X-Ray Absorption and NMR Spectroscopic Studies of CopZ, a Copper Chaperone in *Bacillus subtilis*: The Coordination Properties of the Copper Ion†

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ABSTRACT: XAS studies have been performed, under various experimental conditions, on a copper(I)-transporting protein, CopZ, of *Bacillus subtilis*. The copper(I) ion, reduced with dithiothreitol, is three-coordinate with three sulfur donor atoms, two of which presumably provided by the protein and one by dithiothreitol. If a molar excess of acetate (15 mM; 5:1 respect to CopZ) or citrate (6 mM; 2:1 respect to CopZ) is present in solution, the EXAFS spectra suggest the presence of a dimeric form involving a close contact between Cu(I) ions from two molecules, where Cu is still three-coordinate. ^{1}H and ^{15}N NMR data provide further structural details. If copper reduction is accomplished with ascorbate, the data indicate that one oxygen of ascorbate enters in the first-coordination sphere of copper, together with two sulfur atoms, in a dimeric form of the protein. These results are instructive and have been discussed with respect to the molecular basis of copper trafficking.

Several solution structures of copper(I)-bound proteins involved in copper trafficking, obtained through NMR, are now available (*1*−*5*). X-ray crystal structures of some of these systems are also available (*6*−*9*). However, it is recognized that some of them are difficult to crystallize in the copper-bound form (*10*) or in the reduced apo form and therefore NMR is particularly suitable for structural characterization. Sometimes these systems can be also stabilized in solution as demetalized proteins (*2*, *4*, *5*) or coordinated by metal ions such as mercury (*11*) or silver (*12*). However, NMR does not provide any information about the position and coordination properties of the metal ion and constraints about its location can be derived from X-ray structures obtained on analogous systems (*13*). In the case of the known copper(I) chaperones and ATPases, the copper ligands provided by the proteins are two cysteines (*14*, *15*) whose conformation can be defined through NOEs. The possible coordination of other exogenous ligands is difficult to tackle. On the other hand, the number of coordination bonds around copper, and consequently its reactivity, are key information to understanding the mechanism of copper trafficking.

X-ray absorption spectroscopy (XAS)[1] is a technique that provides independent structural information on the coordination site of a metal ion (*16*−*18*) and is ideal for use in conjunction with NMR studies. Therefore, we have performed a XAS study on the copper transporting protein, Cu(I)−CopZ, from *B. subtilis* in an attempt to learn about copper(I) coordination under different experimental conditions. *B. subtilis* Cu(I)−CopZ is a cytosolic protein which belongs to a family of small proteins (about 8 kDa) involved in the copper homeostasis of *B. subtilis*, a gram positive bacterium. Copper trafficking proteins homologous to CopZ have been described for yeast (Atx1) (*19*), plants (Cch) (*20*), and humans (Hah1) (*21*). All these proteins are characterized by the presence of the putative metal-binding site MXCXXC, suggesting a similar mechanism of metal binding and transfer. The methionine residue of this consensus motif, however, is never found to be bound to copper.

The NMR solution structure of the present Cu(I)−CopZ protein has been recently solved in our laboratory (*3*), with good resolution also in the Cu-coordination loop. The present study, which combines XAS data with further NMR characterization, provides meaningful information on the copper coordination and on its ligands as well as on protein−protein aggregation in a variety of experimental conditions.

EXPERIMENTAL SECTION

Sample Preparations. The CopZ protein of *B. subtilis* has been expressed and purified as previously reported (*3*). Several Cu(I)−CopZ samples were prepared for recording XAS spectra, all under inert atmosphere at 4 °C, in 50 mM Tris/Mes buffer at pH 8.0, using different copper reductants

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[1] Abbreviations: *B. subtilis*, *Bacillus subtilis*; *E. hirae*, *Enterococcus hirae*; DTT, dithiothreitol; GSH, reduced glutathione; XAS, X-ray absorption spectroscopy; EXAFS, extended x-ray absorption fine structure; DWF, Debye−Waller factor; Tris, tris(hydroxymethyl)-aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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and other added compounds. The following protein samples were characterized by XAS:
(1) DTT-reduced 3 mM Cu(I)−CopZ containing 3 mM DTT
(2) DTT-reduced 1 mM Cu(I)−CopZ containing 1 mM DTT
(3) DTT-reduced 3 mM Cu(I)−CopZ containing 3 mM DTT and 15 mM sodium acetate
(4) DTT-reduced 1 mM Cu(I)−CopZ containing 1 mM DTT and 5 mM sodium acetate
(5) DTT-reduced 1.5 mM Cu(I)−CopZ containing 1.5 mM DTT and 3 mM sodium acetate
(6) 3 mM Cu(I)−CopZ where Cu(I) has been delivered to the protein as $[Cu(I)(CH_3CN)_4]^+$ complex and kept reduced under anaerobic conditions. The protein was previously reduced with dithionite.
(7) 1.7 mM Cu(I)−CopZ prepared as sample 6 with addition of 13.6 mM sodium acetate
(8) ascorbate-reduced 3 mM Cu(I)−CopZ
(9) DTT-reduced 3 mM Cu(I)−CopZ, with 6mM reduced glutathione (GSH)
(10) DTT-reduced 3 mM Cu(I)−CopZ containing 3 mM DTT with 6 mM sodium citrate

The metalation of the samples reduced with DTT or ascorbate was performed incubating the apo protein with a Cu(II) salt (2.5 equiv) in the presence of the reductant (20 equivalents). The excess of metal ion and reductant was removed by ultrafiltration and the final reductants concentrations were maintained equal to that of the protein.

For the dithionite-reduced samples, the apo protein was maintained in reducing conditions with dithionite for 1 h; then the reductant was removed by ultrafiltration and Cu(I) was added slowly as $[Cu(I)-(CH_3CN)_4]PF_4$ complex (1 equiv) dissolved in CH_3CN. The excess of metal ion was removed by ultrafiltration.

All the NMR samples were prepared with the methodology reported above, using DTT as reductants in 50 mM Tris/Mes buffer (pH 8.0).

XAS Measurements. The samples, loaded in 1 mm thick plastic holders with Kapton windows, prefrozen in a dry ice/acetone mixture, were transferred into liquid nitrogen. All the spectra were collected at 20 K. XAS measurements were performed at the EMBL EXAFS beamline (c/o DESY, Hamburg). The DORIS III storage ring was operating in dedicated mode at 4.5 GeV with ring currents ranging from 90 to 150 mA. A Si(111) double crystal monochromator with an energy resolution of 1.6 eV at 8980 eV was used. The first monochromator crystal was detuned to 60% of its peak intensity in order to reject higher harmonics. The monochromator angle was converted to an absolute energy scale by using a calibration technique (*22*). Data were collected at the copper edge (~8984 eV assigned to the 1s → 4p transition in Cu(I) compounds) from 8475 to 9796 eV with variable step widths. In the XANES and EXAFS regions steps of 0.3 and 0.5−1.2 eV were used, respectively. Data collection and reduction were performed following previously reported procedures (*23*). Series of 10−40 spectra for each sample were collected. All the spectra were truncated at $k = 13$ Å^{-1}, giving a resolution limit of $\Delta R = \pi/2\Delta k = 0.14$ Å for the different shells of atoms in the EXAFS spectra. The whole experimental spectra were compared with theoretical simulations obtained by the set of programs EXCURVE 9.20 (*24*). The edge energy E_0 was adjusted at the beginning of the refinement in order to bring the experiments and the simulations on the same scale. A fixed amplitude factor of 0.85 was used to compensate for amplitude reduction of the signal due to multiple excitations. The k^3 weighted full spectra were simulated by varying the atom types and the coordination numbers (as integers) and iteratively refining the distance (R) and the Debye−Waller factor ($2\sigma^2$) for each atomic shell. The quality of the fit obtained was assessed by the goodness of fit function:

$$\epsilon^2 = 1/(N_{\mathrm{ind}} - p)(N_{\mathrm{ind}}/N) \sum_i^N w_i(\chi_i^{\mathrm{exp}}(k) - \chi_i^{\mathrm{th}}(k))^2$$

where N_{ind} is the number of independent data points ($N_{\mathrm{ind}} = (2\Delta k \Delta r)/\pi$), p is the number of parameters, N is the number of data points, and w is the weight of the spectrum, and by the R-factor as defined within EXCURVE 9.20 (*24*):

$$R_{\mathrm{exafs}} = \sum_i^N 1/\sigma(|\chi_i^{\mathrm{exp}}(k) - \chi_i^{\mathrm{th}}(k)|) \times 100$$

New shells of scatterers introduced in the simulations have been maintained when their contribution increased the quality of the fit as estimated by the goodness of fit criterion.

NMR Measurements. NMR experiments for the determination of ^{15}N longitudinal and transversal relaxation rates are recorded on a 600 Bruker Avance spectrometer with a 5 mm triple-resonance probe on samples enriched in ^{15}N. The ^{15}N backbone longitudinal relaxation rates, R_1, were measured as previously described (*25*) using delays in the pulse sequence of 10, 20, 40, 160, 320, 1280, and 2500 ms for all samples. The ^{15}N backbone transverse relaxation rates, R_2, were measured with the CPMG sequence using delays (T) in the pulse sequence of 7.8, 46.6, 62.1, 77.6, 155.2, 248.3, and 357.0 ms and a refocusing delay (τ_{CPMG}) of 450 μs (*25*). The recycle delay was set to 2.5 s for all rate measurements. Water signal suppression was achieved with the flip back sequence (*26*). All the NMR experiments are carried out at 298 K with 1024 (^{1}H) × 256 (^{15}N) data points for windows of 15 (^{1}H) × 40 (^{15}N) ppm.

RESULTS AND DISCUSSION

Edge Region. Figure 1 shows the K-edge region of the XAS spectrum for sample 1, together with the spectra of the samples 5, 6, and 8. The edges of samples 1 and 5 have identical shapes and differ from the edges of samples 6 and 8, which, in turn, are very similar each other (Figure 1). The edges of all the other DTT reduced samples (samples 2−4 and 9−10) are identical to that of sample 1 and 5, while the dithionite reduced samples (samples 6 and 7) are equal to each other and different from the above set. For all the samples both the energy and shape of the edges are indicative of copper in +1 oxidation state. No sign of either sample denaturation and Cu(I) dispoportionation to Cu(0) and Cu(II) is seen to occur in any of the samples. Overall the DTT reduced samples (samples 1−5 and 9−10) show three absorptions at 8983.9 eV (inflection point from 1st derivative at 8984.3 eV), 8991.4 eV, 8995.4 eV, and a broad maximum at 9006.0 eV. The absorption at 8984.3 eV is assigned to a 1s → 4p absorption that is typical of Cu(I) compounds (*27*, *28*). A number of studies have shown that the Cu(I) coordination number and/or geometry are correlated to the intensity of this transition (*27*, *29*). In Cu(I)−thiolate

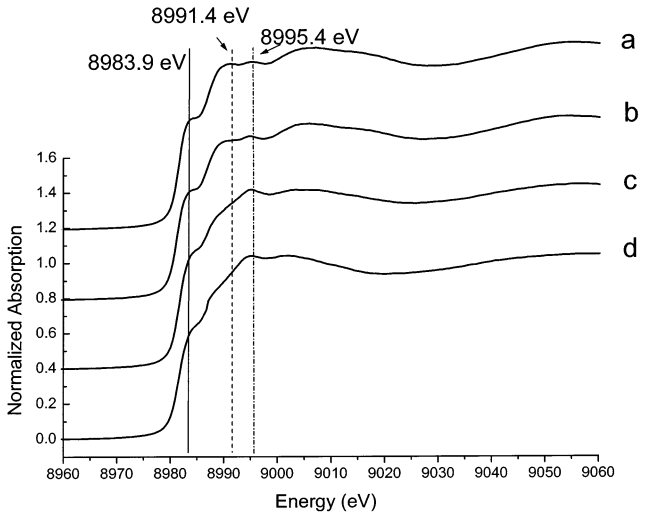


FIGURE 1: Comparison of the Cu K-edge regions of (a) DTT-reduced 3 mM Cu(I)−CopZ (samlple 1); (b) DTT-reduced 1 mM Cu(I)−CopZ (sample 5); (c) dithionite-reduced 3 mM Cu(I)−CopZ (sample 6); (d) ascorbate-reduced Cu(I)−CopZ (sample 8).

complexes, the intensity and sharpness of the peak decreases from linearly two-coordinated Cu(I) sites to tetrahedral ones (*17*, *30*−*32*), although other factors, like distortions from ideal geometry and Cu−Cu direct interactions, influence the transition intensities (*28*). In DTT-reduced Cu(I)−CopZ, the 1s → 4p absorption appears definitely less intense and resolved than that observed in bi-coordinated Cu(I) sites (*32*). When compared to the trigonal model compounds reported in the literature (*18*, *30*, *31*), this feature appears very similar indicating the presence of tri-coordinated Cu(I) centers in all the samples. The same holds for samples reduced with dithionite (samples 6, 7) or ascorbate (sample 8). However, in these latter samples the overall shape of the edge changes: the maximum at 8991.4 eV disappears, a shoulder at lower energy becomes evident (∼8988 eV) and the absorption at 8995.4 eV shows a sharp increase in intensity becoming the predominant feature of the edge. The changes in the edge reflect changes occurring in the Cu(I) coordination, although the coordination number is the same (vide infra).

DTT-Reduced Cu(I)−CopZ. The k^3 weighted experimental EXAFS spectrum of sample 1 and its Fourier transform (FT), reported in Figure 2, are compared with their theoretical simulations. The spectrum is dominated by the first shell contribution that is well simulated by three sulfur atoms at 2.25(2) Å from Cu(I), a distance which, consistently with the edge features, indicates that the copper(I) ion in sample 1 is tri-coordinate. Linear two-coordinated Cu(I)−thiolate complexes have Cu−S distances of 2.14−2.15 Å while three-coordinated complexes have Cu−S distances of 2.26−2.28 Å and four-coordinated Cu(I) complexes show even longer distances (2.28−2.37 Å) (*28*). A study on Cu(I)−thiolate cluster model compounds has shown that the measure of the Cu−S distance correlates with the Cu(I) coordination going from 2.28 Å in pure trigonal Cu (Cu_t) to 2.16 Å in pure diagonal Cu (Cu_d) complexes [17].

When reduced glutathione (GSH) is added to the DTT-reduced Cu(I)−CopZ solution (sample 9) no changes are observed in the copper coordination as measured from XAS data (see Table 1 in Supporting Information), although it is impossible to establish if one of the sulfur atoms coordinated

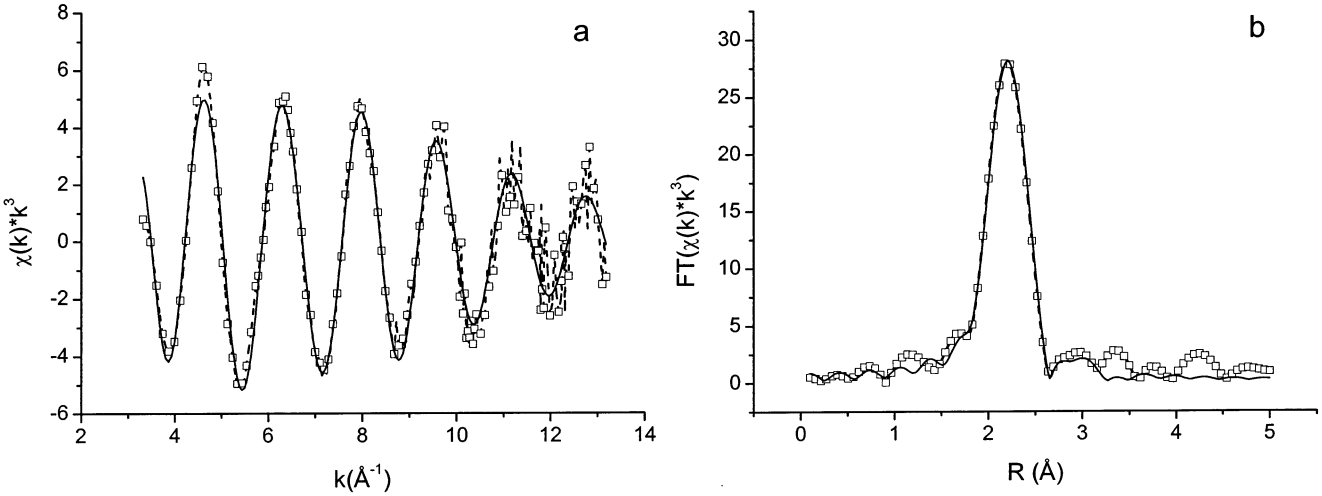


FIGURE 2: (a) EXAFS spectrum and (b) Fourier Transform of DTT-reduced 3 mM Cu(I)−CopZ (sample 1). The best fit (continuous line) and the experimental (dashed line with □) EXAFS data are reported.

Table 1: Cu-Edge EXAFS Best Fitting Results for the Samples Discussed

	DTT reduced 3 mM Cu(I)−CopZ (sample 1)	DTT reduced 3 mM Cu(I)−CopZ + Na acetate 1:5 ratio (sample 3)	ascorbate reduced 3 mM Cu(I)−CopZ (sample 8)	DTT-reduced 3 mM Cu(I)−CopZ + 1:2 sodium citrate (sample 10)
ϵ^2	0.41	0.44	1.05	0.60
R-factor	24.3	23.4	39.2	26.7
atom shells	3 S	3 S; 1 Cu	2 S; 1 O; 1 Cu; 1 S	3 S; 1 Cu
r (Å)	2.25	2.25; 2.81	2.25; 1.95; 2.62; 3.53	2.26; 2.67
$2\sigma^2$(Å^2)·10^3	10	12; 25	12; 5; 15; 11	9; 19
EF (eV)	−6.49	−6.76	−6.21	−5.80

to Cu(I) belongs to one GSH molecule replacing the DTT sulfur. Any of the other potential donors present in the GSH molecule (carboxylate oxygens, carbonyls or amino-groups) does not coordinate the Cu(I) ion in Cu(I)−CopZ. Interestingly, such groups did not bind Cu(I) also in a Cu(I)−GSH adduct, where copper is coordinated by three sulfur atoms, with Cu−S distances of about 2.22 Å (*33*).

A very recent EXAFS report on a sample of CopZ from *E. hirae* in 30% glycerol displays a very similar EXAFS spectrum (*34*). The authors suggest a trigonal coordination with 2 S atoms and an atom of similar size. Apparently no DTT had been added to the sample.

The possible interaction of CopZ with the reductant molecule of DTT has been also analyzed through NMR analysis. The ^{15}N HSQC spectrum of a sample prepared as sample 1, but exhaustively exchanged with buffer using ultrafiltration system (Amicon) to remove part of the reductant, shows the splitting of a few NH signals for residues located in the metal binding loop or in loops located in the surrounding of the metal ion indicating the presence of two species in solution. The fraction of the minor species increases with the duration of the exchange treatment. When DTT is added to this sample the signals of the second, minor species disappear, and the normal spectrum is obtained again. This is consistent with XAS data and suggests that DTT does bind to the copper ion becoming its third ligand. Consistently with this picture, the NMR solution structure shows that the S−Cu−S angle, where the S atoms are due to the Cys ligands, is about 115° ± 26° (*3*).

The FT of the EXAFS spectrum of sample 1 features a small outer shell peak at ∼2.7 Å just at the noise level. This peak might be due to the presence of outer shell atoms. Indeed, introduction of one copper atom at 2.74(2) Å can reproduce this peak quite well although the fit results in a very large Debye−Waller factor ($2\sigma^2 = 0.037$) and the *R*-factor and ϵ^2 improvement with respect to the one shell fit are barely significant (*R* from 24.3 to 22.6; ϵ^2 from 0.41 to 0.38). Attempts to model the outer shell peak using either sulfur or low Z backscatterer atoms (O/N/C) always give lower quality fits. The detection of a possible Cu−Cu interaction may suggest the presence in the solution of a very small fraction of CopZ dimers in which the intermolecular interaction involves the copper coordination sphere.

To check the last hypothesis, the effect of dilution on Cu(I)−CopZ was analyzed by measuring sample 2 where the protein concentration is 1 mM. The Cu(I) ion is still coordinated by 3 S atoms at 2.26(2) Å. The weak second shell peak is still present in the FT spectrum that can be simulated by a Cu atom at 2.86(2) Å with a large DW-factor (0.038 Å^2), indicating that varying the sample concentration in the 1−3 mM range does not cause appreciable changes in the Cu(I) coordination shell (see Table 1).

The aggregation state of CopZ has been also characterized through ^{15}N relaxation rates. The latter are modulated by the correlation time for the molecular tumbling. This time is directly proportional to the molecular weight of the protein, thus providing a direct and simple way to determine the aggregation state of the protein. ^{15}N relaxation measurements, R_1 and R_2, of the backbone amide nitrogens were performed in different sample conditions for those ^{15}N resonances that do not show overlap and/or bad signal/noise ratio and therefore can be accurately integrated. The sets of experiments on samples in Tris/Mes buffer (50 mM, pH 8.0) are carried out at two different protein concentrations: 3.0 and 0.6 mM. For the 3.0 mM sample, equal to the XAS sample 1 conditions, 70 ^{15}N amide nitrogens were analyzed. The average values for the relaxation rates, R_1 and R_2, result in 1.37 ± 0.10 and 12.40 ± 2.80 s^{-1}, respectively. Using only the R_2/R_1 ratios within one standard deviation of the mean value, the rotational correlation time, τ_m, for the protein is calculated equal to 9.08 ± 0.75 ns. This value is about the double of that expected on the basis of the protein molecular weight (*35*) and of that found for proteins of analogue size (*36*). For the sample with a Cu(I)−CopZ concentration of 0.6 mM, the average R_1 and R_2 values are 1.79 ± 0.35 and 10.70 ± 2.36 s^{-1}, respectively, providing a τ_m value of 7.18 ± 0.65 ns. Thus, with decreasing protein concentration the content of the dimeric species decreases. However, upon change of concentration no meaningful change in the ^{15}N chemical shift (≥0.05 ppm) is observed for any residue of the protein, suggesting that the protein−protein interactions are not localized in a specific region of the protein but might produce multiple interexchange adducts involving different regions of the surface. These data indicate that aggregation of CopZ is concentration dependent and involves nonspecific intermolecular interactions. The metal free protein at high concentration has R_1 and R_2 values similar to those of the copper bound form at the same concentration, thus indicating that the protein aggregation is not modulated by the metal ion. The R_1 and R_2 values for apo CopZ (at a concentration of 2.5mM) are 1.50 ± 0.15 and 11.50 ± 2.13 s^{-1}, respectively, which provide a τ_m value of 8.07 ± 0.60 ns.

B. subtilis CopZ has only four sulfur-containing residues in its molecule: two cysteines (Cys13 and Cys16, the copper binding ones) and two methionines (Met1 and Met11). The structure of Cu(I)−CopZ (*3*) shows that Met1 is located at the opposite end of the molecule with respect to the copper binding site, at about 30 Å from it (*3*). The sulfur of Met11 residue is similarly located too far away from copper (∼5 Å, Met 11 being well defined with rmsd values of 0.51 and 1.30 Å for the backbone and the side chains, respectively) to account for the EXAFS results. Furthermore, if Met 11 is imposed to bind copper in the structure calculations, a few NOEs involving residues in the copper surrounding do violate, increasing the average target function of the family of about three times. The formation of dimeric species, where a cysteine from a second Cu(I)−CopZ molecule is involved in intermolecular coordination of copper (see Scheme 1), has been proposed for other copper transporting proteins which have the same two cysteine ligands to copper (*18*) and imply a short Cu−Cu distance (*31*). The EXAFS analysis of the present DTT-reduced Cu(I)−CopZ (sample 1) does not give substantial evidence of the presence of CopZ dimeric species which interact in such a way to form a complex with a short Cu−Cu distance. If this were the case, a much stronger second shell (Cu−Cu) contribution to the EXAFS spectrum would have been observed. Overall, the EXAFS and NMR characterization for DTT reduced proteins indicate that the protein is essentially in a dimeric state but with no specific interactions between the two copper sites and that DTT provides one sulfur donor to complete the copper coordination shell.

Further evidence on the tendency of the CopZ bound Cu(I) ion to achieve tri-coordination by binding exogenous

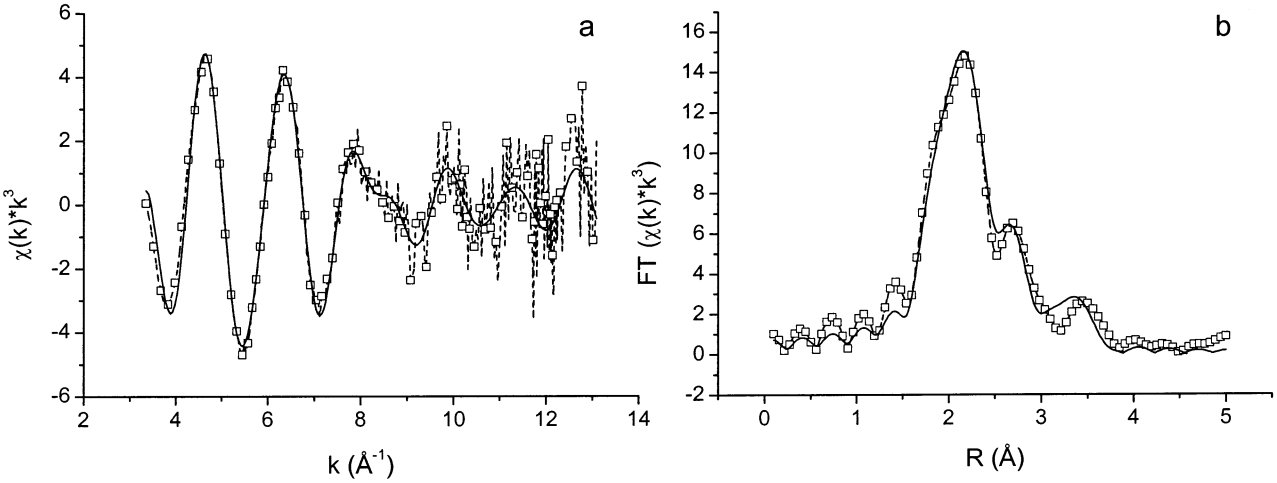
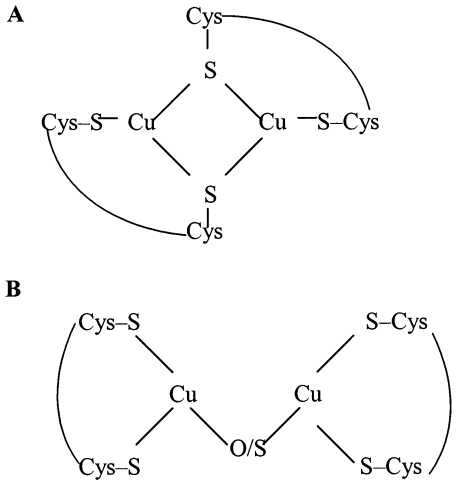


FIGURE 3: (a) EXAFS spectrum and (b) Fourier Transform of ascorbate-reduced 3mM Cu(I)−CopZ (sample 8). The best fit (continuous line) and the experimental (dashed line with □) EXAFS data are reported.

Scheme 1



molecules is obtained from the EXAFS analysis of the ascorbate (see Figure 3) and dithionite reduced samples (samples 8−6). When copper reduction is performed with ascorbate instead of DTT, a huge change is observed both in the edge and in the EXAFS regions of the spectrum (Figures 1 and 3). The analysis shows that oxygen atoms have entered in the Cu(I) coordination shell. The amplitude of the EXAFS signal is much lower than in the other spectra, and it shows (Figure 3a) a beat node at about 8.5 $Å^{-1}$, indicating the presence of oxygen/nitrogen atoms in the first coordination shell of copper. The FT of the spectrum has a main peak of low height at about 2.0−2.2 Å, with a second prominent peak at ∼2.7 Å that is indicative of the presence of a heavy scatterer. In the analysis the main peak is well reproduced by a combination of S and O ligands giving two almost equivalent fits. The best fit is obtained with one oxygen atom at 1.95 Å and two sulfur atoms at 2.25 Å (Table 1, sample 8, fit 4; $\epsilon^2 = 1.05$, $R = 39.2$). The FT feature at 2.7 Å can be reproduced either by a further sulfur atom at 2.77 Å or by a copper atom at 2.62 Å, the latter providing a slightly better fit. The spectrum may also be fitted with 2 O and 2 S atoms at 1.96 and 2.25 Å, respectively, giving a four-coordinated Cu(I) ion (Table 1, sample 8, fit 5; $\epsilon =$ 1.16, R factor = 43.3). In this case the Debye−Waller factor of the O atoms rises to 0.015 $Å^2$, but the quality of the fit is comparable to fits 4 and 5. The Cu−Cu distance of 2.62 Å found here compares well with that found in copper containing proteins where clustering of the metal centers occur (*18*, *30*, *31*). In conclusion the EXAFS spectra are consistent with two sulfur and one oxygen atoms with a Cu atom at longer distance suggesting the occurrence of a CopZ dimer in this system. In samples 7 and 8, where the Cu(I) was delivered to the protein as acetonitrile complex, the spectra simulations suggest that one light atom, possibly N from the acetonitrile molecule, has entered the copper first coordination shell (see Table 1 SI)

Cu(I)−CopZ in the Presence of Carboxylate Salts. The EXAFS spectra of 3 mM Cu(I)−CopZ reduced with DTT have been measured also in the presence of acetate (in different CopZ/acetate molar ratios and concentrations, samples 3−5) and of citrate (sample 10). The spectra bear similarity with the EXAFS spectrum of sample 1, as far as the first coordination shell of copper is concerned (Figure 4). Indeed, these spectra are fitted in both cases with three sulfur atoms at 2.25(2) Å from copper (Figure 4a−d). However, the FTs spectra show an evident outer shell peak at ∼ 2.7 Å that can be well simulated by a copper ion at 2.59 Å and at 2.64 Å, for samples 5 and 10, respectively. The addition of the Cu−Cu interaction brings a noticeable improvement of the fits for the two samples (R from 32.3 to 28.5 and ϵ^2 from 0.69 to 0.59 for sample 5; R from 32.5 to 36.7 and ϵ^2 from 0.78 to 0.60 from sample 10, see Table 1). Cu−Cu distances in the 2.6−2.7 Å range have been found either in model compounds where two trigonal Cu(I) atoms were in contact [17] and in other Cu(I) binding proteins [19, 22, 23]. The EXAFS spectra of acetate and citrate containing Cu(I)−CopZ samples closely resemble the EXAFS spectra of the chaperone for human superoxide dismutase (hCCS) (*18*) and of other Cu-transporting proteins, such as those of the yeast chaperone Cox17 (*31*), of the copper-activated transcription factor Amt1 (*30*), and of the N-terminal domain of the Wilson disease Cu-ATPase (*17*), which all showed a second outer shell peak in their EXAFS Fourier transforms. This peak was interpreted as due to protein dimerization which involves a close approach of the copper binding sites. Such close contact might occur through two different arrangements, as shown in Scheme 1.

We have further tested the effects of varying the protein concentration in the presence of acetate (samples 3 and 4)

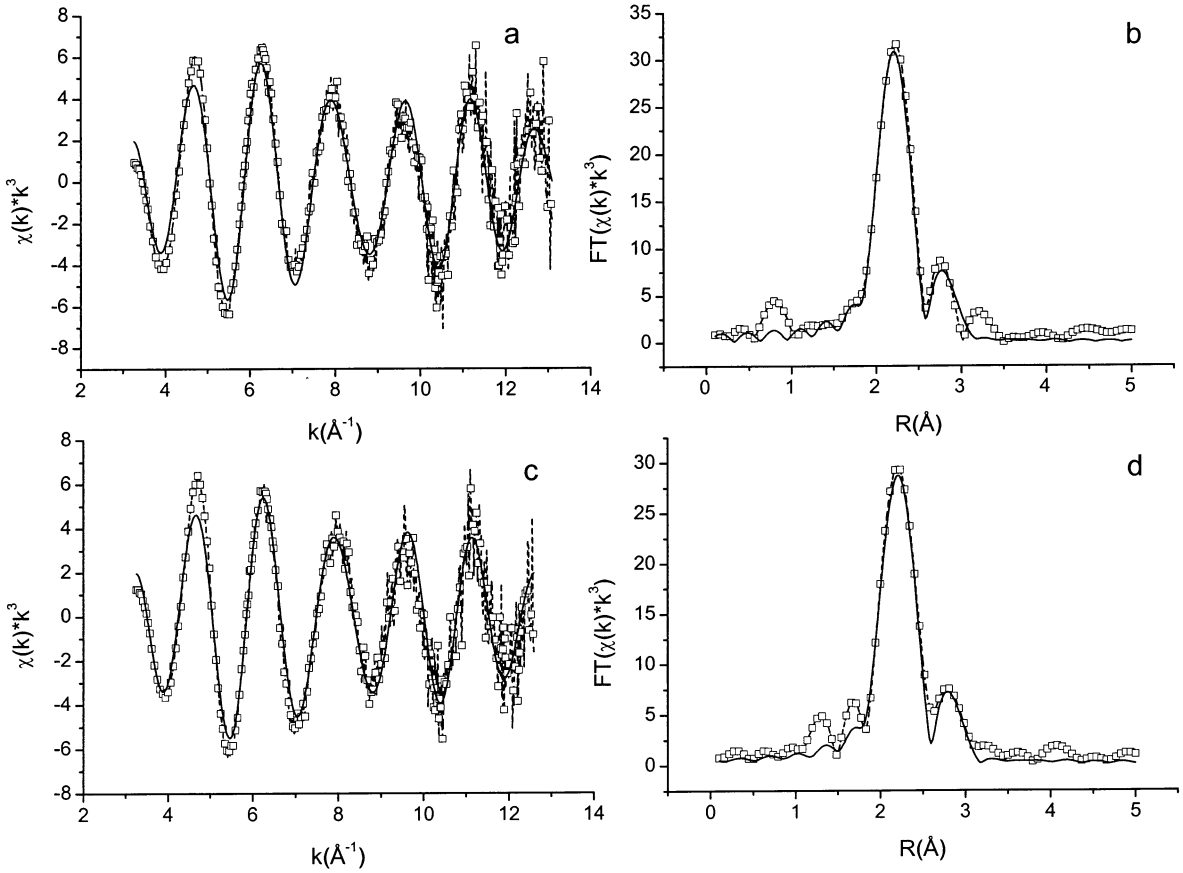


FIGURE 4: (a) EXAFS spectrum of DTT-reduced 1.5 mM Cu(I)−CopZ containing 3 mM sodium acetate (sample 5) and (b) its FT. (c) EXAFS spectrum of DTT-reduced 3 mM Cu(I)−CopZ containing 6 mM sodium citrate (sample 10) and (d) its FT. The best fit (continuous line) and the experimental (dashed line with □) EXAFS data are reported.

and of varying the CopZ/salt molar ratio (sample 5) going from 1:5 down to 1:2 on the EXAFS spectra. Both spectra are identical to that of sample 3, and no significant difference in the copper environment is observed.

The above findings indicate that, when the protein is in the presence of an excess of acetate and citrate, the occurrence of Cu(I)−CopZ dimers which involve the copper site is favored as evidenced by the strong Cu−Cu scattering. However, the potentially metal-coordinating carboxylate anions do not displace sulfur atoms from the Cu(I) coordination shell, which still maintains three sulfur atoms in the first coordination sphere. The above results indicate that in such conditions CopZ dimers, completely different from those in Tris/Mes, are present in solution. In the presence of acetate or citrate, intermolecular interactions become specific and entail the copper binding sites. It is conceivable that the carboxylate salts interact with the positively charged Lys and Arg side chains present on the surface of the CopZ molecule (7 residues in total) disrupting the intermolecular salt bridges with Asp and Glu residues (15 in total) which may be responsible for the aspecific aggregation of CopZ so favoring a specific interaction between two CopZ molecules that involves the copper site. This interaction mode would be consistent with the model shown in Scheme 1b.

Copper Coordination. The EXAFS data clearly show that in all our samples the copper ion is always at least three-coordinate and provide evidence that the reducing agent binds the copper ion. The same EXAFS data also indicate the occurrence of protein dimerization under a number of cases.

The analysis of the XAS and NMR data on Cu(I)−CopZ in several different experimental conditions reveal a large flexibility in the coordination of the copper ion, which cannot be completed by the protein donors. When the copper ion is reduced with DTT, a sulfur atom of the latter molecule enters the copper coordination sphere. The tendency of copper(I) to reach three-coordination is also confirmed by the behavior of the protein when copper is reduced with ascorbate and dithionite. In this case one oxygen/nitrogen is the third donor atom and short Cu−Cu distances implying specific CopZ aggregation have been observed.

At high protein concentrations and low ionic strength, the protein aggregates in a non specific way, giving rise to a significant concentration of dimeric species. Still no evident copper−copper interaction is detected by the EXAFS analysis. Decreasing the protein concentration leads to an increase in the monomeric form, as shown by the values of the NMR reorientational correlation times. The decrease in the protein tumbling correlation time with increasing dilution in Tris/Mes buffer does not induce chemical shift changes thus suggesting that the protein−protein interaction is unspecific. When acetate and citrate are present in solution, dimeric species involving copper−copper short distances are formed.

Biological Implication. The EXAFS spectra on the copper transporting protein Cu(I)−CopZ here reported are particularly instructive on the copper trafficking mechanism. ATPases and cytosolic metal transporting proteins, in both eukaryotic and prokaryotic organisms, have only two cysteins

involved in the copper coordination (*14*). Evidently the two-coordination does not make the copper(I) ion thermodynamically stable, as samples prepared with DTT indicate that the latter brings copper to three-coordination. Such coordination is reached in the present system also by dimerization, when anions such as acetate and citrate are added to the solution (see Scheme 1). The dimerization was also found by X-ray crystallography to occur in similar systems, in the presence of substoichiometric copper content, where tetracoordination of the copper ion can be achieved by two protein molecules binding one copper ion (*9*). The flexibility in the Cu(I) coordination, that is by now well documented, is needed in the translocation of copper from the donor to the acceptor molecule.

Finally, our results point out that exogenous ligands may have access to the copper first coordination sphere in Cu(I)−CopZ (and possibly also in the other copper transporting proteins of similar structure) and that acetate and citrate favor the formation of homodimers in a fashion that is reminiscent of the formation of the donor−acceptor adduct in intermolecular copper transport (*36*).

These new findings explain the EXAFS results obtained for similar copper transport proteins where Cu(I) is coordinated by three sulfur atoms, by providing evidence that the third S ligand may either come from one exogenous molecule or being provided by a second protein molecule in a homodimer complex. Our data also point out that the presence of exogenous thiols such as DTT and GSH migh prevent the intermolecular interactions between Cu(I)−loaded molecules that involve the copper coordination sphere. On the contrary, the homodimer formation through copper bridges appears to be favored by the presence of carboxylate salts and when the copper is reduced by ascorbate or dithionite or provided as acetonitrile Cu(I) complex.

This aspect may imply some new insights about the copper transport mechanism. In a recent paper, Huffman and O'Halloran (*37*) demonstrate that, despite the high affinity for Cu(I), physiological concentration, or even high excess of GSH, does not influence the exchange equilibrium of copper between Atx1 and the partner *Ccc*2. They further suggest that Atx1 may prevent adventitious copper release by preventing exchange reactions with nonprotein ligands or nonpartner proteins. Our data seem to point out that physiological thiols may be needed to complete the Cu(I) coordination sphere in order to prevent homodimer formation that is indeed favored by the presence of other physiological carboxylates (e.g., citrate). The Cu(I)−S_3 arrangement may be the stable adduct that allow Cu(I) to be transported to the partner proteins avoiding the formation of possible dead-end products such as the homodimer. On the other hand, our results also suggest that such arrangement is not thermodynamically so stable as to prevent the interaction with the partner protein when appropriate conditions are met (i.e., presence of carboxylates in our case).

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SUPPORTING INFORMATION AVAILABLE

Relaxation rates, spectra, and significant fits for samples studied. This material is available free of charge via the Internet at http://pubs.acs.org.

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