates are higher for the first domain than for the second (at 500 MHz the observed values were 2.4 ± 0.3 s⁻¹ vs. 1.8 ± 0.5 s⁻¹ for R₁ rates; 9.3 ± 1.6 s⁻¹ and 7.1 ± 2.0 s⁻¹

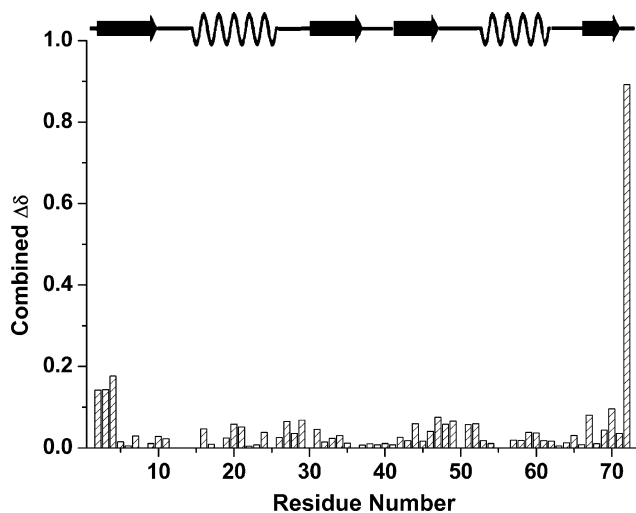


Fig. 1. Combined NMR chemical shift differences of backbone amide moieties between apo-Ccc2a and the first domain of apo-Ccc2ab. Secondary structure elements predicted from the CSI index are shown.

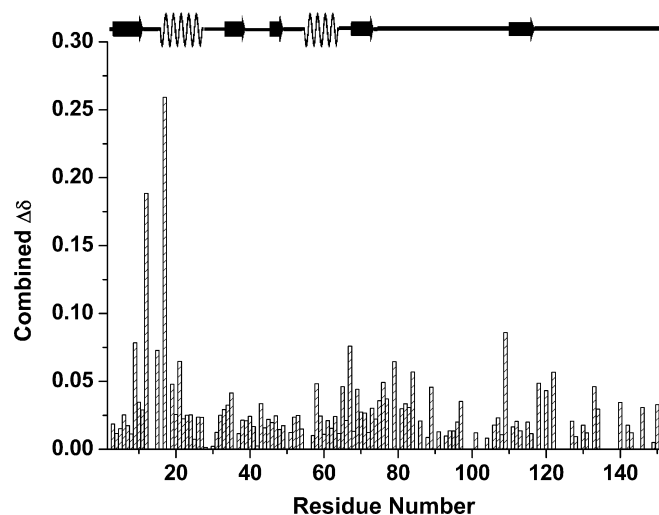


Fig. 2. Combined NMR chemical shift differences of backbone amide moieties between apo-Ccc2ab and holo-Ccc2ab. Secondary structure elements predicted from the CSI index are shown.

for R_2 rates). Even more strikingly, the heteronuclear NOEs are positive for the first domain but negative for the second one (Fig. S2). Altogether, these data indicated that the second domain of the protein experienced significant motions on the sub-nanosecond time scale.

The two domains exhibit a different behavior also with respect to their ability to acquire copper either from a small copper(I) complex or from the yeast copper(I) chaperone, Atx1. When Ccc2ab was presented with two equivalents of the acetonitrile complex of copper(I), spectral changes in the NMR spectra were detected only for some residues of domain 1, in particular for the metal-binding loop.

As described in Materials and methods, Ccc2ab was metallated by adding to the protein an acetonitrile complex of copper(I) at a 2:1 copper:protein ratio. The frequencies of the backbone atoms of Ccc2ab in the presence of two equivalents of copper(I) could be assigned for 127 out of 149 non-proline residues assignments. The per-residue plot of combined chemical shift differences observed between apo- and holo-Ccc2ab (Fig. 2) indicates that copper(I) binding affects only the cysteine-containing loop (loop I) of the first domain, suggesting that the present construct could bind a single copper(I) ion. This stoichiometry was confirmed also through competition assays with BCS.

We then studied the interaction of apo-Ccc2ab and copper(I)-Atx1 by titrating the former, enriched in ^{15}N , with the unlabeled partner up to a 2.2:1 Atx1:Ccc2ab molar ratio. The titration was monitored by ^{15}N - ^1H HSQC spectra. The major chemical shift changes for the signals of ^{15}N Ccc2ab took place in the regions spanning amino acids 8–23 and 55–66 in the first domain (combined $\Delta\delta > 0.025$ ppm). There were no significant changes in the signals of second domain even at highest molar ratio (Fig. 3). The titration has been observed also looking at the Atx1 protein, by reversing the labelling scheme and

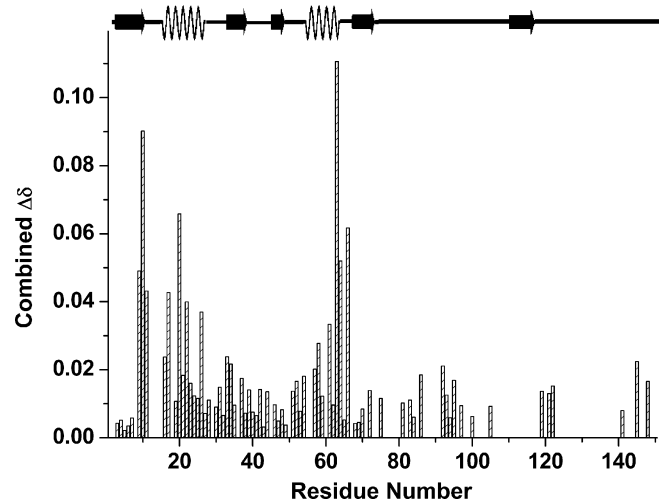


Fig. 3. Combined NMR chemical shift differences of backbone amide moieties between ^{15}N apo-Ccc2ab in the absence or in the presence of copper(I)-Atx1 at a 2.2:1 molar ratio.

acquiring the ^{15}N - ^1H HSQC spectra of Atx1. The chemical shifts of several peaks showed increasing variations with increasing amounts of unlabelled apo-Ccc2ab. Fig. 4 shows that that the major chemical shift changes for copper(I)-Atx1 signals were localized in two regions of the protein corresponding to 15–30 and 59–70 (loop I and loop V), similarly to what observed for the interaction of Atx1 with the single-domain construct Ccc2a [25].

To obtain further insight into the formation of the complex, we also inspected the backbone dynamics of ^{15}N copper(I)-Atx1 in the presence of unlabeled apo-Ccc2ab at 500 MHz. The correlation time for Atx1 tumbling in solution was estimated using from the R_2/R_1 ratio, as previously reported, yielding a value of 10 ± 1 ns which is significantly higher than the tumbling value of ^{15}N cop-

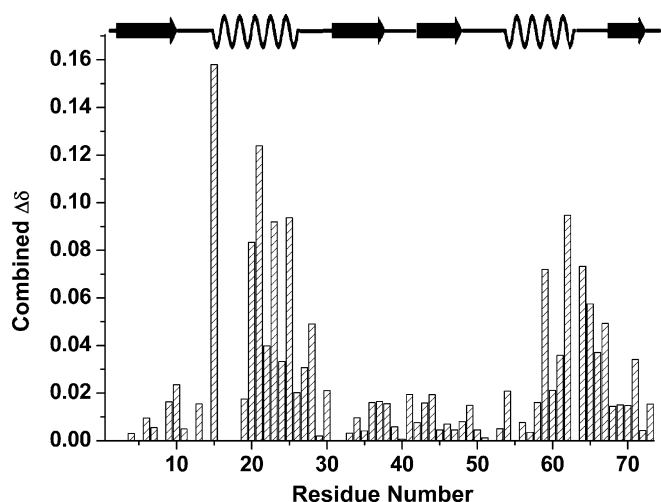


Fig. 4. Combined NMR chemical shift differences of backbone amide moieties between ^{15}N copper(I)-Atx1 in the absence or in the presence of apo-Ccc2ab at a 1:1.3 molar ratio.

per(I)-Atx1 alone (5.1 ± 0.2 ns [25]). This result indicates formation of an intermolecular adduct.

Discussion

The present data showed that *in vitro* there is a substantial differentiation between the two domains from the cytoplasmic metal-binding region of the Ccc2 ATPase. Upon refolding of the Ccc2ab construct, in fact only the N-terminal domain gave rise to a fully folded compact structure. This does not imply that the second domain is not folded *in vivo*, but does point to a different behavior of the two domains in the construct under the same conditions. The first domain had essentially the same overall features [25] as when analysed in a single-domain construct, which was expressed in *E. coli* as a soluble protein [29]. It could bind copper(I) and interact with the partner protein Atx1 in a copper-dependent manner. The changes observed upon presenting either apo-Ccc2ab or apo-Ccc2a with copper(I)-Atx1 are similar, also with respect to the combined chemical shift changes observed. Thus, the presence of the second domain does not alter substantially the regions of intermolecular interaction of the first domain. The second domain of Ccc2ab appears to be poorly folded and to experience significant conformational dynamics, as shown by the smaller dispersion of its chemical shift values with respect to the first domain and by the reduced ^{15}N R_1 , R_2 values as well as by the negative heteronuclear NOE values. A different compactness of the two domains in a two-domain ATPase construct has been reported also for the copper-transporting ATPase CopA from *Bacillus subtilis*, where domain 1 was less structured than domain 2 [30,31]. Notably, if a mutation is introduced that makes the first domain of CopA structured, its affinity for copper(I) becomes higher than that of domain 2 [31]. In the human ATP7A protein, which contains six domains, the

fifth domain is much more mobile than the sixth, both when the two are analysed in isolation [32,33] or within the context of the entire N-terminal region [22,23]. In the latter, where signals of domains 5 and 6 can be easily directly compared, the peaks corresponding to amino acids from domain 5 are often weak and broad or even not detectable, indicative of the presence of significant conformational averaging whereas the signals of residues in domain 6 are sharp (except a few in the metal-binding loop, as observed for most copper(I)-binding domains) [23].

The two domains of Ccc2ab are connected in a relatively tight manner through a stretch of sequence that is short with respect to what typically observed for the domains that are further from the transmembrane region. However, it must be noted that the length of this stretch is quite variable. The alignment of the regions spanning the fifth and sixth domains of the ATP7A and ATP7B proteins with Ccc2ab shows that the latter has a linker sequence that is three amino acids longer than that of the two human proteins, extending it from six to nine residues. The cellular role of the domains thus does not depend on their exact spacing either, which is again in line with the view that they function independently. Notably, the only maintained feature seems to be that they are spaced closely enough that their copper(I)-binding sites cannot interact with one another. Even if Ccc2ab has two sites for potential interaction with Atx1, formation of an intermolecular adduct could be observed only for the first domain. Indeed, NMR data indicated that the second domain did not bind copper(I) when at stoichiometric metal:protein ratio. We confirmed this observation by metallating the protein with a large excess of copper(I), which was then removed by washing the protein with metal-free buffer. The remaining copper(I) content was then measured spectrophotometrically using an excess of the copper(I) chelator BCS. Only one copper(I) ion per molecule of protein was present. This behaviour may suggest that copper-binding is tuned by the conformation of the ligand residues, even though their separation in sequence is fixed. In turn, this points at the role of the protein structure in controlling the processes of copper transport, by tuning the affinity of the site and possibly its capability of receiving copper(I) from other proteins.

In vivo, two-hybrid experiments as well as Δatx1 complementation experiments point to Ccc2b being able to bind and transfer copper(I) [13,14]. Overall, the present data reinforce the view that the two copper(I)-binding domains that are closest to the transmembrane region are essentially two functionally independent units. In fact they can be independently metallated, they can independently interact with the physiological partner(s), they can have different features in terms of stability and dynamics. The short linker separating the domains therefore may be important to prevent potential impairments in the uptake of the metal ion of each domain from the partner metallo-chaperone and/or in the delivery to the metal-binding site in the transmembrane region of the protein. This might be achieved by ensuring that the two domains cannot get

one in the way of the other, providing steric restrictions along the copper(I)-transport pathway.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2007.10.045](https://doi.org/10.1016/j.bbrc.2007.10.045).

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