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Cd^{2+}- or Hg^{2+}-binding proteins can replace the Cu^{+}-chaperone Atx1 in delivering Cu^{+} to the secretory pathway in yeast

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Abstract Copper delivery to Ccc2 – the Golgi Cu^{+}-ATPase – was investigated in vivo, replacing the Cu^{+}-chaperone Atx1 by various structural homologues in an *atx1-Δ* yeast strain. Various proteins, displaying the same ferredoxin-like fold and (M/L)(T/S)C*XX*C metal-binding motif as Atx1 and known as Cu^{+}-, Cd^{2+}- or Hg^{2+}-binding proteins were able to replace Atx1. Therefore, regardless of their original function, these proteins could all bind copper and transfer it to Ccc2, suggesting that Ccc2 is opportunistic and can interact with many different proteins to gain Cu^{+}. The possible role of electrostatic potential surfaces in the docking of Ccc2 with these Atx1-homologues is discussed.

Keywords: Metallo-chaperone; Cu^{+}-homeostasis; Heavy-metal; C*XX*C; P1-type ATPase; Ccc2

1. Introduction

Copper is a transition metal shuttling between the Cu^{+} and Cu^{2+} redox states in the cell. Although toxic, it is required for life. To overcome copper toxicity, organisms have developed various means to have virtually no free copper in their cytoplasm, for instance intracellular sequestration by thiol-rich proteins or extrusion out of the cytoplasm by membrane transport proteins. Copper is also a key element for cell life because it participates in many enzyme activities. Among the Cu^{+}-binding proteins in the cytoplasm, the Cu^{+}-chaperone Atx1 delivers Cu^{+} to Ccc2, a P1-type ATPase imbedded in the *trans*-Golgi membrane, which in turn transfers Cu^{+} to newly synthesised proteins [1,2]. Transportation of Cu^{+} through the Atx1-Ccc2 route is therefore responsible for Cu^{+} delivery to the secretory pathway.

Yeast two-hybrid experiments showed an interaction between Atx1 and Ccc2 N-terminus [2]. One interesting feature of the Atx1-Ccc2 route is that the Ccc2 N-terminus encloses two Atx1-like metal-binding domains (Mbds), denoted as Mbd1 and Mbd2 in this study. Structural studies of Atx1 and Mbd1 have demonstrated that these proteins have the same ferredoxin-like fold with the same C*XX*C metal-binding motif [3,4]. NMR studies of Atx1 and Mbd1 interactions have suggested that their electrostatic potential surface (EPS) may optimize their relative orientation for Cu^{+}-transfer to occur [5]. The capacity for Atx1 and Mbd1 to exchange Cu^{+} was evidenced in vitro and a mechanism was proposed for this exchange [2,6]: (i) in Atx1, Cu^{+} is coordinated by two sulfurs belonging to the C*XX*C thiols and by a third atom (sulfur or oxygen) presumably exogenous; (ii) while docking with Mbd1, an event that is favoured by complementary electrostatic interactions at the surfaces of the proteins [7], the exogenous ligand is replaced by one C*XX*C thiol of Mbd1, linking Atx1 and Mbd1 in a Cu^{+}-bridged heterodimer; (iii) Cu^{+}-transfer is then ensured by its coordination by the second C*XX*C thiol of Mbd1 and (iv) finally apo-Atx1 is released.

Genes for Atx1-like proteins and target Cu^{+}-ATPases with one to six Mbds at their N-terminus have been identified in many genomes. Up to now, all metallo-chaperones and Cu^{+}-ATPase Mbds that have been studied as soluble proteins, display the same ferredoxin-like fold and the same (M/L)(T/S)C*XX*C metal-binding motif (Table 1). Many other metal transporting proteins and ATPase Mbds have or are predicted to have similar folds and metal-binding sites, even though they are not Cu^{+}-proteins [8]. This was actually proved for MerP from *Shigella flexneri* [9], a periplasmic protein involved in Hg^{2+}-resistance [10] and for the Mbd of ZntA from *Escherichia coli* [11], an ATPase involved in Zn^{2+}-resistance [12]. From these similarities arose the idea that all these proteins or Mbds could possibly be functional homologues of Atx1. A similar idea is found in a phylogenetic analysis of Mbds from Cu^{+}-chaperones and -ATPases [13].

This question was addressed herein by determining whether Cu^{+} could be delivered to Ccc2 by various metallo-chaperones or ATPases' Mbds, which would all act as Atx1. For this purpose, we performed complementation assays in an *atx1-Δ* yeast strain, i.e., a strain in which the *ATX1* gene was disrupted, and investigated the ability of different proteins to replace Atx1 in Cu^{+}-transfer to the secretory pathway. We chose both Mbd1 and Mbd2 from Ccc2, the Cu^{+}-chaperone CopZ from *Bacillus subtilis*, the Hg^{2+}-binding protein MerP from

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Abbreviations: Mbd, metal-binding domain; EPS, electrostatic potential surface

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Table 1
Alignment of various Atx1-like proteins. MerP, Mbd2 and Ntk sequences were aligned with those of 12 apo-proteins with available structures

```
1FES  ---maeikhyqfn---vvMTCSGCsgavnkvltklepdvskid-islekqlvdvytt---lpydfilekikktgkevrsgkql------ Atx1
1FEE  -------pkhefs---vdMTCGGCaeavsrvlnklgg--vkyd-idlpnkkvciese---hsmdtllatlkktgktvsylgle------ Atox1
1P8G  ------meqktlq--vegMSCQHCvkavetsvgeldg-vsavh-vnleagkvdvsfdadkvsvkdiadaiedqgydvakiegr------ CopZ
1CPZ  --------aqefs--vkgMSCNHCvarieeavgrisg-vkkvk-vqlkkekavvkfdeanvqateicqainelgyqaevi--------- EhCopZ
1AFI  -----atqtvtla--vpgMTCAACpitvkkalskveg-vskvd-vgfekreavvtfddtkasvqkltkatadagypssvkq-------- SfMerP
1FVQ  ------arevila--vhgMTCSACtntintqlralkg-vtkcd-islvtnecqvtyd-nevtadsikeiiedcgfdceilrds------ Mbd1
1KVI  mdpsmgvnsvtis--vegMTCNSCvwtieqqigkvng-vhhik-vsleeknatiiydpklqtpktlqeaiddmgfdavihnpd------ Mnk1
1Q8L  -gsmaqagevvlkmkvegMTCHSCtstiegkigklqg-vqrik-vsldnqeativyqphlisveemkkqieamgfpafvkkqpkylk-- Mnk2
1AW0  -----ltqetvin--idgMTCNSCvqsiegviskkpg-vksir-vslansngtveydplltspetlrgaiedmgfdatlsd-------- Mnk4
1JWW  -----vtekaefd--iegMTCAACanriekrlnkieg-vanap-vnfaletvtveynpkeasvsdlkeavdklgyklklkgeqdsiegr CopAb
1MWY  -----sgtryswk--vsgMDCAACarkvenavrqlag-vnqvq-vlfateklvvdadn--diraqvesalqkagyslrdeqaae----- ZntAa
1QUP* ----ttndtyeat-yaipMHCENCvndikaclknvpg-insln-fdieqqimsvess---vapstiintlrncgkdaiirga------- Ccs-dI
      -----atqtvtls--vpgMTCSACpitvkkaiskveg-vskvd-vtfetrqavvtfddaktsvqkltkatadagypssvkq-------- MerP
      ----mstkeglls--vqgMTCGSCvstvtkqvegieg-vesvv-vslvteechviyepskttletaremiedcgfdsniimdg------ Mbd2
      -----maektvyr--vdgLSCTNCaakfernvkeieq-vteai-vnfqaskitvtqeas-iqqveqaqafehlkiipekea-------- Ntk
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Left column, PDB ID of the structure, right column, protein name as defined in the text. Ccs-dI structure 1QUP* was extracted from Ccs-Sod heterodimer structure 1QUP.

Ralstonia metallidurans CH34 and the Mbd of the Cd^{2+}-ATPase from *Listeria monocytogenes*, denoted here as Ntk.

We show here that these proteins are all functional homologues of Atx1 in yeast. Therefore, whether they are eukaryotic or prokaryotic, metallo-chaperones or Mbds initially found in a Cu^{+}- or a Cd^{2+}-ATPase, they all restore Cu^{+}-homeostasis in yeast. Our results support the idea that the interactions between the metallo-chaperone and Ccc2 are not highly specific in vivo. Rather, Ccc2 is an opportunistic protein, interacting with many different proteins having the right fold and the right (M/L)(T/S)C*XX*C metal-binding motif. Interestingly enough, the proteins that were able to act as Atx1 displayed various EPSs, from predominantly positive as Atx1 [5] to predominantly negative as CopZ [14], raising the issue of the validity of EPS calculations to predict in vivo interactions.

2. Materials and methods

An *atx1-Δ* yeast strain denoted ΔATX1 was created by disrupting the *ATX1* gene with a deletion cassette bearing the *TRP1* gene as a selection marker. ΔATX1 was grown in yeast extract-peptone-dextrose medium.

All the constructions for expressing Atx1, Mbd1, Mbd2, CopZ, MerP and Ntk were generated by PCR amplifying the nucleotide sequences encoding the amino acid sequences shown in Table 1 from yeast genomic DNA, from *B. subtilis* genomic DNA (given by A.-E. Foucher and J.-M. Jault), from pET-3a (given by E. Rossy and J. Coves) and from pQE60NTK (given by P. Catty), respectively. The products were checked and subcloned in a multi-copy vector pYEp181, denoted as pY [15] or a low-copy vector pLeu (derived from pRS315 [16]). Both pYEp181 and pLeu vectors without insert were used as controls. Another control was obtained by changing the cysteines of the C*XX*C metal-binding motif into serines in Atx1, Mbd1 and Ntk.

ΔATX1 was transformed with construction vectors encoding Atx1 or homologues and transformants were selected on DO-Leu plates. Randomly selected clones were grown for 3 days (pY-) or 4 days (pLeu-) at 30 °C in iron- and copper-limited medium [17]. For each protein, three independent transfections were carried out and the phenotypic tests were performed on at least five different clones.

2.1. Atx1 detection

Yeast cells transformed by pY- or pLeu-Atx1 were grown overnight in DO-Leu and collected 24 h later. Harvested cells were washed and broken by glass bead homogenization in 60 mM Tris–HCl (pH 7.5), 2 mM EDTA, 0.3 M sorbitol, 60 mM NaCl and Roche complete protease inhibitor mixture. The homogenate was spun down for 1 h at 90 000 × *g*, the supernatant collected and reacted with DEAE Sepharose for 2 h at 4 °C. The mixture was spun down, the supernatant concentrated and submitted to SDS–PAGE. Protein expression was assessed by Coomassie staining.

2.2. Molecular modelling by homology and electrostatic calculations

MerP, Mbd2 and Ntk sequences were aligned using Clustalw [18] against the sequences of 12 homologous apo-proteins (Table 1) with known structures and ferredoxin-like folds (Fig. 4A). Starting from these alignments, 10 models of each protein were built with the program Modeller [19]. The model with the lowest energy was retained (see the secondary structures in Table 2) and used in the following electrostatic calculations. Partial charges were assigned to all atoms of the proteins listed in Table 1 using the CHARMM force field [20]. Next, instead of calculating the electrostatic potential at the surface of the proteins, we designed a calculation allowing to get the overall electrostatic potentials at one glance, as a plani-sphere allows to look at the whole earth surface at one glance. To do so, proteins were all oriented with their centre of mass in position (0, 0, 0) and their axes aligned in the *x*, *y* and *z* directions of the coordinate system, hence defining the maximum length of the proteins in each direction ($-a/2 < x < a/2$, $-b/2 < y < b/2$, $-c/2 < z < c/2$). Then, each protein was enclosed in a box 2 Å larger than its maximum dimensions ($-a/2 - 2$ and $a/2 + 2$ on *x*, $-b/2 - 2$ and $b/2 + 2$ on *y*, $-c/2 - 2$ and $c/2 + 2$ on *z*). The electrostatic potential was calculated on the faces of the box and the box was "opened" as sketched in Fig. 4B, which reveals the whole EPS at one glance. For the sake of comparison, the electrostatic potential values (−298 to 189 kcal/mol) are all shown with the same scale, from black for the most negative potentials to white for the most positive potentials.

3. Results

3.1. The reliability of complementation assays on ΔATX1

In yeast, Fet3, an iron multi-copper-oxidase involved in high-affinity iron uptake at the plasma membrane incorporates copper in the Golgi, prior to be sent to the plasma membrane. Because yeast cells lacking Atx1 are defective in delivering Cu^{+} to the Golgi, Fet3 is not activated and an *atx1-Δ* yeast strain such as ΔATX1 cannot grow in an iron- and copper-limited medium. However, this phenotype can be rescued by adding excess copper or iron [1]. This is illustrated by the phenotypic tests in Fig. 1 which show that the ΔATX1 strains transformed with the empty plasmids, denoted pY- and pL-, did not grow under copper- and iron-limiting conditions and were rescued upon addition of iron. Another negative control was obtained by producing Atx1-S*XX*S, which bears serines instead of cysteines in its metal-binding motif and was previously shown not

Table 2
The ferredoxin-like fold obtained by homology modelling

```
                β1      11        α1      12  β2      13  β3   14      α2      15  β4
1FES    maeikhyqfn---vvMTCSGCsgavnkvltklepdvskid-islekqlvdvytt---lpydfilekikktgkevrsgkql      Atx1
1FVQ    ---arevila--vhgMTCSACtntintqlralkg-vtkcd-islvtnecqvtyd-nevtadsikeiiedcgfdceilrds      Mbd1
1P8G    ---meqktlq--vegMSCQHCvkavetsvgeldg-vsavh-vnleagkvdvsfdadkvsvkdiadaiedqgydvakiegr      CopZ
        --atqtvtls--vpgMTCSACpitvkkaiskveg-vskvd-vtfetrqavvtfddaktsvqkltkatadagypssvkq-----   MerP
        -mstkeglls--vqgMTCGSCvstvtkqvegieg-vesvv-vslvteechviyepskttletaremiedcgfdsniimdg----  Mbd2
        --maektvyr--vdgLSCTNCaakfernvkeieg-vteai-vnfgaskitvtgeas-iqqveqagafehlkiipekea-----   Ntk
```

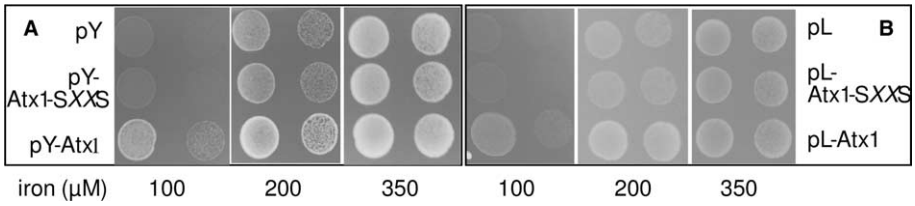

Fig. 1. Atx1 expression rescues the growth of ΔATX1 in an iron- and copper-limited medium: 0.1 μM $CuSO_4$, 2 mM ferrozine and iron ammonium sulfate as indicated. Under each condition, the drops shown in the first column contained 10^5 cells and 10^4 in the next column. ΔATX1 was transformed with: (A) high-copy plasmids and (B) low-copy plasmids. pY- and pL- are strains transformed with empty plasmids, pY- and pL-Atx1-S*XX*S express Atx1-S*XX*S, pY- and pL-Atx1 express wild type Atx1.

to interact with the Ccc2 N-terminus [7]. Finally, overexpression of wild-type Atx1 (pY- and pL-Atx1) did restore yeast growth under copper- and iron-limiting conditions. This shows the expected requirement of Atx1 cysteines for recovery of Cu^+-transfer, hence the reliability of these phenotypic tests.

Complementation was therefore achieved by Atx1, even at a low expression level. Atx1 and Atx1-S*XX*S were detected by SDS–PAGE analysis (Fig. 2). Although pY-Atx1 and pY-Atx1-S*XX*S produced high levels of proteins (Fig. 2; lanes 3 and 4), only Atx1 expression enabled ΔATX1 to grow under iron-limiting conditions (Fig. 1). pL-Atx1 produced Atx1 at such a low level that it was hardly detected on a Coomassie-stained gel (Fig. 2, lane 5). This was also the case with endogenous Atx1 in YPH499 (data not shown). Although low, the expression level of pL-Atx1 was enough to restore the growth defect of ΔATX1 in 4 days (Fig. 1). The reliability of the phenotypic tests led us to use them further to assess the ability of various proteins to act as Cu^+-chaperones.

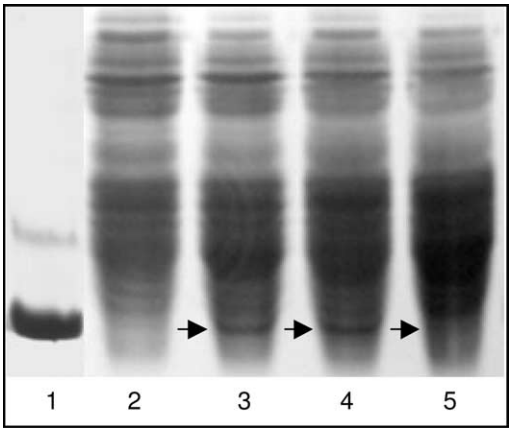

Fig. 2. Protein expression in yeast. Cytosolic proteins were isolated from various cells and purified: Atx1 from *E. coli* is shown in lane 1 to identify Atx1 in the yeast extracts (black arrow, lanes 2–5): lane 2, ΔATX1 shown as a negative control; lane 3 pY-Atx1; lane 4 pY-Atx1-S*XX*S; lane 5 pL-Atx1.

3.2. Both metal-binding domains of Ccc2 are efficient copper-chaperones in yeast

In vivo, Cu^+-transfer is thought to occur through direct interactions between Atx1 and the Ccc2 N-terminus. Indeed, no interaction has been detected between Atx1 and other cytosolic domains of Ccc2 by the yeast two-hybrid system [2]. The Ccc2 N-terminus can be divided into two Atx1-like domains, Mbd1 and Mbd2, which are 34% and 29% identical to Atx1, respectively (Table 1). To check their potential role as Atx1-homologues, both domains were expressed as soluble proteins in ΔATX1. Overexpression of Mbd1 and Mbd2 rescued the defect of growth in an iron- and copper-limited medium (Fig. 3, pY-Mbd1 and pY-Mbd2), whereas Mbd1-SXXS did not (data not shown). Therefore, both Mbd1 and Mbd2 can be considered as Atx1 functional homologues.

The three-dimensional structures of Atx1 and Mbd1 have already been described (Table 1) and that of Mbd2, which is 43% identical to Mbd1, was calculated here by molecular modelling (Table 2). The corresponding EPS were calculated as described in Section 2 and the patterns of Atx1, Mbd1 and Mbd2 EPS are shown in Fig. 4. The whole Atx1 EPS is positive, whereas Mbd1 appears negatively charged, in agreement with previous work [5] and Mbd2 displays the same pattern as Mbd1, i.e., almost entirely negatively charged. Therefore, we can assume that Mbd1 and Mbd2 can be considered as Atx1-homologues in yeast, despite their different EPS.

3.3. The Cu^+-chaperone does not need to be eukaryotic

In bacteria, Cu^+-homeostasis is regulated by the *cop* operon which encodes among other proteins the metallo-chaperone CopZ and the Cu^+-ATPase CopA [21]. These two proteins have been shown to interact with each other [22] and the structures of CopZ and CopAb, the second Mbd of CopA, have

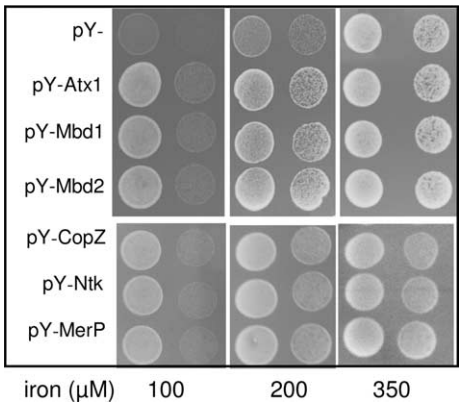

Fig. 3. Expression of Atx1 structural homologues rescues the growth of ΔATX1 in the same iron- and copper-limited medium as in Fig. 1. ΔATX1 was transformed with various high-copy plasmids: pY- and pY-Atx1 as in Fig. 1, pY-Mbd1, pY-Mbd2, pY-CopZ, pY-Ntk and pY-MerP are ΔATX1 strains expressing Mbd1, Mbd2 (from Ccc2), CopZ, Ntk and MerP, respectively. Similar results were obtained with the low-copy plasmids.

been described. CopZ binds copper, has a ferredoxin-like fold and its EPS pattern is predominantly negative (see Fig. 4) [14,23]. However, overexpression of CopZ in ΔATX1 restored the growth in an iron- and copper-limited medium (pY-CopZ in Fig. 3), showing that CopZ is another Atx1 functional homologue.

3.4. The Atx1 functional homologue does not need to be originally involved in Cu^+-homeostasis

Among the P1-type ATPases which are specific for heavy-metal transport and have an N-terminus made of one or more Mbds, some were shown to be responsible for bacterial resistance to cations other than Cu^+. For instance, in *Escherichia coli*, the activity of the P1-type ATPase ZntA is stimulated by Cd^{2+}, Zn^{2+}, Pb^{2+} and Hg^{2+} [24] and its N-terminus Mbd has a ferredoxin-like fold [11]. Herein, we focused on CadA, a Cd^{2+}-ATPase from *Listeria monocytogenes* which has one single Mbd at its N-terminus. Ntk is made of the first 71 amino acids of CadA (Table 1) and shares enough homologies with the metallo-chaperones to allow molecular modelling (Table 2). The EPS pattern of Ntk is a mixture of negative and positive patches, markedly different from that of CopZ, Mbd1 and Mbd2 (Fig. 4). To assess the ability of Ntk to act as Atx1, it was expressed in ΔATX1 and found to restore ΔATX1 growth in a copper- and iron-limited medium (Fig. 3). We also checked the requirement for the C*XX*C thiols in Ntk by expressing Ntk-S*XX*S, which did not restore ΔATX1 growth (data not shown). Thus, Ntk, the N-terminal Mbd of CadA, produced as a soluble protein in yeast, was able to restore Cu^+-homeostasis in ΔATX1.

Finally, MerP, a protein involved in Hg^{2+}-resistance which also displays a ferredoxin-like fold and the right metal-binding motif [9], has been predicted to be a metallo-chaperone [13]. The ability of MerP from *Ralstonia metallidurans* CH34 [25] to act as a Cu^+-chaperone was indeed demonstrated here, as its expression in ΔATX1 restored Cu^+-homeostasis (Fig. 3). Therefore, we found that both Ntk and MerP, which are not involved in Cu^+-homeostasis in their native organism, were able to act as a Cu^+-chaperone in yeast.

4. Discussion

Over the past 10 years, considerable interest has been expressed in the literature for the C*XX*C motif as a heavy-metal binding motif. On the one hand, Cu^+-chaperones bearing this motif were identified for their ability to interact with their Cu^+-ATPase targets in eukaryotes as well as in prokaryotes and on the other hand, the target Cu^+-ATPases [1] were identified as having the same C*XX*C motif at their N-terminus. In addition, non-Cu^+ heavy-metal ATPases were also found, which bear the same C*XX*C motif at their N-terminus and are involved in Cd^{2+}, Zn^{2+} or Pb^{2+} resistance of prokaryotes [26,27]. It was therefore tempting to examine whether exogenous metallo-chaperones and Mbds could fulfil the role of Atx1 in yeast, regardless of their origin.

Since Atx1 was discovered, the role of putative metallo-chaperones from various organisms was demonstrated because they were able to replace Atx1 in *atx*1-*Δ* strains. This method has been used in plants [28], fungi [29], worms [30], rats [31] and humans [32,33] and the involvement of these proteins in Cu^+-homeostasis in their own organism was confirmed. Therefore, the well-established *atx*1-*Δ* phenotype is an appropriate tool for assessing the ability of various proteins to act as Atx1, allowing ΔATX1 to recover Cu^+-homeostasis.

4.1. Proteins of different specificity and origin can play the role of Atx1 in Cu^+-homeostasis

We show here that Mbd1 and Mbd2 were able to replace Atx1 when individually expressed as cytosolic proteins in ΔATX1 (Fig. 3). This demonstrates that the same domain can play both roles in Cu^+-transfer, that of the soluble metal-binding protein and that of the Mbd tethered to an integral membrane protein, in agreement with phylogenetic studies [13]. CopZ from *B. subtilis* was also able to replace Atx1 in ΔATX1 (Fig. 3), suggesting that the Cu^+-chaperone function is independent of eukaryotic-prokaryotic separation. However, Mbd1, Mbd2 and CopZ were expected to bind Cu^+, as genuine Cu^+-binding proteins. Ntk, the Mbd of a cadmium ATPase and MerP, a Hg^{2+}-binding protein were both able to replace Atx1 when expressed as a cytosolic protein in ΔATX1 (Fig. 3), suggesting that they both act as functional Cu^+-chaperones in yeast, as well as Mbd1, Mbd2 and CopZ.

At this point, it is tempting to generalize and suggest that all known metallo-chaperones and P1-type ATPase Mbds could mimic Atx1 in yeast. If it were the case, they would all be able to bind Cu^+-and transfer it to Ccc2. Inasmuch as Cu^+-binding is concerned, this is not surprising, because of the high affinity of C*XX*C thiols for Cu^+. In the same way, but in vitro, Atx1 and Mbd1 were shown to bind Hg^{2+} [3], Atox1, Hg^{2+} and Co^{2+} [34], the Wilson ATPase N-terminus, Zn^{2+} [35] and *Enterococcus hirae* CopZ, Cd^{2+} [36]. Ntk and MerP used in this study are probably also able to bind various cations and hence, Cu^+ in the yeast cytoplasm. However, the next step, that is Cu^+-transfer from any of these proteins to Ccc2 is not as straightforward.

[1] See Axelsen's database on P-type ATPases at http://biobase.dk/~axe/patbase.html.

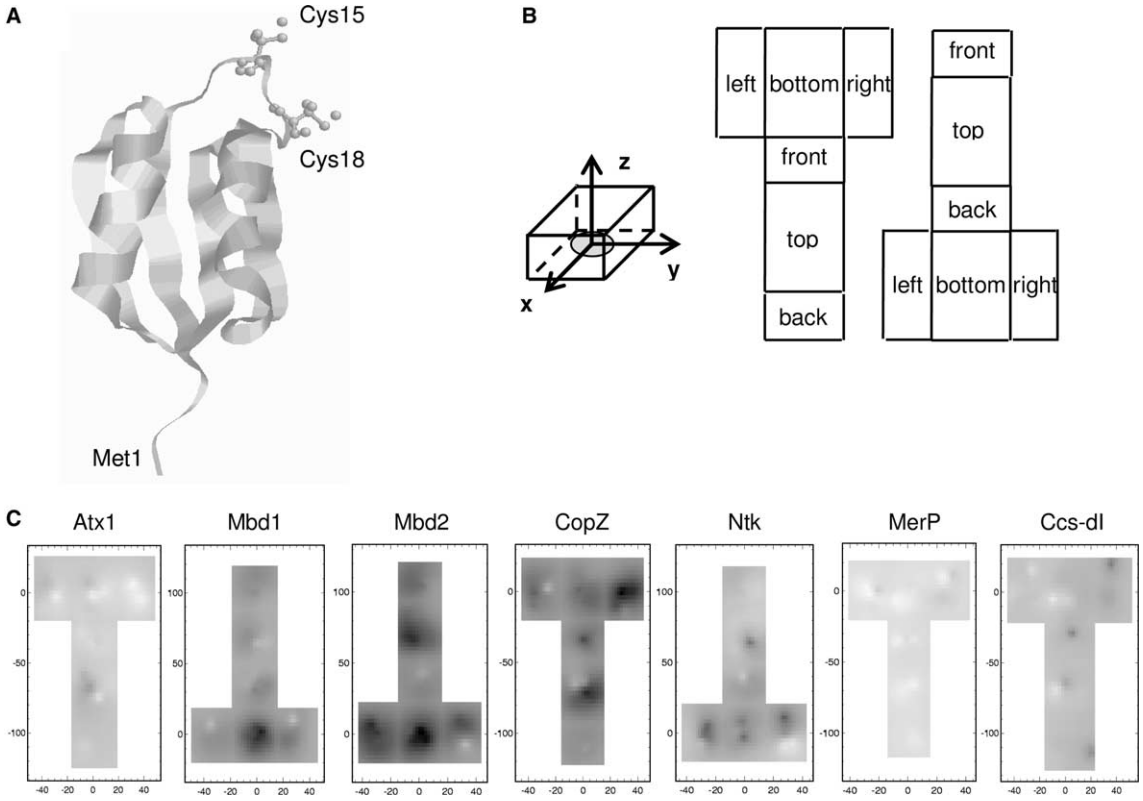

Fig. 4. (A) Atx1 ferredoxin-like fold, successively β1 α1β2β3α2β4. The two Cys of the C*XX*C motif are shown. (B) The box enclosing the protein (see definition in Section 2) is opened following two different patterns, as a reminder of the protein origin (T for genuine chaperones, up-side down T for Mbds). (C) EPS patterns of various Atx1-like proteins (units are Å). Positive potentials – hence positive charges – appear in white and negative charges in black. EPS were calculated from experimental structures when available, otherwise from structures obtained from homology modelling. EPS patterns were also calculated from the Cu^+-bound structures of Atx1 (1FD8) and Mbd1 (1FVS) and found to be similar to those of the apo-proteins, Atx1 being still positive and Mbd1 still negative.

4.2. Cu^+-transfer between the metallo-chaperones and their targets: the role of the EPS

Based on the original proposition from O'Halloran's laboratory [2], Cu^+-transfer from Atx1 to Mbd1 is thought to occur via an interaction which allows Cu^+ to exchange its coordinations by the thiols of Atx1 for those of Mbd1 through a Cu^+-bridged heterodimer. Solution structures of Atx1 and Mbd1 on the one hand [5] and of CopZ and CopAb on the other hand [14] showed that in both pairs, docking could be favoured by electrostatic forces allowing the C*XX*C motifs to face each other. Such docking would therefore favour heterodimer formation and hence Cu^+-transfer. In addition, NMR studies of the whole N-terminus of CopA which comprises two Mbds have led to the idea that although linked to each other, each Mbd could be independently folded and, therefore, be a target for CopZ and Cu^+-transfer [37].

Given these similarities, the Ccc2 N-terminus can be seen as two similar Mbds, displaying predominantly negative EPS patterns (Fig. 4). The Atx1 EPS pattern being predominantly positive, it can be assumed that docking is favoured by electrostatic attraction. This also holds for MerP, which displays a predominantly positive EPS pattern and might hold for Ntk, which has a mixed EPS (Fig. 4). However, we face a major difficulty in explaining docking of Mbd1, Mbd2 or CopZ with tethered-Mbd1 or -Mbd2, according to their EPS. The diversity of the EPS displayed by these molecules suggests that complementary electrostatic interactions may not be the predominant property for docking of any of these Atx1 functional homologues with Ccc2. A reasonable explanation is that Cu^+-transfer involving these homologues does not depend on electrostatic interactions in vivo. Recent results on the Wilson ATPase which N-terminus bears 6 Mbds show that complementary electrostatic interactions do not allow to predict which Mbd Atox1 interacts with [38].

Up to now, the characteristics of a functional Atx1-like protein can be summarized as having a ferredoxin-like fold and containing an intact (M/L)(T/S)C*XX*C motif in the first loop (Table 2). All the proteins tested here succeeded in replacing Atx1. The only protein that was previously shown to fail to replace Atx1 is Ccs-dI (Table 1), the first domain of the yeast superoxide dismutase Cu^+-chaperone [39], and the reasons for this are still unclear. Indeed, Ccs-dI has a predominantly positive EPS pattern (Fig. 4). In conclusion, straightforward growth tests of an *atx1-Δ* strain showed that Atx1 can be functionally replaced by several proteins which all display a ferredoxin-like fold and a common metal-binding motif. Interestingly enough, there was no requirement for eukaryotic origin or involvement in Cu^+-homeostasis for these proteins to act as Atx1, once expressed in yeast. According to their EPS, the variety of the proteins acting as Atx1 precludes for most

of them that electrostatic interactions favour docking with Ccc2. Ccc2 thus appears as an opportunistic protein, able to scavenge Cu^+ from a large variety of partners by a mechanism which remains to be elucidated.

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