Solution Structure of the Yeast Copper Transporter Domain Ccc2a in the Apo and Cu(I)-loaded States*

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Ccc2 is an intracellular copper transporter in Saccharomyces cerevisiae and is a physiological target of the copper chaperone Atx1. Here we describe the solution structure of the first N-terminal MTCXXC metal-binding domain, Ccc2a, both in the presence and absence of Cu(I). For Cu(I)-Ccc2a, 1944 meaningful nuclear Overhauser effects were used to obtain a family of 35 structures with root mean square deviation to the average structure of 0.36 ± 0.06 Å for the backbone and 0.79 ± 0.05 Å for the heavy atoms. For apo-Ccc2a, 1970 meaningful nuclear Overhauser effects have been used with 36 JENHs to obtain a family of 35 structures with root mean square deviation to the average structure of 0.38 ± 0.06 Å for the backbone and 0.82 ± 0.07 Å for the heavy atoms. The protein exhibits a βαββαβαβ structure similar to that of its target Atx1 and that of a human counterpart, the fourth metal-binding domain of the Menkes protein. The overall fold remains unchanged upon copper loading, but the copper-binding site itself becomes less disordered. The helical context of the copper-binding site, and the copper-induced conformational changes in Ccc2a differ from those in Atx1. Ccc2a presents a conserved acidic surface which complements the basic surface of Atx1 and a hydrophobic surface. These results open new mechanistic aspects of copper transporter domains with physiological copper donor and acceptor proteins.

Saccharomyces cerevisiae Ccc2 is a member of a class of proteins that transport heavy metals across vesicular membranes (1). Members of this family, referred to as P-type or CPx-type ATPases, have been identified in a variety of bacteria, yeast, nematodes, and mammals. The cytoplasmic N-terminal copper-binding domain, CXXC motif. In humans, this intracellular copper pump is encoded by the Wilson’s and Menkes’ disease genes (2–6), and a functional homologue (Ccc2) has been characterized in yeast (7). Copper is incorporated into trans-Golgi vesicles via the action of Ccc2 and ultimately into the multicopper oxidase Fet5p, which translocates to the plasma membrane and works in conjunction with an iron permease to mediate high affinity iron uptake (8, 9). Both the Wilson’s and Menkes’ disease proteins complement the function of Ccc2 in this pathway (10–12). The Menkes CPx-type ATPase contains six N-terminal GMXCXXC motifs (2–4), is located in the trans-Golgi network, and translocates copper across intracellular membranes into the secretory pathway (13). Metal binding studies on the complete N-terminal cytoplasmic region have established that this region binds Cu(I) selectively (relative to cadmium, cobalt, or zinc) with a stoichiometry of one copper per metal-binding domain (14).

The yeast metallochaperone Atx1 is a cytosolic Cu(I) receptor that delivers its metal ion cargo to Ccc2 (15). The thermodynamic gradient for metal transfer between Atx1 and the first metal-binding domain of Ccc2 (Ccc2a) is shallow, yet copper transfer is facile, suggesting that Atx1 works like an enzyme to catalyze the rate of copper transfer between partners (16). A high resolution (1.02-Å) x-ray crystallographic structure of Hg(II)-Atx1 reveals that the mercury is coordinated in a bidentate fashion from two cysteine sulfurs with a S–Hg–S bond angle of 167° (17). Mutation of several conserved lysines on the surface of Atx1 greatly reduces the copper-dependent interaction of Atx1 and Ccc2 in vivo (15, 18). The Atx1 metallochaperone (17), domain I of the copper chaperone for superoxide dismutase (CCS) (19), and the fourth metal-binding domain of the Menkes protein (20) all adopt a βαββαββ structural fold. This same structural fold is found in the mercury-binding protein MerP (21, 22) and the putative copper chaperone CopZ (23). In all of these domains, the cysteine ligands are located within the first loop and the first helix.

The mechanism of copper transfer between Atx1 and Ccc2 is proposed to involve a series of two- and three-coordinate intermediates (15, 16). The factors responsible for facile and reversible copper transfer between Atx1 and Ccc2a are probably mediated in part by metal-dependent conformational changes in Atx1 and Ccc2. In Atx1, copper release involves a series of structural changes, in which both cysteines change conformation (46). In apo-Atx1, the loop is not well defined. We report here the solution structures of Cu(I)-Ccc2a from S. cerevisiae obtained for a nonlabeled sample and its reduced apo form, obtained for a 15N-labeled sample. Relative to Atx1, the first loop is well defined, and Cu(I) binding induces fewer changes in the structural ensemble of Ccc2a. This ensemble is not only well defined but also reproducible, with root mean square deviation of 0.06 Å for the backbone and 0.79 Å for the heavy atoms. The overall fold remains unchanged upon copper loading, but the copper-binding site itself becomes less disordered. The helical context of the copper-binding site, and the copper-induced conformational changes in Ccc2a differ from those in Atx1. Ccc2a presents a conserved acidic surface which complements the basic surface of Atx1 and a hydrophobic surface. These results open new mechanistic aspects of copper transporter domains with physiological copper donor and acceptor proteins.
the conformation of Ccc2a. Unlike Cu(I)-Atx1, the metal binding residues in Cu(I)-Ccc2a are on the surface of a helix, and the copper cargo is more accessible to incoming ligands in the latter. Finally, a negative patch is observed on the surface of Ccc2a in a site that corresponds to a positive patch on Atx1, suggesting that a complementary docking interface is employed in the copper transfer mechanism.

**EXPERIMENTAL PROCEDURES**

Sample Isolation and Preparation—Ccc2a was uniformly labeled with $^{15}$N by expressing the protein in *Escherichia coli* strain BL21(DE3) (Novagen), transformed with pDLHV021, in minimal media supplemented with $^{15}$NH$_4$Cl. Unlabeled Ccc2a was isolated as described previously (16). Ccc2a($^{15}$N) was purified from the cell pellet by freeze-thaw extraction with 20 mM MES/Na, pH 6.0, followed by streptomycin sulfate precipitation (5%, w/v) of the extract. The supernatant was further purified by repetitive runs on Superdex 75 (Amersham Pharmacia Biotech). The yield of labeled protein was $\frac{2}{9}$ mg/liter of culture.

Protein samples were purified and stored in the presence of reducing agent. NMR samples were prepared in a Vac Atmospheres nitrogen atmosphere chamber at 12 °C. Protein concentrations were determined by the Bradford assay and calibrated as described previously (16). Copper concentration was determined by ICP-AES. The NMR sample of apo-Ccc2a and apo-Ccc2a($^{15}$N) was prepared by exchanging the reduced form of the protein into 100 mM sodium phosphate, pH 7, 90% H$_2$O, 10% D$_2$O via ultrafiltration; the final concentrations were 2.7 and 3.3 mM, respectively. Cu(I)-Ccc2a was prepared as previously described (16), and the buffer was exchanged by ultrafiltration into 100 mM sodium phosphate, pH 7, 90% H$_2$O, 10% D$_2$O. The metal/protein ratio was 1.2 with a protein concentration of 1.2 mM. No exogenous thiols were added to the sample buffers. Approximately 0.6 ml of sample was loaded into 535-PP 5-mm quartz NMR tubes (Wilmad), which were capped with a latex serum cap in the VacAtmospheres chamber.

NMR Spectroscopy—The NMR spectra were acquired on Avance 800 and 600 Bruker spectrometers operating at a proton nominal frequency of 800.13 and 600.13 MHz, respectively. A QXI probe has been used on the Avance 800 spectrometer, and a triple resonance (TXI) 5-mm probe has been used on the 600 spectrometer. All probes were equipped with pulsed field gradients along the $z$ axis. Total correlation spectroscopy (25, 26) spectra were recorded on the 600-MHz spectrometer with a spin-lock time of 100 ms, a recycle time of 1 s and a spectral window of 14 ppm. Two-dimensional NOESY maps (27, 28) were acquired on the 800-MHz spectrometer with a mixing time of 100 ms, a recycle time of 1 s, and a spectral window of 14 ppm.

On the $^{15}$N apo-Ccc2a sample, a two-dimensional $^{15}$N-$^1$H heteronuclear single quantum coherence (29–31) map was obtained at 800 MHz with an INEPT delay of 2.66 ms, a recycle time of 1 s and spectral windows of 14 and 33 ppm for the $^1$H and $^{15}$N dimensions, respectively. A three-dimensional NOESY-$^{15}$N heteronuclear multiple quantum coherence experiment (32) was recorded with 290 ($^1$H) $\times$ 96 ($^{15}$N) $\times$ 2048 ($^1$H) data points on the 600-MHz spectrometer. The INEPT delay was set to 5.4 ms, the mixing time was 100 ms, and the carrier frequency was set in the center of the amide proton region, at 7.35 ppm. Spectral windows of 14, 14, and 29 ppm were used for the direct $^1$H dimension and the indirect $^1$H and $^{15}$N dimensions. A HNHA experiment (33) was performed at 600 MHz to determine $^3$J$_{HNH}$, coupling constants. The spectrum was recorded as a 128 ($^1$H) $\times$ 80 ($^{15}$N) $\times$ 2048 ($^1$H) data set using pulsed field gradients along the $z$ axis. The mixing time was 100 ms. Spectral windows of 14, 14, and 29 ppm were used, respectively, for direct $^1$H dimension and the indirect $^1$H and $^{15}$N dimensions.

The abbreviations used are: MES, 4-morpholineethanesulfonic acid; r.m.s., root mean square; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; INEPT, insensitive nuclei enhanced by polarization transfer; WATERGATE, water suppression by gradient-tailored excitation; REM, restrained energy minimization; mbd4, metal-binding domain 4.
For all the experiments, quadrature detection in the indirect dimensions was performed in the time-proportional phase incrementation mode (28), and water suppression was achieved through WATERGATE sequence (34). All two-dimensional data consisted of 4K data points in the acquisition dimension and of 1K experiments in the indirect dimension. All three- and two-dimensional spectra were collected at 298 K, processed using the standard Bruker software (XWINNMR), and analyzed on IBM RISC 6000 computers through the XEASY program.

Constraints Used in Structure Calculations—The peaks used for the structure calculations were integrated in the two-dimensional NOESY map acquired at 298 K in H2O. Intensities of dipolar connectivities were converted into upper distance limits, to be used as input for structure calculations, by using the approach provided by the program CALIBA (35). The calibration curves were adjusted iteratively as the structure calculations proceeded. Stereospecific assignments of diastereotopic protons were obtained using the program GLOMSA (35). 3JHNH coupling constants were correlated to the backbone torsion angle $\phi$ by means of the appropriate Karplus curve (33). These angles were used as constraints in the DYANA calculations and restrained energy minimization (REM) refinement.

Hydrogen bond constraints were introduced for backbone amide protons that were found to be within hydrogen bond distance and to have the correct orientation with respect to hydrogen bond acceptors in structural models obtained without inclusion of these constraints. The distance between the NH proton and the hydrogen bond acceptor was constrained to be in the 1.8–2.4 Å interval by inclusion of the corresponding upper and lower distance limits in structure calculations. In addition, upper and lower distance limits of 3.0 and 2.7 Å between the N and the acceptor atoms were also included.

Structure Calculations—The structure calculations were performed using DYANA (36). 200 random conformers were annealed in 10000 steps using NOE and 3J values (when available) constraints. The 35 conformers with the lowest target function constitute the final family. The copper ion was included in the calculations by adding a new residue in the amino acid sequence, formed by a chain of dummy atoms that have their van der Waals radii set to 0 so that it can freely penetrate into the protein and one atom with a radius of 1.4 Å, which mimics the copper ion. The sulfur atoms of Cys13 and Cys16 were linked to the metal ion through upper distance limits of 2.5 Å. This approach does not impose any fixed orientation of the ligands with respect to the copper.
REM was then applied within the molecular mechanics and dynamic module of SANDER (37). The force field parameters for the copper(I) ion were adapted from similar systems (38). In particular, no constraint on the S(Cys13)-Cu-S(Cys16) angle was used. The values of NOE and torsion angle potentials were calculated with force constants of 50 kcal mol$^{-1}$ Å$^{-2}$.

The program CORMA (39), which is based on relaxation matrix calculations, was used to back calculate the NOESY cross-peaks from the calculated structure to check the consistency of the analysis. The quality of the structure was evaluated in terms of deviations from ideal bond lengths and bond angles and through Ramachandran plots, obtained using the programs PROCHECK (40) and PROCHECK-NMR (41). Structure calculations and analyses were performed on IBM RISC 6000 computers.

RESULTS

Sequence-specific Assignment of Apo-Ccc2a and Cu(I)-Ccc2a—The $^1$H NMR spectra of apo-Ccc2a and Cu(I)-Ccc2a are reported in Fig. 1, A and B, respectively. The most relevant difference between apo and Cu(I) form is observed in the HN region. In particular, the HN resonance of Thr12 is broader in the apo form than in Cu(I)-Ccc2a and has a change in the shift from 9.72 ppm, in the apo form, to 9.21 ppm, in the Cu(I) form. In the $^1$H NMR spectrum of the Cu(I) form, it is also possible to identify the HN resonance of Ala15 at 9.50 ppm, a residue very close to the cysteine binding motif, that cannot be detected in the apo form.

Assignments of the resonances of apo-Ccc2a started from the analysis of the $^{15}$N heteronuclear single quantum coherence map, which allowed the identification of the $^{15}$N and $^1$HN resonances. Then through the analysis of the three-dimensional NOESY-heteronuclear multiple quantum coherence and of two-dimensional NOESY and total correlation spectroscopy, the sequence-specific assignment was performed. The assignment of the Cu(I)-Ccc2a derivative was performed through the analysis of two-dimensional NOESY and total correlation spectroscopy maps only. Resonances for all 72 residues both for the apo and Cu(I) forms of Ccc2a have been assigned. In the apo and in the Cu(I) protein, about 97 and 98% of the proton resonances, respectively, could be located in the maps, and all of the $^{15}$N resonances have been assigned, with the exception of Ser14 and Ala15 in the apo form and with the exception of Ser14 in the Cu(I) form. The $^1$H and $^{15}$N resonance assignments of the apo and Cu(I) forms are reported in Tables I and II of the supplementary materials, respectively.2

Secondary Structure from NMR Data—The elements of secondary structure were identified by analyzing the pattern of assigned NOEs. Backbone short, medium range NOEs were used to generate Fig. 2, A and B. From their analysis, it is apparent that the secondary structure is not significantly af-

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2 Supplementary materials may be accessed at the CCRM site, under Structural Biology, on the World Wide Web.
REM indicates the energy-minimized family of 35 structures; <REM> is the energy-minimized average structure obtained from the coordinates of the individual REM structures.

| Parameters                                      | REM   | <REM>  |
|-------------------------------------------------|-------|-------|
| r.m.s. violations per experimental distance constraint (Å) | 0.0092 ± 0.0025 | 0.0074 |
| Intrasidue (257)                                 |       |       |
| Sequential (453)                                 | 0.0066 ± 0.0010 | 0.0063 |
| Medium range' (511)                              | 0.0082 ± 0.0010 | 0.0073 |
| Long range (706)                                 | 0.0058 ± 0.0013 | 0.0047 |
| Total (1970)                                     | 0.0073 ± 0.0008 | 0.0062 |

Average number of violations per structure

|                      |       |
|----------------------|-------|
| Intrasidue           | 3.6 ± 1.3 |
| Sequential           | 5.5 ± 1.4 |
| Medium range'        | 6.5 ± 1.2 |
| Long range           | 6.2 ± 2.2 |
| Total                | 21.7 ± 2.7 |

Average no. of NOE violations larger than 0.3 Å

|                      |       |
|----------------------|-------|
| Intrasidue           | 0.00 ± 0.00 |
| Sequential           | 0.00 ± 0.00 |
| Medium range'        | 0.00 ± 0.00 |
| Long range           | 0.00 ± 0.00 |
| Total                | 0.00 ± 0.00 |

Average NOE and torsion deviations (Å²)

|                      |       |
|----------------------|-------|
| Average r.m.s. deviations (Å²) | 0.14 ± 0.02 |
| Residues in most favourable regions (%) | 80.0 |
| Residues in generously allowed regions (%) | 17.3 |
| Residues in disallowed regions (%) | 2.0 |
| Residues in disallowed regions (%) | 0.8 |

The number of experimental constraints for each class is reported in parenthesis. Medium range distance constraints are those between residues (i, i + k), (j, j + k). As it results from the Ramachandran plot analysis.

N-terminal

C-terminus

Cys 13

Cys 16

**Fig. 5.** Backbone atoms for the solution structure of apo-Ccc2a as a tube with variable radius, proportional to the backbone r.m.s. deviation value of each residue. The side chains of Cys13 and Cys16 are also shown. The figure was generated with the program MOLMOL (45).

Solution Structure Calculations and Analysis of apo-Ccc2a—A total of 3785 NOESY cross-peaks were assigned, integrated, and transformed in upper distance limits with the program CALIBA (35). They corresponded to 2314 unique upper distance limits, of which 1970 were found to be meaningful (nonmeaningful distance constraints are those that cannot be violated in any structure conformation and those involving proton pairs at fixed distance). The number of NOEs per residue is reported in Fig. 4A. The average number of NOEs per residue is 32 for apo-Ccc2a, of which 27 are meaningful. 35 $^3$J_HH, couplings were obtained from the HHNHA three-dimensional spectrum, which were translated into dihedral angles through the standard equation. For $^3$J_HH values of >8 and <4.5 Hz, the dihedral angle was assumed to be between −30° and −30°, respectively (42, 43). Hydrogen bond constraints for all amide protons were used at later stages of structural calculations. A total of 38 proton pairs were stereospecifically assigned through the program GLOMSA (35). The constraints used for structure calculations and the stereospecific assignments are reported in the supplementary materials.

The 35 conformers constituting the final DYANA family had an average target function of 0.64 ± 0.10 Å² and average r.m.s. deviation values over all of the 72 residues with respect to the mean structure of 0.39 ± 0.05 Å for the backbone and of 0.80 ± 0.07 Å for the heavy atoms. The family of conformers was then subjected to further refinement through energy minimization (37). The REM family has an average target function of 0.14 ± 0.02 Å², to which the NOE contribution is 0.13 Å², while the r.m.s. values for the family with respect to the mean structure are 0.38 ± 0.06 Å for the backbone and 0.82 ± 0.07 Å for the heavy atoms for all of the amino acids in the sequence. The r.m.s. deviation values per residue of the final REM family to the mean structure are shown in Fig. 4B.

The final family of conformers was analyzed with PROCHECK-NMR (41), and results are reported in Table I. According to this program, the secondary structure elements in the energy-minimized mean structure involve residues 2–9 (a1), 13–26 (a2), 29–36 (b2), 40–47 (b3), 51–63 (a2), and 65–70 (b4). Analysis of the NOE patterns has led to somewhat similar
conclusion (see above). It is worth noting that in contrast to NOE secondary structure analysis above, Cys13 is assigned to helix α1 in the energy-minimized mean structure of the apo form. In this energy-minimized average structure, 82.1% of the residues are in the most favored regions of the Ramachandran plot, and 17.9% of the residues are in the allowed regions. No residues are in the disallowed regions (Table I).

The structure of apo-Ccc2a, shown in Fig. 5, is well defined all over its sequence. All of the α-helices and antiparallel β-sheet are very well defined, with an average backbone r.m.s. deviation lower than 0.38 Å. The largest backbone r.m.s. deviation values are obtained for residues 12–14 and residue 48. The high r.m.s. deviation values of residues 12–14 (0.74 Å) are due to the paucity of NOEs in this region (Fig. 4A) that constitutes loop 1 and the beginning of helix α1. Indeed, this region contains Ser14 and Ala15, whose HN resonances have not been identified and are a break in the sequential connectivities. This region includes Cys13, one of the copper ligands. The side chain of Cys13 is disordered in the apo state and spans different conformations due to the small number of NOEs. On the contrary, the other copper ligand Cys16, which belongs to the first α-helix, is very well defined (r.m.s. deviation BB, 0.22 Å; r.m.s. deviation HA, 0.37 Å). The side chains of Cys13 and Cys16 are very close; the distance between the two sulfur atoms in the average structure is 5.6 Å.

Solution Structure Calculations and Analysis of Cu(I)-Ccc2a—A total of 3866 NOESY cross-peaks were assigned, integrated, and transformed in upper distance limits with the program CALIBA (35). They corresponded to 2338 upper distance limits, of which 1944 were found to be meaningful. The number of NOEs per residue is reported in Fig. 6A. The average number of NOEs per residue is 32, of which 27 are meaningful. Hydrogen bond constraints for 22 amide protons were used at later stages of structural calculations. A total of 47 proton pairs were stereospecifically assigned through the program GLOMSA (35). The constraints used for structure calculations and the stereospecific assignments are reported in the supplementary materials.2

The 35 conformers constituting the final DYANA family had an average target function of 0.48 ± 0.11 Å2 and an average r.m.s. deviation value over all of the 72 residues with respect to the mean structure of 0.39 ± 0.06 Å for the backbone and 0.79 ± 0.04 Å for the heavy atom. The family of conformers was then subjected to further refinement through energy minimization (37). The REM family has an average target function of 0.26 ± 0.03 Å2. The average r.m.s. deviation values for the family with respect to the mean structure are 0.36 ± 0.06 Å for the backbone and 0.79 ± 0.05 Å for the heavy atoms for all of the amino acids in the sequence. The r.m.s. deviation values per residue of the final REM family to the mean structure are shown in Fig. 6B.

The final family of conformers was analyzed with PROCHECK-NMR (41), and results are reported in Table II. The secondary structure elements in the energy-minimized mean structure involve residues 2–9 (β1), 13–26 (α1), 29–36 (β2), 40–47 (β3), 51–62 (α2), and 65–70 (β4). Analysis of the NOE patterns has led to a similar conclusion (see above). In the energy-minimized average structure, 83.6% of the residues are in the most favored regions of the Ramachandran plot, 13.4% of the residues are in the allowed regions, and 3.0% are in the generously allowed regions. No residues are in the disallowed regions (Table II).

The structure of Cu(I)-Ccc2a, shown in Fig. 7, is also well defined all over its sequence. All of the α-helices and antiparallel β-sheet are very well defined and also the loop that includes the copper binding site is more defined than in the apo form. Indeed, the backbone r.m.s. deviation values with respect to the mean structure for residues in the less well defined region of apo (10–14) decrease from 0.64 Å in the apo form to 0.27 Å in the Cu(I) form, and the side chain of Cys13 that in the apo form is disordered (r.m.s. deviation BB, 0.80 Å; r.m.s. deviation HA, 1.17 Å) is very well defined in the Cu(I) form (r.m.s. deviation BB, 0.25 Å; r.m.s. deviation HA, 0.62 Å).

DISCUSSION

Comparison between the Structures of Apo- and Cu-Ccc2a—The solution structure of S. cerevisiae Ccc2a exhibits the βαβαβ folding pattern typical of copper chaperones (44) and the fourth metal-binding domain of the Menkes’ disease protein (20). In the Cu-Ccc2a structure, the copper ion coordinates two of the six cysteine residues, Cys13 and Cys16. The global r.m.s. deviation values between the Cu(I)-bound and apo-averaged minimized structures are 0.86 and 1.35 Å for backbone and heavy atoms, respectively. The r.m.s. deviation per residue is reported in Fig. 8 (dotted dashed line) and is compared with the r.m.s. deviation values per residue for each family (apo-Ccc2a (solid line) or Cu(I)-Ccc2a (dotted line)) and the sum of the r.m.s. deviation values of the two families (dashed line). The structure definition within each family of structures is overall very good and comparable between the two families; thus, a meaningful comparison can be undertaken. The highest backbone r.m.s. deviation value between the two structures is 1.21 Å.
Solution Structure of Cu(I) and Apo Forms of Ccc2a

**TABLE II**

Statistical analysis of the final REM family and the mean structure of Cu(I)-Ccc2a from S. cerevisiae

REM indicates the energy-minimized family of 35 structures; <REM> is the energy-minimized average structure obtained from the coordinates of the individual REM structures.

| Parameters                                      | REM (35 structures) | <REM>   |
|-------------------------------------------------|---------------------|---------|
| r.m.s. violations per experimental distance constraint (Å)¹ | 0.0153 ± 0.0016     | 0.0160  |
| Intraresidue (272)                               | 0.0072 ± 0.0013     | 0.0087  |
| Sequential (511)                                 | 0.0103 ± 0.0015     | 0.0102  |
| Medium range ² (490)                             | 0.0102 ± 0.0012     | 0.0106  |
| Long range (671)                                 | 0.0105 ± 0.0008     | 0.0106  |
| Total (1944)                                     | 0.0105 ± 0.0008     | 0.0106  |
| Average number of violations per structure       | 10.4 ± 2.4          | 11      |
| Intraresidue                                     | 5.1 ± 1.5           | 4       |
| Sequential                                       | 9.3 ± 1.9           | 11      |
| Medium range ²                                   | 12.2 ± 2.0          | 13      |
| Long range                                       | 37.0 ± 4.2          | 39      |
| Total                                            |                     |         |
| Average no. of NOE violations larger than 0.3 Å   | 0.00 ± 0.00         | 0       |
| Largest residual NOE violation (Å)                | 0.20                | 0.13    |
| Average NOE deviations (Å²)                       | 0.26 ± 0.03         | 0.25    |
| Structural analysis                              | 79.0                | 83.6    |
| Residues in most favourable regions              | 18.6                | 13.4    |
| Residues in allowed regions                      | 2.0                 | 3.0     |
| Residues in generously allowed regions           | 0.3                 | 0.0     |
| Residues in disallowed regions                   | 0.3                 | 0.0     |

¹ The number of experimental constraints for each class is reported in parenthesis.
² Medium range distance constraints are those between residues (i, i + 2), (i, i + 3), (i, i + 4), and (i, i + 5).
³ As it results from the Ramachandran plot analysis.

(490) 0.0103 0.0102
(511) 0.0102 0.0106
(671) 0.0105 0.0106
(1944) 0.0105 0.0106

Average number of violations per structure

Average no. of NOE violations larger than 0.3 Å

Average NOE deviations (Å²)

Structural analysis

Residues in most favourable regions

Residues in allowed regions

Residues in generously allowed regions

Residues in disallowed regions

These numbers indicate a meaningful difference between the two structures. Analysis of side chains reveals a significant r.m.s. deviation difference for Thr17, which is in the copper binding pocket. Indeed, when copper is bound, the side chain of Thr17 rotates closer to the backbone oxygen of Cys13, and a hydrogen bond interaction can be readily formed, since it is found in many conformers of the family. This interaction can presumably be important to determine the optimal conformation of Cys13 in the copper-bound state of the protein.

The backbone r.m.s. deviation values for each secondary structure element are obtained either superimposing all residues (global r.m.s. deviation) or three residues at a time (local r.m.s. deviation) and are reported in Table III. The highest r.m.s. deviation values are found for strand β2, helix α2, and loops 1, 3, and 4. The r.m.s. deviation values for all of these regions but the last drop when the two structures are superimposed locally, indicating that the structural differences in these regions originate from some global translational displacements, since the local conformations are well maintained. On the contrary, loop 4 shows the highest difference in the local r.m.s. deviation, and these local difference are due to variations of dihedral angles.

Comparing the copper binding region in apo- and Cu(I)-Ccc2a, helix α1 (containing Cys16 and Cys13) is very well defined in both forms, while loop 1, which is involved in the copper binding pocket, is less defined in the apo form (see Fig. 8). The conformation of Cys16 is well defined in both structures, being the same in both the apo and metal-bound proteins, while Cys13 is more disordered in the apo form than in the metal-bound protein. Indeed, in the apo-Ccc2a structure, loop 1 becomes more disordered (Fig. 5), and the amide NHs of residues 14 and 15, belonging to helix α1, are not detected anymore, probably due to solvent exchange or to an increased mobility. The lower number of NMR constraints in the metal-binding pocket (i.e. residues 12–17) of the apo form might be the result of increased conformational flexibility in this region with respect to the metal-bound state. Indeed, different NOE intensity for proton pairs at fixed distances of Cys13 and Cys16 of Ccc2a has been observed in the two forms; Cys13 experiences a decrease in intensity, while those of Cys16 show roughly the same intensity, suggesting that the binding of copper produces higher order in this region.

Upon copper release, the most important change in the shallow binding pocket is observed for the side chain of Cys13; the sulfur flips away from the hydrophobic interior toward the surface (Fig. 9). Calculations of the solvent accessibility on the apo-Ccc2a structure show that exposure of Cys13 is remarkably increased (from 29% in the copper-bound form to 36% accessible surface in the apo form), while Cys16 is always more buried (with 7% in the copper-bound form and 11% accessible surface in the apo form).

The changes in HN and Hα chemical shifts observed between the apo and the Cu(I)-bound forms are plotted for each residue in Fig. 10, A and B, respectively, and confirm that only residues

![Fig. 7. Backbone atoms for the solution structure of Cu(I)-Ccc2a as a tube with variable radius, proportional to the backbone r.m.s. deviation value of each residue. The side chains of Cys13, Cys16, and the Cu(I) ion are also shown. The figure was generated with the program MOLMOL (45).](image-url)
very close to the metal binding pocket are affected by the presence of the copper ion. Indeed, several highly conserved hydrophobic residues appear to play a role in maintaining an optimal metal-ligand conformation. These include Phe64 and Ile20, which are in Van der Waals contact with Cys16, and Leu37, which contacts Cys13 and is highly conserved between the Ccc2, Wilson, and Menkes domains as well as Atx1 and CCS. While these side chains can stabilize the metal binding loop, no significant conformational changes are detectable either as differences between their chemical shifts (Fig. 10, A and B) or as differences between the two average structures (Fig. 9).

The methionine in the GM\textsuperscript{X}CXX metal binding loop (Met\textsuperscript{11}) is highly conserved between other N-terminal, membrane-tethered domains of heavy metal ATPases and small metallochaperones alike. Of all of the residues in the protein, Met\textsuperscript{11} shows the greatest change in \textsuperscript{1}H\textalpha resonances upon copper binding.
(Fig. 10, A and B), but it is not directly involved in metal ion coordination. Instead, it points toward the hydrophobic core of the protein (Fig. 9). The contacts between this side chain and the residues surrounding it change little between the apo and Cu(I)-bound forms (Fig. 9) as judged from the observed medium and long range NOEs; however, copper occupancy does change the conformation of this residue. These results suggest that Met\textsuperscript{11} acts as a hydrophobic tether that anchors the metal-binding loop via hydrophobic interactions with Leu\textsuperscript{37}, Phe\textsuperscript{64}, and Ile\textsuperscript{20}. The slight movements in the region are probably coupled to stabilization of the GMXCCXC domain in the presence of bound metal ion.

Comparison between the Solution Structures of the Fourth Metal Binding Cytosolic Domain from Menkes Copper-transporting ATPase and Cu(I)-Ccc2a—The structure of Cu(I)-Ccc2a is similar to the Ag(I)-bound solution structure of the fourth metal-binding domain (mbd4) of Menkes ATPase (20), the human homologue of \textit{S. cerevisiae} Ccc2. A sequence alignment of Ccc2a with this protein (Fig. 11A) reveals 29% identity. The structures were superimposed according to the sequence alignment. Both proteins show the same ferrodoxin-like fold (Fig. 11B). The overall backbone r.m.s. deviation value is 1.15 Å between Cu(I)-Ccc2a and Ag(I)-mbd4. The major structural differences are represented by simple translations of the secondary structure elements. All \(\alpha\)-helices and \(\beta\)-strands are well superimposed or show slight displacements except for the short C-terminal \(\beta\)-strand. The conserved hydrophobic residues Ile\textsuperscript{20}, Leu\textsuperscript{37}, and Phe\textsuperscript{64} presumably important for maintaining optimal metal-binding loop conformation, have the same conformation in the two structures. The biggest differences are observed in loop 1 and loop 4. Indeed, loop 1 differs in the vicinity of Cys\textsuperscript{13}, which does not superimpose with Cys\textsuperscript{14} of mbd4. The conformation of the other cysteine (Cys\textsuperscript{16} and Cys\textsuperscript{17}, respectively) is very similar between the two structures. In Cu(I)-Ccc2a, Cys\textsuperscript{13} is the first residue of helix \(\alpha1\), while in mbd4, Cys\textsuperscript{14} belongs to the metal binding loop 1 (Fig. 11B). The stabilization of the helix and the variation in the conformation of loop 1 in Cu(I)-Ccc2a with respect to Ag(I)-mbd4 may be dictated by the different chemical properties of Cu(I) versus Ag(I) and by the different kinds of residues in the vicinity of the loop 1 region.

It is worth noting that in the refinement of the Ag(I)-mbd4 solution structure, a linear digonal coordination was imposed by modifying the AMBER force field (20). In the data refinement for the Cu(I)-Ccc2a family of conformers, no S-Cu-S angle constraints were included. The resulting value of the S-Cu-S angle is 119 ± 29°. When the S-Cu-S angle is constrained to linearity, a digonal copper thiolate center can be refined. This suggests that coordination is not rigid but that Cu(I) in this environment may represent a mixture of coordination numbers of two and higher or that a bent two-coordinate S-Cu-S geometry is adopted. No other protein atoms appear close enough to be the third ligand in Ccc2a. A third coordinating atom, if there is one at all, can come only from an exogenous ligand, such as a buffer component. While no low molecular weight thiol (e.g. DTT, GSH) are present in the sample, other buffer components could be coordinating to the Cu(I). The NMR data here do not allow us to distinguish between these possibilities.

Comparison between the Solution Structures of Ccc2a and Atx1 for both Apo and Cu(I) States—The solution structures of Cu(I)- and apo-Atx1 from \textit{S. cerevisiae} have been recently solved (46). The global folding of Atx1 is very similar to that of Ccc2a. When superimposing Cu(I)-Ccc2a and Cu(I)-Atx1 structures according to the sequence alignment (see Fig. 11A), the overall backbone r.m.s. deviation value is 2.8 Å. Helix \(\alpha2\); \(\beta\)-strands 1, 2, 3; and loops 3 and 5 are well superimposed (Fig. 11C). The major structural differences are translations or changes in length of the secondary structure elements and of the loops. The largest differences are found for helix \(\alpha1\), \(\beta\)-strand 4, and loops 1, 2, and 4. The length of helix \(\alpha1\) in the Cu(I) form of both proteins is the same, but in Cu(I)-Atx1 this helix spans from residue 17 to 30, while in the Cu(I)-Ccc2a the same helix is slightly offset, starting from residue 13 and ending at 26. Loop 1 exhibits conformational differences, in particular close to Cys\textsuperscript{13}. This cysteine in Cu(I)-Atx1 belongs to loop 1, whereas in Cu(I)-Ccc2a it belongs to helix \(\alpha1\). These differences in secondary structure lead to distinct positioning of the metal-binding cysteines (see Fig. 11C) and allow the copper ion to be more exposed to solvent in Ccc2a with respect to Atx1 (see below). In addition, the offset of helix \(\alpha1\), the different loop size, and the presence of Pro\textsuperscript{31} in the Atx1 sequence (see Fig. 11A) determine a large conformational difference in loop 2, which is more extended toward the surface in the Cu(I)-Atx1 structure.

The structures of apo-Ccc2a and apo-Atx1 show an overall backbone r.m.s. deviation value of 2.93 Å (Fig. 12) and exhibit more differences than do the two copper-loaded forms (Fig. 11C). The \(\beta\)-strands 1, 2, and 3 and the loops 3 and 5 are well superimposed for the apoproteins; however, differences in other secondary structure elements are apparent. For example, helix \(\alpha1\) is one full turn shorter in apo-Atx1 than in apo-Ccc2 (Fig. 12). Helix \(\alpha2\), which superimposes well in both forms of Atx1 (46), is translated away from the copper-binding site in apo-Atx1 relative to its position in apo-Ccc2a (Fig. 12). While the structure of Atx1 undergoes changes as a function of copper capture and release, the Ccc2a structure remains relatively invariant, suggesting that the metal site in apo-Ccc2a is more preorganized than in apo-Atx1. This is one of the key structural differences between the Atx1 metallochaperone family and the...
homologous metal-binding domains of the copper-transporting P-type ATPases.

Another important difference between these copper donor and acceptor proteins is apparent in Fig. 13, A and B, namely the accessibility of the copper to incoming nucleophiles. The solvent-accessible surface of the Cu(I) center in Cu-Atx1 is 9% but over 18% in Cu-Ccc2a. Access to the Cu(I) in Atx1 is partially obscured by Lys65, which is highly conserved among copper chaperones. In Ccc2a, this residue corresponds to Phe64, and the structure reveals that it extends into the hydrophobic core, away from the surface. In fact, a Phe (or a Tyr) is highly conserved among all of the domains of the CPx ATPases, while Lys65 is conserved among the diffusible Atx1 and CCS copper chaperone proteins (44). The Phe64 side chain packs adjacent to the conserved Met11 and is anticipated to contribute to the stability of the metal binding loop in Ccc2a. In contrast, the lysine at this position in Atx1 (Lys65) can access several conformations and may play a role in partner recognition and control of metal ion access.

**Electrostatic Surface and Structural Implications for Interaction with Physiological Partners—Partnership between Atx1 and Ccc2 in vivo requires several basic residues, which cluster in several sites on the surface of Atx1 (18). The structure of Ccc2a reveals a complementary set of acidic residues. The surface electrostatic potential distribution was generated with the program MOLMOL (45) using the refined coordinates of the Cu(I)-Ccc2a structure (Fig. 13A). This protein has a region comprising several glutamate and aspartate residues that generate a negatively charged face on the protein surface in the proximity of the copper binding region. These residues are Glu67, Glu57, Glu60, Asp65, Asp61, and Asp63. In particular, Asp65, Asp61, and Glu60 are conserved in the metal-binding domain of a large number of metal-transporting ATPases. The surface electrostatic potential distribution was also generated for human mbd4 (Fig. 13B). A very similar negative patch, formed by Asp67, Glu62, and Asp63, which are conserved in the homologous yeast Ccc2a, and Asp10 is observed on one surface of the protein. The electrostatic potential distribution of Cu(I)-Atx1 is almost complementary and shows the presence of 7 lysines (46), in positions 24, 28, 59, 61, 62, 65, and 71, which generate a positively charged face on the molecule, as shown in Fig. 13C. It is known that the ATPase Ccc2 is the target of copper delivery by Atx1 (15, 16, 24). The interaction between Atx1 and Ccc2a could be therefore determined by the complementary attractions between the positive cluster in Atx1 and the negative region in Ccc2a.

Finally, a large “nonpolar” area is present for both Ccc2a and mbd4 proteins adjacent to the metal binding site in a region composed of residues Leu37, Val39, and Ile40. In fact the side chain of Val38 extends into the solvent, and in Atx1 this residue is replaced with Glu (Fig. 11A). These solvent-exposed nonpolar

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**FIG. 11.** A, sequence alignment of the Ccc2a amino acid sequence from *S. cerevisiae* with the sequences of the fourth metal-binding domain from Menkes-transporting ATPase (mbd4) (Protein Data Bank accession number 1aw0) (20) and of Atx1. The positions of the Ccc2a secondary structure elements (as found in the mean Cu(I) structure) are shown at the top, β-strands are in blue, α helices are shown in orange, and loop regions are in yellow. Each sequence is color-shaded according to secondary structure element, as found in their metal-bound structures. Residues that are highly similar or conserved are indicated, respectively, by the ° and * below the sequences. B, comparison of the backbone of Cu(I)-Ccc2a (blue) and Ag(I)-mbd4 (green) structures (20). C, comparison of backbone of Cu(I)-Ccc2a (blue) and Cu(I)-Atx1 (green). The copper ion and the cysteine ligands are also shown. The secondary structure elements are indicated.

**FIG. 12.** Comparison of the backbone of apo-Ccc2a (blue) and apo-Atx1 (green). The cysteines involved in the copper binding are indicated in blue and green for apo-Ccc2a and apo-Atx1 structures, respectively.
regions may be important after transfer of copper from Atx1 to Ccc2a. For instance, in the subsequent steps of the original copper-trafficking mechanism, Cu(I) is transferred to a cytosolic face of the membrane-spanning ATPase (15). The fate of the Cu(I) bound by Ccc2a is not known, but it is speculated to be transferred to a cation translocation site within the membrane portions of Ccc2, such as the canonical CPC motif. In an extension of this model, we propose that a hydrophobic patch on mobile N-terminal domains of Ccc2a or mdb4 can serve as a site for interaction with other hydrophobic sites essential for copper movement across the membrane. After transfer and ATP-induced conformational changes, the Cu(I) would be subsequently released to the interior of the ATPase-containing vesicle. Tests of this model are under way.

CONCLUSION

This paper presents the first report of the solution structure of a copper-transporting ATPase domain bound to the native metal ion Cu(I). The fold is similar to its physiological partner, the cytoplasmic copper chaperone Atx1 (17) and is even more similar to the structure of a metal-binding domain of one of its human orthologs, the Menkes’ disease protein (20). Unlike other structurally characterized members of this family, both of the metal-binding cysteines of Ccc2a are located in a helical region, and the metal is more accessible to solution. Comparisons of the cytosolic domains of the transporters with the chaperones reveal complementary features that are important in their respective copper-trafficking functions. First, unlike Atx1, few conformational changes are observed in Ccc2a upon copper release, and the metal binding region is well defined in both the apo and holo forms of this domain. In the case of apo-Atx1, the amide NH for residues close to the copper binding, in particular Cys13, are not detected, and this disappearance could be ascribed to an increased disorder/mobility of the loop region. In contrast, apo-Ccc2a exhibits a higher degree of order and both amide NH of the metal-binding cysteines are detected. Furthermore, NOE signal intensity is significant for both Cys13 and Cys16 in apo-Ccc2. Together, these results suggest a significantly greater stability of the Ccc2a domain with respect to Atx1.

Finally, there are several mechanistically important aspects of the Ccc2a surface that differ from Atx1. One prominent feature is a negative patch (Fig. 13) formed by glutamate and aspartate residues in the proximity of the copper binding region that could interact with complementary array of lysine and arginine residues on the Atx1 (17). A phenylalanine is found in Ccc2 in place of the pivotal Lys85 residue of Atx1. Side chains at this site abut the metal binding loop and are anticipated to not only play an important role in determining the stability of this region of the protein but are likely to control key steps in the metal transfer mechanism.

REFERENCES

1. Solioz, M. & Vulpe, C. (1996) Trends Biochem. Sci. 21, 237–241
2. Vulpe, C., Levinson, B., Whitney, S., Paekman, S. & Gitschier, J. (1995) Nat. Genet. 3, 7–13
3. Chelly, J., Tumer, Z., Tonnesen, T., Petterton, A., Ishikawa-Brush, Y., Tommerup, N., Monaco, A. P. & Horn, N. (1993) Nat. Genet. 3, 14–19
4. Mercer, J. F., Livingston, J., Hall, B., Paynter, J. A., Bogy, C., Chandrasekharappa, S., Lackhart, P., Grimes, A., Bhave, M., Siemieniak, D. & Glover, T. W. (1993) Nat. Genet. 3, 20–25
5. Buil, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R. & Cox, D. W. (1993) Nat. Genet. 5, 327–337
6. Tanzi, R. E., Petrukhin, K., Chernov, I., Pellequer, J. L., Wasco, W., Ross, B., Romano, D. M., Parano, E., Pavone, L., Braztowicz, L. M., Devoto, M., Peppercorn, J., Bush, A. L., Sternlieb, I., Pirastu, M., Gusella, J. F., Evgrafov, O., Penchaszadeh, G. K., Honig, B., Edelman, J. S., Soares, M. B., Scheinberg, I. H. & Gilliam, T. C. (1993) Nat. Genet. 5, 344–350
7. Fu, D., Beeler, T. J. & Dunn, T. M. (1996) FEBS Lett. 11, 283–289
8. Yuan, D. S., Stearman, R., Dancis, A., Dunn, T., Beeler, T. & Klausner, R. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2632–2636
9. Stearman, R., Yuan, D., Yamaguchi-Iwan, Y., Klausner, R. D. & Dancis, A. (1990) Science 271, 1552–1557
10. Hung, I. H., Suzuki, M., Yamaguchi, Y., Yuan, D. S., Klausner, R. D. & Gitlin, J. D. (1997) J. Biol. Chem. 272, 21461–21466
11. Forbes, J. R., Bai, G. & Cox, D. W. (1999) J. Biol. Chem. 274, 12408–12413
12. Larin, D., Mekins, C., Das, K., Ross, B., Yang, A. S. & Gilliam, C. T. (1999) J. Biol. Chem. 274, 28497–28504
13. Yamaguchi, Y., Heiny, M. E., Suzaki, M. & Gitlin, J. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14030–14035
14. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T. & Kaplan, J. H. (1997) J. Biol. Chem. 272, 14030–14035
15. Pufahl, R., Singer, C. P., Fearis, K. L., Lin, S.-J., Schmidt, P. J., Fahmi, C. J., Ciszewski Calutta, V., Penner-Hahn, J. E. & O’Halloran, T. V. (1997) Science 278, 853–856
16. Huffman, D. L. & O’Halloran, T. V. (2000) J. Biol. Chem. 275, 16161–16164
17. Rosenzweig, A. C., Huffman, D. L., Hou, M. Y., Wernimont, A. K., Pufahl, R. A. & O’Halloran, T. V. (1999) Structure 7, 605–617
18. Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D. L., O’Halloran, T. V. & Ciszewski Calutta, V. (1999) J. Biol. Chem. 274, 15041–15045
19. Lamb, A. L., Wernimont, A. K., Pufahl, R. A., Calutta, V. C., O’Halloran, T. V. & Rosenzweig, A. C. (1999) Nat. Struct. Biol. 6, 724–729
20. Gitschier, J., Moffat, B., Reilly, D., Wood, W. I. & Fairbrother, W. J. (1998) Nat. Struct. Biol. 5, 47–54
21. Steele, R. A. & Opella, S. J. (1997) Biochemistry 36, 6885–6895
22. Eriksson, P.-O. & Sahlman, L. (1993) J. Biomol. NMR 3, 613–626
23. Wimmer, R., Herrmann, T., Solioz, M. & Wuthrich, K. (1999) J. Biol. Chem. 274, 22597–22603
24. Lin, S. J., Pufahl, R., Dancis, A., O’Halloran, T. V. & Calutta, V. C. (1997) J. Biol. Chem. 272, 9215–9220
25. Sax, A. & Davis, D. G. (1985) J. Magn. Reson. 63, 207–213
26. Griesinger, C., Otting, G., Wuthrich, K. & Ernst, R. R. (1988) J. Am. Chem. Soc. 110, 7870–7872
27. Macura, S., Wuthrich, K. & Ernst, R. R. (1982) J. Magn. Reson. 47, 351–357
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28. Marion, D. & Wuthrich, K. (1983) Biochem. Biophys. Res. Commun. 113, 967–974
29. Palmer, A. G., III, Cavanagh, J., Wright, P. E. & Rance, M. (1991) J. Magn. Reson. 93, 151–170
30. Kay, L. E., Keifer, P. & Saarinen, T. (1992) J. Am. Chem. Soc. 114, 10663–10665
31. Schleucher, J., Schwendinger, M., Sattler, M., Schmidt, P., Schedletzky, O., Glaser, S. J., Sorensen, O. W. & Griesinger, C. (1994) J. Biomol. NMR 4, 301–306
32. Kay, L. E., Marion, D. & Bax, A. (1989) J. Magn. Reson. 84, 72–84
33. Vuister, G. W. & Bax, A. (1993) J. Am. Chem. Soc. 115, 7772–7777
34. Piotto, M., Saudek, V. & Sklenar, V. (1992) J. Biomol. NMR 2, 661–666
35. Guntert, P., Braun, W. & Wuthrich, K. (1991) J. Mol. Biol. 217, 517–530
36. Guntert, P., Mumenthaler, C. & Wuthrich, K. (1997) J. Mol. Biol. 273, 283–298
37. Pearlman, D. A., Case, D. A., Caldwell, J. W., Ross, W. S., Cheatham, T. E., Ferguson, D. M., Seibel, G. L., Singh, U. C., Weiner, P. K. & Kollman, P. A. (1997) AMBER, version 5.0, University of California, San Francisco
38. Banci, L., Benedetto, M., Bertini, I., Del Conte, R., Piccioli, M. & Viezzoli, M. S. (1998) Biochemistry 37, 11780–11791
39. Borgias, B., Thomas, P. D. & James, T. L. (1989) Complete Relaxation Matrix Analysis (CORMA), University of California, San Francisco
40. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
41. Laskowski, R. A., Rullmann, J. A. C., MacArthur, M. W., Kaptein, R. & Thornton, J. M. (1996) J. Biomol. NMR 8, 477–486
42. Bax, A. & Wang, A. C. (1995) J. Am. Chem. Soc. 117, 1810–1813
43. Weisemann, R., Ruterjans, H., Schwalbe, H., Schleucher, J., Berman, W. & Griesinger, C. (1994) J. Biomol. NMR 4, 231–240
44. Rosenzweig, A. C. & O’Halloran, T. V. (2000) Curr. Opin. Chem. Biol. 4, 140–147
45. Koradi, R., Billeter, M. & Wuthrich, K. (1996) J. Mol. Graphics 14, 51–55
46. Arnesano, F., Banci, L., Bertini, I., Huffman, D. L., & O’Halloran, T. V. (2001) Biochemistry 40, 1528–1539