Functional and conformational properties of the exclusive C-domain from the *Arabidopsis* copper chaperone (CCH)

Helena MIRA, Marçal VILAR, Enrique PÉREZ-PAYÁ and Lola PEÑARRUBIA[1]

Departament de Bioquímica i Biologia Molecular, Universitat de València, Avda. Dr Moliner 50, E-46100 Burjassot, València, Spain

The *Arabidopsis thaliana* copper chaperone (CCH) is a small copper binding protein involved in copper trafficking. When compared to homologues from other eukaryotes, CCH has two different domains; the conserved N-domain and the plant-exclusive C-domain, a C-terminal extension with an unusual amino-acid composition. In order to characterize this extra C-domain, the CCH protein, the N-domain and the C-domain were all expressed separately in heterologous systems. While the N-domain retained the copper chaperone and antioxidant properties described for the yeast Atx1 and human HAH1 counterparts, the C-domain displayed particular structural properties that would be necessary to optimize copper homoeostasis in plant cells where it could be responsible for the metallochaperone plant-exclusive intercellular transport. The whole CCH protein and the C-domain, but not the N-domain, displayed altered SDS/PAGE mobilities. CD spectroscopy showed that the N-domain fold is representative of an α/β protein, while the C-domain adopts an extended conformation.

Key words: metallochaperone, extended structure, protein domain.

INTRODUCTION

Copper is essential, yet toxic, therefore appropriate levels are crucial for eukaryotic cells and must be maintained. Intracellular copper distribution is carried out by copper chaperones, a novel family of copper carriers that bind Cu(I) in the cytosol and mediate its delivery to copper-dependent target proteins [1]. In the yeast *Saccharomyces cerevisiae*, the molecular mechanisms underlying the activities of metallochaperones are being clarified, and three members of the protein family have been described so far: Cox17, which is involved in copper delivery to mitochondrial cytochrome oxidase [2]; Ccs, which inserts copper into Cu/Zn superoxide dismutase [3]; Atx1, which targets copper to Ccc2, a P-type ATPase, which pumps the ion into Golgi vesicles of the secretory pathway [4,5]. Both Atx1 and Ccc2 are essential factors for copper delivery to Fet3, a plasma membrane multi-copper oxidase required for high-affinity iron uptake in yeast [4].

Structural studies have shown that most copper chaperones share a similar fold ascribed to the ferredoxin-like structural family [6]. Recently, the crystal structure of Atx1 solved at 1.02 Å resolution [7], has revealed a global 'open-faced β-sandwich' fold, and has located the conserved metal-binding motif (MXCXXC) of Atx1 in an external loop where it is easily accessible to the receptor Ccc2. The interaction between Atx1 and Ccc2 has been demonstrated through the two-hybrid system [8], and it has been proposed that this protein–protein interaction is mediated by electrostatic attraction between a conserved Lys-rich region on Atx1 and a cluster of acidic residues on the surface of Ccc2 [7]. Such an interaction has also been proved to exist between the corresponding human homologues [9]. Moreover, Atx1 and its human homologue HAH1 have intrinsic antioxidant properties which are unrelated to their interaction with Ccc2 [10–12].

Current evidence suggests that copper homoeostasis in plants could follow a similar pathway to the one described in other eukaryotes. In this sense, the copper chaperone (CCH) from *Arabidopsis thaliana* is the functional homologue of Atx1 [13], and the RAN1 ATPase is the plant counterpart of Ccc2 [14]. RAN1 has been implicated in the ethylene-signalling pathway, since binding of this gaseous hormone to its receptor is copper-dependent [15]. If plants have a chaperone-mediated delivery of copper similar to the one described in yeast, CCH would transfer the metal to RAN1, that in turn would pump copper ions into the secretory pathway making them accessible to the ethylene receptor. CCH is located mainly in plant vascular tissues where its concentration increases greatly during senescence [16]. This suggests that CCH plays a role in copper mobilization in senescing organs to direct copper towards nutrient-demanding reproductive structures.

We have focused our attention on the *Arabidopsis* CCH. CCH (121 residues) has two different domains (Figure 1); a conserved N-terminal region (amino acids 1–71, referred herein as the N-domain) containing the copper-binding motif MXCXXC, and a

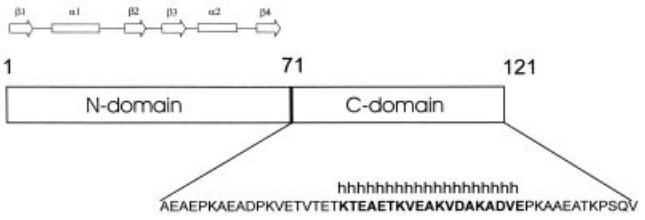

Figure 1 Representation of the CCH protein

The left segment represents the CCH N-domain [the Atx1-like part, with the secondary structure elements of yeast Atx1 shown as the following: β-sheet, arrows (β); α-helix, rectangles (α); loops, single lines]. The right segment shows the amino-acid sequence of the exclusive C-terminal domain. The stretch of 'h' and the motif in **bold** indicate the putative α-helix predicted by secondary-structure algorithms.

Abbreviations used: CCH, copper chaperone; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside.

[1] To whom correspondence should be addressed (e-mail lola.penarrubia@uv.es).

C-terminal region (amino acids 72–121, the C-domain), which is absent in all the identified non-plant metallochaperones [13]. The purpose of this study was to gain insight into the functional and conformational properties of this plant-exclusive C-domain, which has not yet been described in any detail.

EXPERIMENTAL

Sequence analysis

The *Arabidopsis* CCH cDNA nucleotide sequence corresponds to GeneBank® accession number U88711. Secondary structure predictions were determined using the PHD software (EMBL, Heidelberg, Germany).

Yeast strains, vectors and culture conditions

The *S. cerevisiae* strain used was SL215 (*atx1*Δ, derivative of strain YPH250; [4]). To create the vectors for the expression of *CCH* and the N-domain of *CCH* in yeast, cDNA fragments were directionally subcloned into p416GPD generating p416-CCH(1-121) and p416-N(1-68). Other plasmids used were p413-A1 for expression of yeast *ATX1*, and pRS413 as the control vector without the insert [4]. The SL215 strain was transformed with the corresponding vectors, and to test for iron-uptake restoration in this strain, transformants were grown for six days at 30 °C as described previously [4]. The antioxidant function was tested by growing transformants in 1.5 mM H_2O_2 [10]. CCH (residues 1–121) and the N-domain (residues 1–68) were fused in frame to the C-terminus of the green fluorescent protein (GFP). The coding sequences of the fusion proteins were subcloned into p416GPD, and the strain SL215 was transformed with the resulting plasmids. Cells were incubated with 4,6-diamidino-2-phenylindole (DAPI) for nuclei staining, and were revealed by fluorescence microscopy using the appropriate filter settings. In some experiments, cells were treated with increasing concentrations, in the μM range, of copper sulphate, bathocuproine disulphonic acid or menadione.

Expression and purification of recombinant proteins

Glutathione S-transferase (GST) fusion constructs were created by subcloning appropriate fragments of the CCH cDNA into pGEX vectors. The insert DNA for construction of GST-CCH(1-121) was prepared by digesting the cDNA with *Nco*I, 'filling-in' with the Klenow fragment of DNA polymerase I and redigesting with *Not*I. The fragment was then subcloned into pGEX-5X-3. *Bpu*1102I and *Not*I digestions of this plasmid were followed by a fill-in reaction to generate blunt-ends, and then by religation of the backbone DNA to create GST-N(1-73). For the C-domain construct, GST-C(69-121), the *Sau*3A–*Not*I cDNA insert was subcloned into pGEX-5X-2. All the constructs were sequenced to verify the frame conservation. *Escherichia coli* DH5α cells were transformed with the plasmids, grown at 37 °C and induced with isopropyl β-D-thiogalactoside (IPTG). Cultures were harvested, resuspended in PBS, lysed by sonication, and the fusion proteins were purified from the soluble fraction by glutathione–Sepharose affinity chromatography. The GST-tag was removed by digestion of the matrix-bound fusion proteins with Factor Xa. All the recombinant proteins were analysed for purity and integrity by SDS/PAGE. Identity was confirmed by matrix-assisted laser-desorption ionization-time-of-flight ('MALDI-TOF') MS.

Before ultracentrifugation and CD analysis, the proteins were further purified by analytical HPLC on a Hibar Lichrosorb RP-18 (7 μm) column (Merck). They were subjected to a refolding protocol based on Wingfield et al. [17] which is described briefly as follows: proteins were denatured in 8 M guanidinium chloride, dialysed for 16 h at 4 °C in buffer A [50 mM glycine/10 % (w/v) sucrose/1 mM EDTA/1 mM GSH/0.1 mM GSSG/4 M urea (pH 9.6)], and dialysed again in buffer B [20 mM 4-morpholine-ethanesulphonic acid/10 % sucrose/100 mM NaCl/0.1 mM GSH/0.01 mM GSSG (pH 6.0)]. The concentration of the protein solution was determined by UV spectroscopy for the CCH and the N-domain (using $\epsilon^{280} = 6850\ M^{-1} \cdot cm^{-1}$), and by Coomassie staining of SDS/PAGE gels for the C-domain (using different concentrations of BSA as a standard).

Anti-CCH antibody production and plant crude extracts analysis

Recombinant-CCH protein (0.15 mg) was purified, lyophilized and sent to Eurogentec (Seraing, Belgium) for rabbit immunization. Pre-immune and immunized serum (dilution 1:1000) were used in a Western blot under standard conditions [using an Immune-blot PVDF membrane (Bio-Rad, Hercules, CA, U.S.A.)], the secondary antibody (diluted 1:3000) was added and the blot developed with the ECL® (enhanced chemiluminescence system) (Amersham International).

Mature leaves from *Arabidopsis* were ground in 2 volumes of extraction buffer [100 mM Tris/HCl (pH 7.5)/1 mM EDTA/2 mM PMSF/5 mM β-ethylthiol/1 μg/ml leupeptin/2 μg/ml apoprotinin/1 μg/ml pepstatin], centrifuged at 6000 ***g*** for 10 min, and the supernatant was used as crude extract.

CD spectroscopy

CD spectra were obtained with a Jobin Yvon spectropolarimeter (Paris, France). Samples were measured at 10 °C in 5 mM 4-morpholine-propanesulphonic acid (pH 7.0) buffer solution, unless indicated, in a 1 mm-path-length cell. An average of five scans were performed on the resulting spectra. The temperature was maintained with a circulating water bath.

RESULTS AND DISCUSSION

Yeast complementation experiments using the N-domain and the whole CCH protein

In order to study the influence of the C-domain on the CCH protein function, both the entire protein and the N-domain were expressed in the corresponding yeast mutant for functional complementation and localization studies. The *S. cerevisiae* strains used lack cytosolic Atx1 copper chaperone (*atx1*Δ), and are defective in both high-affinity iron uptake and antioxidant defence [10]. First, we questioned whether the N-domain of CCH is sufficient for functional complementation, as implied by the high sequence homology, and how the presence of the C-terminal domain influences the function and/or folding of CCH. Thus *atx1*Δ yeast cells were transformed separately with the CCH and CCH N-domain constructs, grown in the absence of iron (Figure 2A) and in the presence of 1.5 mM H_2O_2 (results not shown). Recovery of growth was observed in all cases. Moreover, CCH and N-domain fused to the C-terminus of GFP, that complemented *atx1*Δ viability defects (Figure 2A), were localized throughout the yeast cell cytoplasm (Figure 2B) in the same way as the yeast Atx1 and human HAH1 metallochaperones [4,9]. Alteration of the intracellular copper concentration by incubation in excess copper or bathocuproine disulphonic acid (a copper chelator) had no effect on the localization of CCH and N-domain fusions, and neither had the addition of the oxidant menadione (results not shown). Therefore, the N-domain by itself retains the antioxidant activity and the ability to deliver

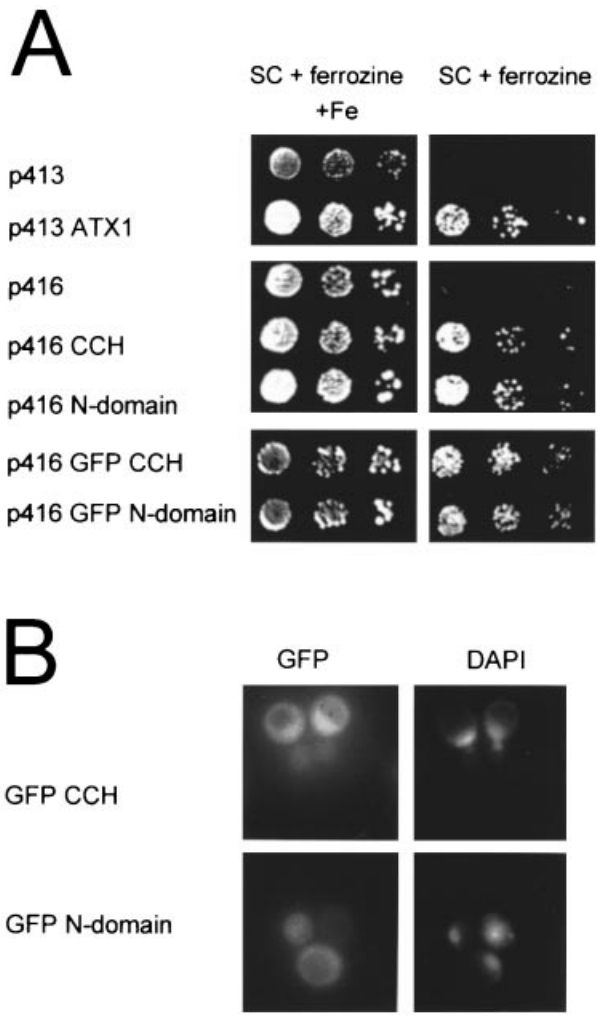

Figure 2 Functional complementation and localization of the CCH protein and N-domain in yeast

(**A**) Functional complementation of the high-affinity iron uptake in the *atx1*Δ mutant yeast by CCH and its N-domain. The *atx1*Δ mutant strain was transformed with vectors p413 and p416 (negative controls), p413 ATX1 (ATX1 expression, positive control), p416 CCH, p416 GFP CCH, p416 N-domain and p416 GFP N-domain, and grown at different dilutions on synthetic complete medium plates supplemented with either histidine or uracil (SC-his or SC-ura) containing either 3 mM ferrozine, an iron chelator, or 3 mM ferrozine plus 50 mM ferrous ammonium sulphate. Plates were photographed after incubation at 30 °C for 6 days. (**B**) Cytoplasmic localization of functional CCH and N-domain–GFP fusion proteins in yeast cells. Subcellular distribution was revealed by fluorescence microscopy, and DAPI was used for DNA staining.

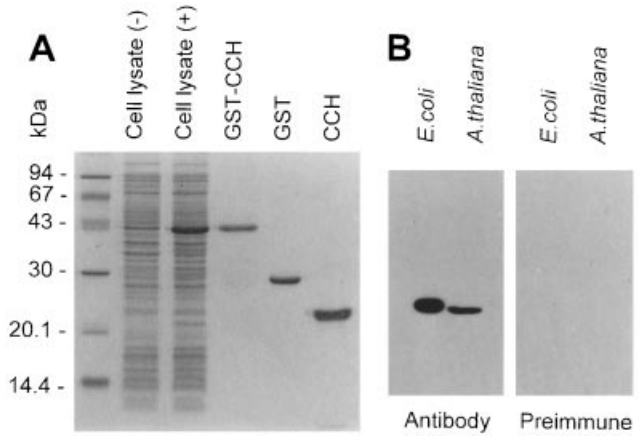

Figure 3 CCH protein expression in *E. coli* cells, purification and antibody reaction

(**A**) Electrophoretic profile of *E. coli* cell lysates before (−) and after (+) IPTG induction, purified GST–CCH fusion protein (GST-CCH) and the separate products obtained after Factor-Xa digestion (GST and CCH). Molecular-mass markers and their sizes in kDa are shown on the left. (**B**) Western immunoblots of the CCH protein produced in *E. coli* cells and from the *Arabidopsis* crude extract tested with the anti-CCH antibody and pre-immune serum.

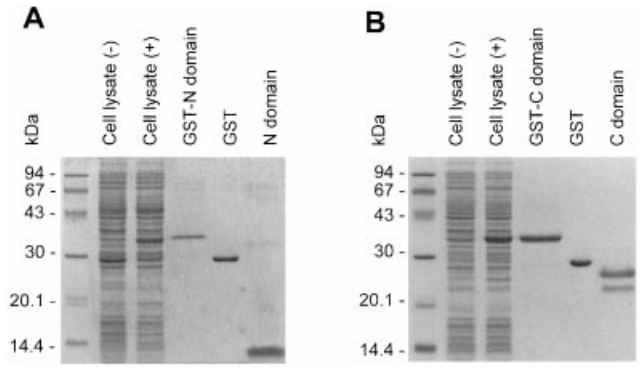

Figure 4 Expression of the CCH domains in *E. coli* cells

The expression of the CCH N-domain (**A**) and C-domain (**B**) protein in *E. coli* cells as GST-fusion proteins. Electrophoretic profiles of the *E. coli* cell lysates before (−) and after (+) IPTG induction. The purified GST-fusion constructs (GST-N domain and GST-C domain) and the products obtained after Factor Xa digestion (GST, N domain and C domain) are shown. Molecular-mass markers are shown on the left.

copper to the Ccc2 transporter in the secretory pathway, and the C-domain does not seem to interfere either with the function or the subcellular distribution of the protein in yeast. Moreover, this C-terminal sequence is not necessary for the correct folding of CCH *in vivo*, because its absence did not affect the folding of the N-terminal domain as shown by the complementation activity experiments. Thus these results suggest that the plant-exclusive C-domain should have a plant-specific function.

Expression in *E. coli* and altered SDS/PAGE mobility of the purified proteins

Arabidopsis CCH and its N- and C-terminal domains were produced as GST-fusion proteins in *E. coli* cells. The CCH protein migrated in SDS/PAGE with the apparent size of 23 kDa instead of its expected 14 kDa molecular mass (Figure 3A). Polyclonal antibodies raised against CCH also detected a 23 kDa band in Western immunoblots from *Arabidopsis* crude extracts (Figure 3B), indicating that the natural CCH protein migrates like its recombinant counterpart.

The electrophoretic mobilities on SDS/PAGE of the CCH domains were 9 kDa for the N-domain, as expected from its molecular mass (Figure 4A), and 28 kDa (major band) or 23 kDa (minor band) for the 5 kDa C-domain (Figure 4B). These results indicate that the unusual migration of the C-domain could be responsible for the altered mobility of the entire CCH protein. Previously, electrophoretic mobilities on SDS/PAGE which are uncorrelated to protein molecular masses have been described as a probable result of intrinsic net charge, protein conformation and/or SDS-resistant oligomerization [18–20].

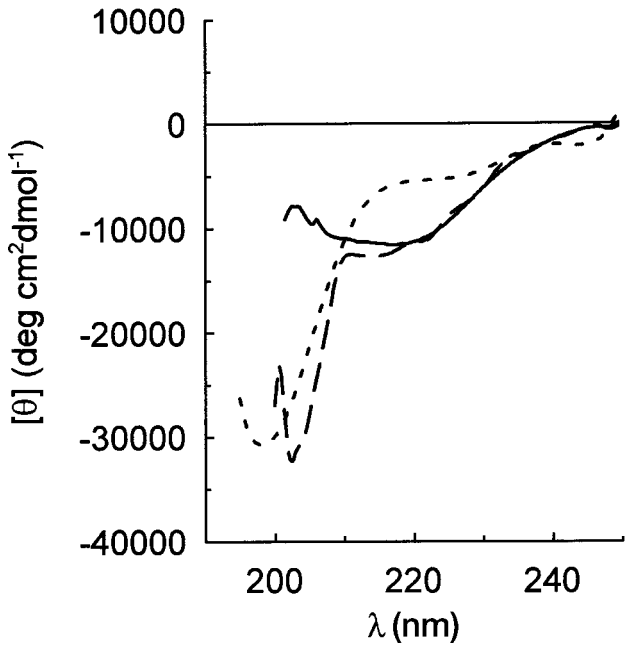

Figure 5 Far-UV CD spectra of the CCH protein and subdomains

The far-UV CD spectra of CCH (– – –), C-domain (-----) and N-domain (—) in refolding buffer solution at 10 °C. The protein concentration was 10 μM.

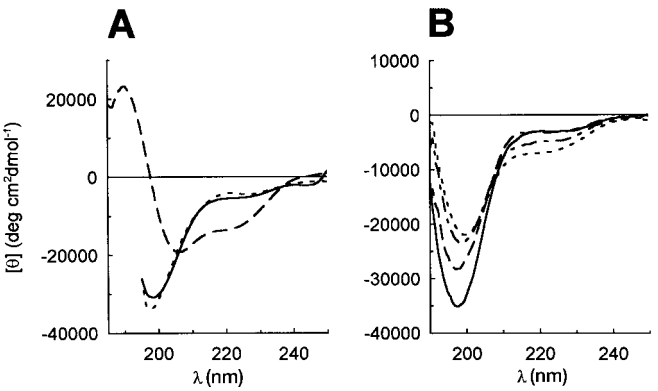

Figure 6 Far-UV CD spectra of C-domain

(**A**) The far-UV CD spectra of C-domain in 5 mM 4-morpholine-propanesulphonic acid, pH 7.0, buffer solution (—), in 40% trifluorethanol (– – –) and in 1 mM of SDS (-----). (**B**) The far-UV CD spectra of the C-domain at increasing temperatures, 5 °C (—), 25 °C (– – –), 55 °C (–·–·–) and 70 °C (-----). The protein concentration was 10 μM.

Secondary structure studies of the CCH protein and its domains

After purification by analytical reverse-phase HPLC, the recombinant CCH protein and its N- and C-domains were completely denatured before being refolded through dialysis, and analysed separately by CD spectroscopy. The far-UV CD spectrum of CCH displayed a minimum negative ellipticity at 203 nm and a 'shoulder' at 210 nm (Figure 5). The spectrum of the N-domain (Figure 5) was typical of an α/β protein, as expected due to its high sequence similarity to the Atx1 protein. The far-UV CD spectrum of the isolated C-domain showed a strong negative band at 198 nm (approx. -30000 degrees $\cdot$ cm$^2\cdot$ dmol^{-1}; Figure 5). This CD spectrum of the C-domain (Figure 5) could be associated with a disordered polypeptide structure, although such a large intensity value could also suggest the contribution, to some extent, of secondary structure elements like a polyproline II-type helix [21] or an extended β-sheet structure [22,23]. The CD spectra of the intact CCH protein could be accounted for by the contribution of both the N- and C-domains, as it is consistent with the presence of two structurally independent domains.

In order to test the propensity of the C-domain and to acquire a more defined secondary structure, the CD spectra were measured in the presence of trifluoroethanol. Under these conditions, the C-domain was induced to partially fold into an α-helix (Figure 6A). The CD analysis was also performed in the presence of submicellar concentrations of SDS, a medium described as a template that would stabilize secondary structures [24]. As shown in Figure 6(A), in the presence of 1 mM SDS the strong negative band at 198 nm of the far-UV CD spectrum obtained in buffer was further increased (approx. -35000 degrees $\cdot$ cm$^2\cdot$ dmol^{-1}), and a small 2 nm blue shift was observed.

The effect of temperature on the CD spectrum of the isolated C-domain was examined. When the temperature was lowered to 5 °C, the strong negative band at 198 nm of the C-domain CD spectrum increased (in absolute value) and a blue shift was observed. As the temperature increased (from 5–75 °C), the spectra show less intense bands with a red shifting. The presence of an isodichroic point implies that there is an equilibrium between two conformational states, which may be interpreted as a defined secondary structure prevailing at low temperatures and a disordered random coil at high temperatures. Although the CD studies globally reflect a complex structural behaviour that deserves further characterization, we suggest that the C-domain spectra could be the result of the contribution of extended structures like type II polyproline and/or antiparallel β-sheets.

Since both the functional and the structural properties are not affected by the loss of the C-terminal part of the protein, the N-domain in CCH behaves as an independent module that retains the overall folding and the biological properties assigned to other metallochaperones. The new C-terminal domain could have been added 'in-line', as postulated for other multimodular proteins [25]. This assembly would explain the lack of interference with the original intracellular function played by this metallochaperone in single-cell organisms, while allowing a novel or more complex role related to pluricellularity in plants. In this sense, we have recently found that [16], while no other metallochaperones leave the cells where they are synthesized, CCH has to be transported from neighbour cells, since the cells where CCH is mainly located lack their nuclei and consequently are unable to transcribe or translate. One could speculate that the extra C-domain in plants could account for this exclusive plant characteristic. Very little is known about this type of transport which is currently a main focus of interest in plant biology, since it has been proved crucial in processes such as plant signalling and pathogen spreading [26]. Furthermore, the observation that SDS probably stabilizes the conformation, points to hydrophobic interactions that may take place with lipids *in vivo*, perhaps affecting CCH function. Although it requires further study, our hypothesis that the C-domain is involved in intercellular transport presents exciting implications where the oligomerization and conformational properties of the C-domain described here could have new and, as yet, undiscovered physiological roles.

We thank Dr Valeria C. Culotta (Department of Environmental Health, John Hopkins University School of Public Health, Bethesda, MD, U.S.A.) and Dr Dennis J. Thiele (Department of Biological Chemistry, University of Michigan Medical School, Ann

Arbor, MI, U.S.A.) for providing the yeast strain and plasmids and for assistance with the yeast experiments, respectively. We are grateful to Dr Joaquín Moreno for critical reading of the manuscript. H.M. and M.V. were supported by long-term predoctoral fellowships from the Conselleria d'Educació i Ciència Generalitat Valenciana and from the University of València, respectively. This work was supported by grants from Dirección General de Investigación Científica y Tecnológica (Spain) (PB95-0029-C02-02), from Conselleria de Cultura, Educació i Ciència Generalitat Valenciana (GV98-3-82) to L.P., and European Union Biotechnology grant (BIO4-CT97-2086) to E.P-P. We also thank F. Barraclough and D. Donnellan (Online English, València, Spain) for their help with the English text.

REFERENCES

1 Harrison, M. D., Jones, C. E., Solioz, M. and Dameron, C. T. (2000) Intracellular copper routing: the role of copper chaperones. Trends Biochem. Sci. **25**, 29–32
2 Glerum, D. M., Shtanko, A. and Tzagoloff, A. (1996) Characterization of COX17: a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. J. Biol. Chem. **271**, 14504–14509
3 Culotta, V. C., Klomp, L. W., Strain, J., Casareno, R. L., Krems, B. and Gitlin, J. D. (1997) The copper chaperone for superoxide dismutase. J. Biol. Chem. **272**, 23469–23472
4 Lin, S. J., Pufahl, R. A., Dancis, A., O'Halloran, T. V. and Culotta, V. C. (1997) A role for the *Saccharomyces cerevisiae* ATX1 gene in copper trafficking and iron transport. J. Biol. Chem. **272**, 9215–9220
5 Yuan, D. S., Dancis, A. and Klausner, R. D. (1997) Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. J. Biol. Chem. **272**, 25787–25793
6 Harrison, M. D., Jones, C. E. and Dameron, C. T. (1999) Copper chaperones: function, structure and copper-binding properties. J. Biol. Inorg. Chem. **4**, 145–153
7 Rosenzweig, A. C., Huffman, D. L., Hou, M. Y., Wernimont, A. K., Pufahl, R. A. and O'Halloran, T. V. (1999) Crystal structure of the Atx1 metallochaperone protein at 1.02 Å resolution. Structure **7**, 605–617
8 Pufahl, R. A., Singer, C. P., Peariso, K. L., Lin, S. J., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E. and O'Halloran, T. V. (1997) Metal ion chaperone function of the soluble Cu(I) receptor Atx1. Science **278**, 853–856
9 Hamza, I., Schaefer, M., Klomp, L. W. and Gitlin, J. D. (1999) Interaction of the copper chaperone HAH1 with the Wilson disease protein is essential for copper homeostasis. Proc. Natl. Acad. Sci. U.S.A. **96**, 13363–13368
10 Lin, S. J. and Culotta, V. C. (1995) The ATX1 gene of *Saccharomyces cerevisiae* encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. Proc. Natl. Acad. Sci. U.S.A. **92**, 3784–3788
11 Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D. L., O'Halloran, T. V. and Culotta, V. C. (1999) Structure-function analyses of the ATX1 metallochaperone. J. Biol. Chem. **274**, 15041–15045
12 Hung, I. H., Casareno, R. L., Labesse, G., Mathews, F. S. and Gitlin, J. D. (1998) HAH1 is a copper-binding protein with distinct amino acid residues mediating copper homeostasis and antioxidant defense. J. Biol. Chem. **273**, 1749–1754
13 Himelblau, E., Mira, H., Lin, S. J., Culotta, V. C., Penarrubia, L. and Amasino, R. M. (1998) Identification of a functional homologue of the yeast copper-binding gene ATX from *Arabidopsis thaliana*. Plant Physiol. **117**, 1227–1234
14 Hirayama, T., Kieber, J. J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J. M., Dailey, W. P., Dancis, A. and Ecker, J. R. (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signalling in *Arabidopsis*. Cell **97**, 383–393
15 Rodriguez, F. I., Esch, J. J., Hall, A. E., Binder, B. M., Schaller, G. E. and Bleecker, A. B. (1999) A copper cofactor for the ethylene receptor ETR1 from *Arabidopsis*. Science **283**, 996–998
16 Mira, H., Martínez-García, F. and Peñarrubia, L. (2001) Evidence for the plant-specific intercellular transport of the *Arabidopsis* copper chaperone CCH. Plant J. **25**, 521–528
17 Wingfield, P. T., Palmer, I. and Liang, S.-M. (1993) Purification of recombinant proteins. In Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), pp. 6.5.1–6.5.27, John Wiley and Sons, New York
18 Hames, B. D. (1981) An introduction to polyacrylamide gel electrophoresis. In Gel Electrophoresis of Proteins: A Practical Approach (Hames, B. D. and Rickwood, D., eds.), pp. 1–86, Oxford IRL Press, Washington DC
19 Kaufmann, E., Geisler, N. and Weber, K. (1984) SDS-PAGE strongly overestimates the molecular masses of the neurofilament proteins. FEBS Lett. **170**, 81–84
20 Jones, D. H., Ball, E. H., Sharpe, S., Barber, K. R. and Grant, C. W. (2000) Expression and membrane assembly of a transmembrane region from Neu. Biochemistry **39**, 1870–1878
21 Rabanal, F., Ludevid, M. D., Pons, M. and Giralt, E. (1993) CD of proline-rich polypeptides: Application to the study of the repetitive domain of maize Glutein-2. Biopolymers **33**, 1019–1028
22 Sieber, V. and Moe, G. R. (1996) Interactions contributing to the formation of a β-hairpin-like structure in a small peptide. Biochemistry **35**, 181–188
23 Koepf, E. K., Petrassi, H. M., Sudol, M. and Kelly, J. W. (1999) WW: An isolated three-stranded antiparallel beta-sheet domain that unfolds and refolds reversibly; evidence for a structured hydrophobic cluster in urea and GdnHCl and a disordered thermal unfolded state. Protein Sci. **8**, 841–853
24 Blondelle, S. E., Ostresh, J. M., Houghten, R. A. and Pérez-Payá, E. (1995) Induced conformational states of amphipathic peptides in aqueous/lipid environments. Biophys. J. **68**, 351–359
25 Baron, M., Norman, D. G. and Campbell, I. D. (1991) Protein modules. Trends Biochem. Sci. **16**, 13–17
26 Thompson, G. A. and Schulz, A. (1999) Macromolecular trafficking in the phloem. Trends Plant Sci. **4**, 354–359

Received 6 June 2000/3 April 2001; accepted 4 May 2001