X-ray Absorption Investigation of a Unique Protein Domain Able To Bind both Copper(I) and Copper(II) at Adjacent Sites of the N-Terminus of *Haemophilus ducreyi* Cu,Zn Superoxide Dismutase†

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Received May 18, 2005; Revised Manuscript Received July 28, 2005

ABSTRACT: The N-terminal metal binding extension of the Cu,Zn superoxide dismutase from *Haemophilus ducreyi* is constituted by a histidine-rich region followed by a methione-rich sequence which shows high similarity with protein motifs involved in the binding of Cu(I). X-ray absorption spectroscopy experiments selectively carried out with peptides corresponding to the two metal binding regions indicate that both sequences can bind either Cu(II) or Cu(I). However, competition experiments demonstrate that Cu(II) is preferred by histidine residues belonging to the first half of the motif, while the methionine-rich region preferentially binds Cu(I) via the interaction with three methionine sulfur atoms. Moreover, we have observed that the rate of copper transfer from the peptides to the active site of a copper-free form of the Cu,Zn superoxide dismutase mutant lacking the N-terminal extension depends on the copper oxidation state and on the residues involved in metal binding, histidine residues being critically important for the efficient transfer. Differences in the enzyme reactivation rates in the presence of mixtures of the two peptides when compared to those obtained with the single peptides suggest that the two halves of the N-terminal domain functionally interact during the process of copper transfer, possibly through subtle modifications of the copper coordination environment.

Copper is an essential element for all aerobic organisms which incorporate this metal ion in several important enzymes. One of these enzymes is Cu,Zn superoxide dismutase (Cu,ZnSOD),[1] which protects cells from the toxic effects of reactive oxygen intermediates by converting the superoxide radical into hydrogen peroxide and molecular oxygen (*1*). In bacteria, Cu,ZnSOD is located in extracytoplasmic compartments where it plays an important role in protecting microorganisms from exogenous sources of superoxide (*2*).

The Cu,ZnSODs from a subset of Gram-negative pathogens possess divalent metal binding N-terminal extensions which likely favor the uptake of the enzyme's prosthetic metals in environments where their concentration is very low (*3*). In particular, the N-terminal extension of the Cu,ZnSOD from *Haemophilus ducreyi*, which is the causative agent of a genital ulcerative disease known as chancroid, shows an unusual sequence (HGDHMHNHDTKMDTMSKDMMSME). The N-terminus of this motif contains a cluster of four histidines interspersed with other residues, which are very similar to the transition metal binding regions already identified in other proteins which are able to bind Ni(II), Zn(II), or Cu(II) (*3*). The second half of the domain contains an unusual methionine-rich sequence that resembles the Cu(I)-binding domains observed in several proteins involved in copper homeostasis in prokaryotic and eukaryotic cells (*4*, *5*). Although for a long time the function of these methionine-rich domains has been unclear, they have been postulated to bind copper (*4*). This hypothesis has been recently confirmed by studies carried out on PcoC and CopC, two soluble periplasmic proteins involved in copper resistance isolated from *Escherichia coli* and *Pseudomonas syringae*, respectively (*5*−*8*). For both of these proteins it has been demonstrated that Cu(II) is bound by at least two histidine residues and two other N- or O-ligand donors, while Cu(I) is bound by two thioethers (i.e., S-Met) and one N/O donor in PcoC (*7*) and by two or three methionine residues and one histidine in CopC (*8*). Biochemical and genetic investigations on the high-affinity copper transporters be-

† This work was partially supported by the Murst Cofin Project "Structure and biological role of Cu,Zn superoxide dismutase involved in bacterial virulence" and by the MURST-CNR program L.95/95 "Biomolecole per la salute umana".

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[1] Abbreviations: Cu,ZnSOD, Cu,Zn superoxide dismutase; XAS, X-ray absorption spectroscopy; XANES, X-ray absorption near edge structure; EXAFS, extended X-ray absorption fine structure.

10.1021/bi050925x CCC: $30.25
Published on Web 09/10/2005

longing to the Ctr family have also demonstrated that methionine-rich motifs are essential for copper uptake in yeast and humans (*9*).

Previous investigations have shown that efficient Cu(II) binding by the N-terminal region of the *H. ducreyi* Cu,ZnSOD requires the presence of the histidine-rich region (*3*). These studies have demonstrated that, under conditions of limited copper availability, oxidized copper is initially bound at the N-terminal region and subsequently slowly transferred to an active site (*3*). However, the growing observations concerning the involvement of methionine-rich sequences in copper binding suggest that both of the two halves of the N-terminal domain of *H. ducreyi* Cu,ZnSOD might be involved in the uptake and the transfer of copper to the active site. To verify this hypothesis and to assess the copper binding ability and coordination geometry of the two metal binding regions, we have carried out X-ray absorption measurements on solutions containing Cu(I), Cu(II), and peptides having the same sequence of the histidine-rich and methionine-rich halves of the N-terminal domain of *H. ducreyi* Cu,ZnSOD.

The here reported results indicate that both the histidine-rich and methionine-rich peptides are able to bind Cu(II) and Cu(I). However, competition binding assays indicate that Cu(II) is preferentially bound by the histidine-rich peptide, whereas Cu(I) is preferentially bound by the methionine-rich peptide. The ability of the N-terminal domain of *H. ducreyi* Cu,ZnSOD to bind efficiently either Cu(II) or Cu(I) could be important to ensure efficient copper uptake by Cu,Zn superoxide dismutase under copper starvation.

MATERIALS AND METHODS

Sample Preparation and X-ray Absorption Spectroscopy Data Collection and Analysis. The His-rich (HGDHMHNHDTK) and Met-rich (KMDTMSKDMMSME) peptides were purchased from Advanced Biotech Italia (Seveso, Italy). Molecular mass and peptide purity (more than 95% homogeneous) were confirmed by mass spectrometry analysis and HPLC analyses, respectively. X-ray absorption measurements were carried out on solutions containing 5 mM peptide and 2.5 mM $CuSO_4$ dissolved in 50 mM Hepes, pH 7.4, after an incubation of 2 h at room temperature. Samples containing Cu(I) were obtained by adding ascorbic acid (250 mM) under nitrogen atmosphere to peptides previously incubated with $CuSO_4$, as described above. The samples used to analyze the ability of the two peptides to compete for Cu(I) and Cu(II) were prepared in the same way, except that the concentration of each peptide was 2.5 mM and copper concentration was 1.25 mM. All samples were combined with 30% glycerol, loaded in plastic cells with Kapton windows, and frozen in liquid N_2. Cu K-edge XAS spectra were collected in the fluorescence mode at the BM30-B (FAME) beamline of the European Synchrotron Radiation Facility. To collect data on the Cu binding peptides, the sample cells were mounted in a helium cryostat, constantly kept at 11K during data collection. The storage ring was running in the two-thirds filling mode with a typical current of 170 mA. The monochromator was equipped with a Si(111) double crystal, in which the second crystal was elastically bent to a cylindrical cross section. The X-ray photon beam was vertically focused by a Rh-coated mirror and dynamically sagittally focused in the horizontal size by the second crystal of the monochromator. The XAS data were recorded by measuring the Cu Kα fluorescence using a 30 Ge-element solid-state detector over the range from 8735 to 9900 eV, with variable energy step widths. For each sample 10−15 spectra were recorded, depending on the Cu concentration, and averaged to obtain a good signal-to-noise statistic. A Cu foil internal energy calibration was measured simultaneously with each spectrum. The energy was defined by assigning the first inflection point of the Cu foil spectrum to 8980.3 eV.

The EXAFS data analysis has been performed using the GNXAS code, which is based on a theoretical calculation of the X-ray absorption fine structure signal and a subsequent refinement of the structural parameters (*10*, *11*). In the GNXAS approach the interpretation of the experimental data is based on the decomposition of the EXAFS χ(k) signal (defined as the oscillation with respect to the atomic background cross section normalized to the corresponding K-edge channel cross section) into a summation over n-body distribution functions $\gamma^{(n)}$, calculated by means of the multiple scattering (MS) theory. Each signal has been calculated in the muffin-tin approximation using the Hedin−Lundqvist energy-dependent exchange and correlation potential model, which includes inelastic loss effects. The theoretical framework of the GNXAS method is described in detail in previous publications (*10*, *11*).

The theoretical EXAFS spectrum was calculated to include contributions from first shell two-body signals and three-atom and four-atom configurations associated with the histidine rings. Previous investigations on model compounds have shown that a quantitative EXAFS analysis of systems containing histidine rings requires a proper treatment of MS four-body terms (*12*). The model χ(k) signal was then refined against the experimental data by using a least-squares minimization procedure in which structural and nonstructural parameters were allowed to float. The structural parameters were the bond distance (R) and bond variance (σ^2_R) for a two-body signal, the two shorter bond distances, the intervening angle (θ), and the six covariance matrix elements for a three-body signal. The four-body configurations are described by six geometrical parameters (*10*) which were allowed to float within a preset range, typically ±0.05 Å and ±5° for distances and angles, respectively, around the average Cu−histidine geometry. Two additional nonstructural parameters were minimized, namely, E_0 (core ionization threshold) and S_0^2 (many body amplitude reduction factor). The quality of the fits was determined by the goodness-of-fit parameters, R_i (*10*), and by careful inspection of the EXAFS residuals and their Fourier transforms.

Copper-Free Enzyme Reconstitution. Wild-type *H. ducreyi* Cu,ZnSOD and the N-deleted mutant form of the enzyme lacking the first 22 amino acids were purified and prepared in a copper-free form as previously described (*3*). The rate of copper binding at the active site was evaluated by measuring the increase in catalytic activity following the addition of 2.9×10^{-6} M Cu(I) or Cu(II), bound to each peptide (5.8×10^{-6} M), to the copper-free N-terminal-deleted enzyme (2.9×10^{-6} M) dissolved in 50 mM Hepes, pH 7.4. The activity assays were carried out by the pyrogallol method (*13*). Control experiments carried out by adding 0.1 mM EDTA [a Cu(II) chelator] or 0.1 mM bathocuproine sulfonate

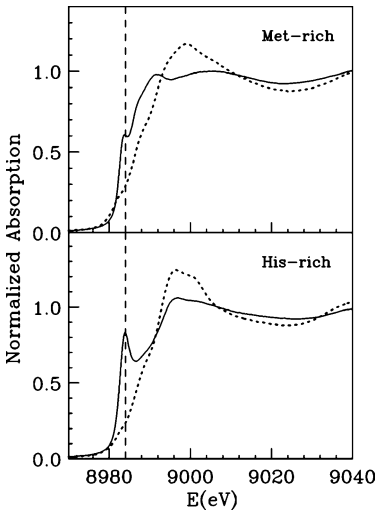

FIGURE 1: Absorption edges of Cu(I) and Cu(II) complexes. Upper panel: Cu K-edge XANES spectra for Cu(I)/Met-rich (solid line) and Cu(II)/Met-rich peptide (dotted line) aqueous solutions. Lower panel: Cu K-edge XANES spectra for Cu(I)/His-rich (solid line) and Cu(II)/His-rich peptide (dotted line) aqueous solutions. Vertical dashed lines are at 8984 eV.

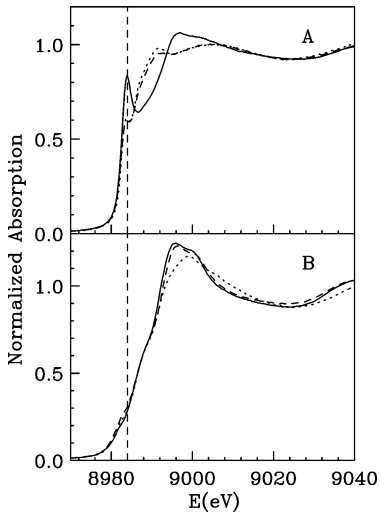

FIGURE 2: Absorption edges of Cu(I) and Cu(II) complexes. (A) Cu K-edge XANES spectra for Cu(I) with Met-rich (dotted line), His-rich (solid line), and an equimolar mixture of the two peptides (dashed line) in water solution. (B) Cu K-edge XANES spectra for Cu(II) with Met-rich (dotted line), His-rich (solid line), and an equimolar mixture of the two peptides (dashed line) in water solution.

[a Cu(I) chelator] to the assay buffer demonstrated that copper bound to the protein surface or to the peptides negligibly contributes to the enzyme activity (*3*). Reconstitution experiments with Cu(I) were carried out by adding ascorbic acid at a final concentration 100-fold higher than the copper amount, under nitrogen atmosphere. Aliquots were always withdrawn under inert atmosphere. The percent activity values were obtained by the ratio between the activity of reconstituted enzymes and that of a proper amount of fully metalated N-terminal-deleted mutant enzyme. All of the activity values obtained during reconstitution experiments were corrected by subtracting the contribution to activity of residual copper present in copper-free enzyme preparations.

RESULTS

XANES Spectra. To characterize the behavior of the interaction of copper with the N-terminal domain of *H. ducreyi* Cu,ZnSOD, we analyzed XAS spectra of different aqueous solutions containing Cu(I), Cu(II), and two peptides having the same sequence of the histidine-rich and methionine-rich halves of the N-terminal domain. Preliminary analyses were carried out by atomic, optical, and EPR spectroscopy to verify the ability of the two peptides to bind copper. These studies revealed that each one of these peptides can bind more than one copper atom in vitro (data not shown). Moreover, we observed that the optical and EPR spectra are identical up to a Cu(II)/peptide ratio of 0.5. At higher Cu(II)/peptide ratios the shape of the spectra changes, possibly due to the presence of heterogeneous copper binding sites and/or to a concentration-dependent modification in the Cu(II) coordination geometry. To simplify the analysis of the XAS spectra and to characterize the metal binding site with the highest copper affinity, all of the here reported analyses have been carried out at a Cu/peptide ratio of 0.5.

Figure 1 shows the low-energy region absorption spectra of Cu(I) and Cu(II) complexes with the Met-rich peptide (upper panel) and the His-rich peptide (lower panel), where the changes in the edge energy and shape clearly identify the two different copper oxidation states. In both cases there is a large shift of the main transition threshold to higher energies, in going from Cu(I) to Cu(II), which is consistent with what is seen in model complexes (*14*). The preedge feature at 8984 eV, assigned to a 1s → 4p transition (*14*), is present in the Cu(I) complex absorption spectra but not in those containing Cu(II). The Cu(I) coordination number and geometry are correlated with the intensity of this transition (*14*, *15*). In contrast, Cu(II) complexes exhibit only a broad low-energy tail in the region below 8985 eV, which originates from electronic transitions between the 1s and 3d atomic orbitals whose intensity is proportional to the deviation from centrosymmetry of the Cu(II) coordination geometry (*15*).

Figure 2A shows the XANES spectra of Cu(I) aqueous solutions containing either the Met-rich or the His-rich peptide or an equimolar amount of the two peptides dissolved simultaneously. The XANES spectrum of the sample containing both peptides is almost identical to that observed for the Met-rich peptide, suggesting that Cu(I) is preferentially bound by this peptide also in the presence of the His-rich peptide. The shape and the intensity of the preedge peak at 8984 eV indicate that in the presence of the Met-rich peptide the Cu(I) ion is coordinated with three sulfur atoms (*14*). In the presence of the His-rich peptide the sharp preedge peak characterized by a large intensity suggests a three-coordinate compound, where Cu(I) is bound by two histidine nitrogens and a third oxygen ligand (*14*). The XAS spectrum of a solution containing the Cu(I) ion and ascorbic acid was also recorded. Both the XANES and EXAFS regions of this spectrum are dramatically different from those obtained in the presence of the Met-rich and His-rich peptides, pointing to a completely different coordination of the Cu(I) ion (data not shown).

Figure 2B shows the XANES spectra of three Cu(II) solutions containing the Met-rich, the His-rich, and an equimolar mixture of the two peptides, respectively. In this case, differences in the low-energy regions are less evident, suggesting that the Cu(II) environment is similar in the three

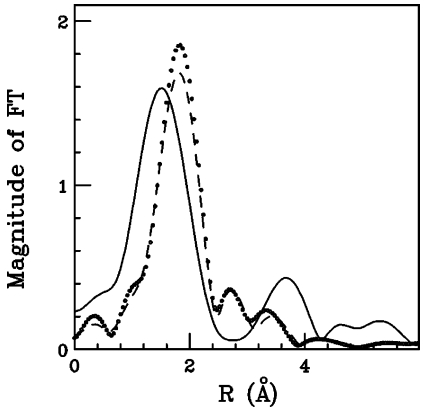

FIGURE 3: Fourier transforms of the k^2-weighted EXAFS experimental data for Cu(I). Data are shown as a dotted line (Met-rich peptide), solid line (His-rich peptide), and dashed line (equimolar mixture of the two peptides).

systems. In all cases, the extremely weak 1s → 3d transition around 8980 eV (Figure 2B) indicates an almost centrosymmetrical coordination environment of the Cu(II) ion, suggesting that both peptides bind Cu(II) with similar coordination geometries. The XANES of the sample containing both peptides is nearly identical to the His-rich spectrum, indicating that the Cu(II) ion is preferentially bound by the His-rich peptide. These results are further corroborated by the EXAFS data analysis.

EXAFS Analysis of Copper(I) Complexes. The Fourier transform (FT) moduli of the EXAFS experimental spectra corresponding to the three Cu(I) solutions containing the Met-rich (dotted line), the His-rich (solid line), and the mixture of the two peptides (dashed line), extracted with a three-segmented cubic spline, are shown in Figure 3. The FTs of the Met-rich and of the mixed solutions have been calculated in the interval $k = 2.8-10.0$ Å^{-1}, while the FT of the His-rich spectrum has been calculated in the interval $k = 2.8-9.0$ Å^{-1}, due to the higher noise of the spectrum, with no phase-shift correction applied. The FT spectra of the Met-rich and of the mixed solutions are very similar and show the presence of a single first shell peak at ~1.8 Å. The higher distance peaks are due to cutoff effects and not to a structural contribution. The FT of the His-rich solution is markedly different from the previous ones, being characterized by a first-shell peak centered at 1.5 Å and by additional outer-shell peaks indicative of histidine binding to the metal ion (*7*, *8*).

A quantitative estimate of the Cu(I) coordination geometry parameters can be obtained from the analysis of the EXAFS data by means of the GNAXS program. The best-fit analysis of the Met-rich EXAFS spectrum is reported in the upper panel of Figure 4. The χ(k) signals are shown multiplied by k^2 for better visualization. The EXAFS experimental spectrum is well reproduced by a coordination sphere constituted by three sulfur atoms at a distance of 2.29 Å. The accuracy of the data analysis can be appreciated by looking at the FTs of the experimental and theoretical signals which are shown in the lower panel of Figure 4. The best-fit values for the full set of structural parameters are listed in Table 1. To establish error limits on the structural parameters, we have applied a statistical analysis methodology using two-dimensional contour plots to selected parameters of the fit. This analysis examines correlations among fitting parameters

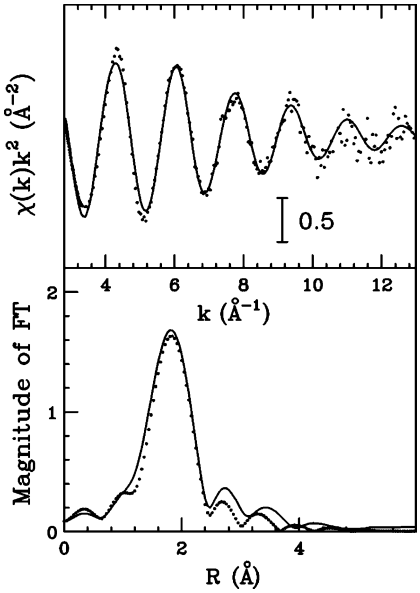

FIGURE 4: EXAFS fitting results of the Cu(I)/Met-rich complex. Upper panel: Comparison of the theoretical signal (solid line) with experimental data (dotted line) of Cu K-edge k^2-weighted EXAFS data of Cu(I)/Met-rich aqueous solution. Lower panel: Nonphase-shift-corrected Fourier transforms of the experimental data (dotted line) and of the total theoretical signal (solid line).

Table 1: Structural Results for the Cu(I)− and Cu(II)−Peptide Complexes Obtained from the EXAFS Data Analysis[a]

complex	ligand	R (Å)	σ^2 (Å^2)
Cu(I)/Met-rich	3 S	2.29 (2)	0.0056 (5)
Cu(I)/His-rich	2 His	1.95 (3)	0.016 (2)
	1 O/N	1.99 (2)	0.018 (2)
Cu(I)/His-rich + Met-rich	3 S	2.28 (2)	0.0061 (4)
Cu(II)/His-rich	4 His	1.99 (2)	0.0045 (5)
	2 O/N	2.54 (3)	0.010 (7)
Cu(II)/Met-rich	4 O/N	1.98 (2)	0.011 (4)
Cu(II)/His-rich + Met-rich	4 His	1.99 (2)	0.0040 (6)
	2 O/N	2.55 (3)	0.006 (4)

[a] The parameters are as follows: the average distance R and the Debye−Waller factor σ^2. Standard deviations obtained from the EXAFS analysis are given within parentheses.

and evaluates statistical errors in the determination of the Cu coordination structure (*10*). The fit of the Met-rich experimental data shows unequivocally the presence of sulfur atoms in the first coordination shell of the Cu(I) ion, and the quality of the fit improves when three, instead of two sulfur atoms, are at a distance of 2.29 Å, in agreement with the shape and intensity of the 1s → 4p transition described above. A similar result is obtained from the EXAFS analysis of the solution containing an equal amount of the two peptides, and the parameters obtained from the fitting procedure are listed in Table 1. The Cu−S coordination distances and Debye−Waller factors are comparable with the values previously found for the PcoC and CopC proteins (*7*, *8*) and are typical for thioether ligation of the Met residues.

The EXAFS data for the His-rich solution are dramatically different. The minimization procedure of the experimental spectrum reveals that the Cu(I) ion is bound to two histidines at 1.95 Å and another O/N ligand at 1.99 Å, in agreement with the shape of the 1s → 4p preedge peak. As previously observed, the presence of peaks in the region between 3 and 4 Å in the FT (see Figure 3) is a clear indication of the Cu

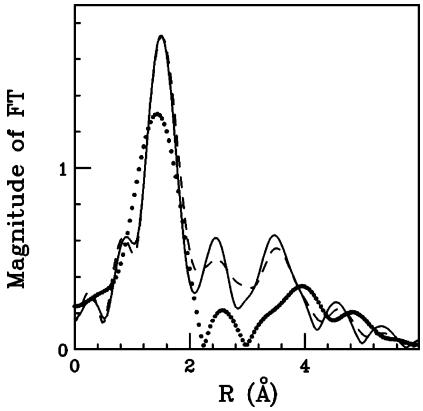

FIGURE 5: Fourier transforms of the k^2-weighted EXAFS experimental data for Cu(II). Data are shown as a dotted line (Met-rich peptide), solid line (His-rich peptide), and dashed line (equimolar mixture of the two peptides).

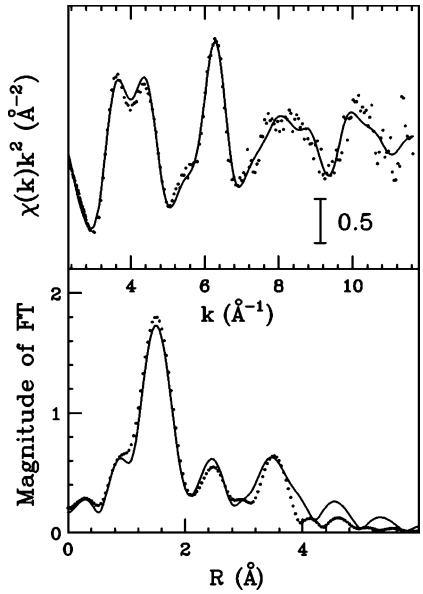

FIGURE 6: EXAFS fitting results of the Cu(II)/His-rich complex. Upper panel: Comparison of the theoretical signal (solid line) with experimental data (dotted line) of Cu K-edge k^2-weighted EXAFS data of Cu(II)/His-rich aqueous solution. Lower panel: Nonphase-shift-corrected Fourier transforms of the experimental data (dotted line) and of the total theoretical signal (solid line).

ion being ligated to histidine imidazole ligands (*7*). Attempts to fit the EXAFS data with three histidines resulted in an increase of the fit index parameter of about 20%.

EXAFS Analysis of Copper(II) Complexes. The magnitudes of the FTs of the three Cu(II) solutions containing the Met-rich (dotted line), the His-rich (solid line), and the mixture of the two peptides (dashed line), extracted with a three-segmented cubic spline, are shown in Figure 5. The FTs have been calculated in the interval $k = 2.8-9.8$ Å^{-1} for the His-rich spectrum and in the interval $k = 2.8-9.4$ Å^{-1} for the other two spectra. All of the FTs have outer-shell peaks, indicating that both a first and a second coordination shell contributes to the EXAFS spectra. The FT of the mixed solution is almost identical to the His-rich one, with a first shell peak centered at 1.5 Å and two additional peaks at ~2.5 and 3.8 Å, which are typical of the histidine carbon backscattering. The intensity of the higher shell peaks of the two spectra is not equal due to the different k interval used in the transformations. The Met-rich FT spectrum is markedly

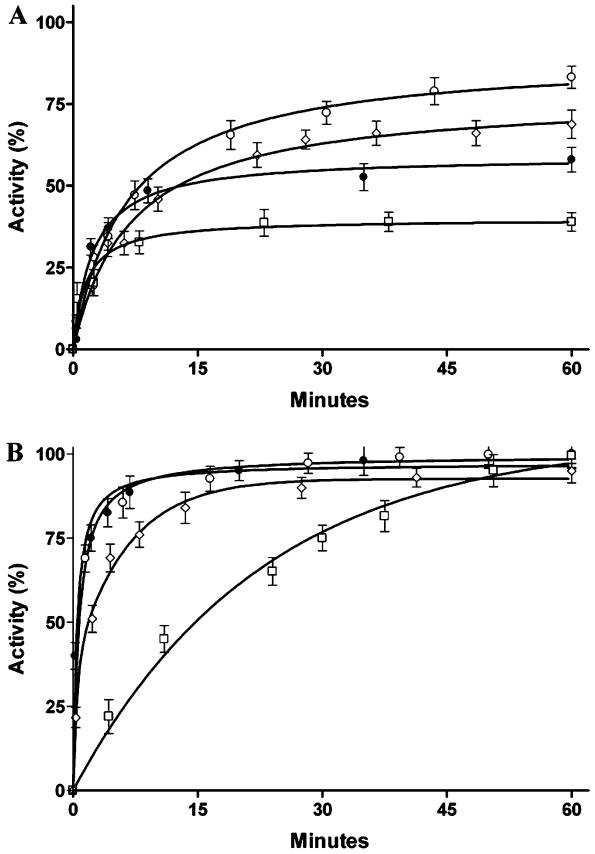

FIGURE 7: Kinetics of regaining of activity by the Cu-free enzyme. Copper-free N-deleted *H. ducreyi* Cu,ZnSOD was incubated with Cu/Met-rich peptide (□), Cu/His-rich peptide (○), or the mixture of Cu and the two peptides (◇). Reconstitution of the Cu-free wild-type enzyme is also reported (●). Panel A: Reconstitution assays carried out with Cu(II). Panel B: Reconstitution assays carried out with Cu(I). The reported results are the mean ± SD of three independent assays.

different from those of the other two Cu(II) samples. In particular, the first shell peak is less intense, while there is a higher distance peak at ~4 Å.

The best-fit analysis of the Cu(II)/His-rich spectrum is reported in the upper panel of Figure 6. The experimental χ(k) signal is characterized by several structured peaks revealing histidine binding to the metal ion (*15*). The structural parameters obtained from the minimization procedure, listed in Table 1, indicate that the Cu(II) ion is bound to four histidines at 1.99 Å. Addition of two further O/N ligands in the axial positions at 2.54 Å improves the agreement between the experimental and theoretical curves with a 20% decrease of the fit index parameter. The consistency of this analysis is confirmed by the perfect agreement between the experimental and theoretical FT spectra, obtained considering four histidines coordinated to Cu(II) in the equatorial positions and two additional O/N ligands at the apical sites (Figure 6). Since the present investigation has been carried out at a Cu/peptide ratio of 0.5, it is possible that the histidine ligands of Cu(II) belong to two different molecules of the His-rich peptide.

Similar results have been obtained from the analysis of the solution containing an equal amount of the two peptides. The full set of structural parameters obtained from the minimization procedure is reported in Table 1. Also, in this case the EXAFS and FT data are well reproduced by a

coordination sphere constituted by four histidines in the equatorial plane and two additional O/N ligands in the equatorial positions, in agreement with the XANES results presented above.

Analysis of the EXAFS spectrum of the Cu(II)/Met-rich complex revealed a completely different coordination sphere around the ion. In this case, four O/N atoms occupy the Cu(II) equatorial sites at 1.98 Å, while the outer-shell sphere cannot be accurately determined on the basis of the EXAFS data.

Reconstitution of Cu,Zn Superoxide Dismutase by Cu−Peptide Complexes. We have previously shown that the N-terminal domain of *H. ducreyi* Cu,ZnSOD binds Cu(II) with high affinity and that the copper ions bound to this protein domain can be subsequently transferred to the active site of the enzyme (*3*). The above-reported XAS studies show that both the His-rich and Met-rich regions of the N-terminal domain can bind copper in the two oxidation states, leaving open the question of which region can more efficiently reconstitute the active site of the copper-free enzyme. The rates of activity recovery by copper-free N-deleted mutant Cu,ZnSOD in the presence of 0.5 equiv of Cu(II)−peptide complexes are shown in Figure 7, panel A. When copper reconstitution is carried out using the Cu(II)/Met-rich peptide, only 40% of the activity is recovered. The Cu(II)/His-rich peptide complex reconstitutes the copper-free N-deleted enzyme more efficiently, 50% of the activity being recovered in 12 min. The reactivation rate of the enzyme obtained with this peptide is faster than that obtained with the native enzyme or with the mixture of Cu(II)/His- and Met-rich peptides. These results indicate that the Met-rich region of the peptide negatively influences the transfer of oxidized copper to the catalytic site of CuZnSOD and that the Met-rich peptide is not a good donor of copper to the enzyme.

When reconstitution experiments are carried out with Cu(I) complexes, the enzyme activity regain is much faster than with copper in the oxidized state, and the enzyme is fully reconstituted under all of the conditions used, although at different rates (Figure 7, panel B). Reconstitution of the enzyme with the Cu(I)/Met-rich peptide complex is slower than that with the Cu(I)/His rich peptide, 50% of activity being achieved after 12.7 min with the Cu(I)/Met-rich peptide, compared to 0.75 min with the Cu(I)/His-rich peptide. The rate of copper reconstitution obtained with Cu(I) and the mixture of both peptides is slightly lower than that obtained with the Cu(I)/His-rich peptide. These results indicate that, as observed in the case of Cu(II), Cu(I) is not efficiently transferred from the Met-rich peptide to the enzyme active site and that the His-rich peptide is needed to facilitate Cu(I) insertion in the catalytic site. The rate of reconstitution obtained with Cu(I) and the mixture the two peptides is similar to that obtained with the wild-type enzyme, indicating that the addition of the two peptides to the N-terminal truncated SOD reproduces fairly well the reconstitution process observed for the full enzyme. However, the slightly faster reconstitution of the wild-type enzyme suggests that there are productive interactions in the native enzyme that do not occur in the N-deleted plus the mixed peptides.

DISCUSSION

The identification of several methionine-rich motifs in bacterial proteins involved in metal homeostasis (*5*−*8*, *16*, *17*), and the likely possibility that copper can be found in the periplasm either as Cu(I) or as Cu(II) due to the opposite activities of efflux systems that pumps Cu(I) from the cytoplasm to the periplasmic space and of a multicopper oxidase promoting Cu(I) oxidation (*18*, *19*), has prompted us to analyze the copper binding propensity of the N-terminal domain of *H. ducreyi* Cu,ZnSOD by X-ray absorption spectroscopy. Our results indicate that the peptides corresponding to the two halves of the N-terminal region of *H. ducreyi* Cu,ZnSOD can form stable complexes with copper in both of the oxidation states. However, competition experiments demonstrate that the His-rich peptide, corresponding to the first half of the N-terminal domain, has a strong propensity for Cu(II) binding, while the Met-rich peptide, corresponding to the second part of the domain, prefers Cu(I) binding. Although the copper coordination geometries determined in the peptides do not necessarily describe the exact metal environment in the full-length protein (because residues belonging to the two halves of the domain might concur to metal binding in the native protein and/or the N- and C-termini of the peptides could participate to metal coordination), our results indicate that Cu(II) coordination involves histidine residues, while methionine residues mediate Cu(I) coordination. Several observations suggest that bacterial Cu,ZnSODs compete with periplasmic metallochaperones for the binding of the active site metal ions when bacteria grow in environments where such elements are scarce (*3*, *20*, *21*). The unprecedented arrangement of two adjacent Cu(I) and Cu(II) binding motifs within the N-terminal metal binding domain of *H. ducreyi* Cu,ZnSOD may favor metal recruitment by the enzyme under conditions of limited copper availability. In particular, the methionine-rich sequence following the cluster of histidine residues may be particularly important in facilitating copper recruitment under anaerobic conditions, when copper rapidly shifts from the oxidized to the reduced state (*18*).

Reconstitution experiments of the N-deleted mutant enzyme, either in the presence of each of the two peptides used in the XAS experiments or in the presence of an equimolar mixture of the two peptides, indicate that Cu(I) is transferred to the active site much more efficiently than Cu(II). This finding is in agreement with previous studies showing that Cu(I) complexes achieve the fast and complete reconstitution of eukaryotic Cu-devoid Cu,ZnSOD (*22*) through a direct transfer of Cu(I) into the empty copper binding site (*23*). This result suggests that Cu(I) is the most likely copper form mediating bacterial Cu,ZnSOD reconstitution in vivo.

The reconstitution experiments also show that, irrespective of the metal oxidation state, the mixture of the two peptides simulates the process of reconstitution of the wild-type enzyme better than the methionine-rich peptide. Moreover, despite XAS studies demonstrating that Cu(I) is preferentially bound by the Met-rich peptide, reconstitution experiments indicate that the presence of the His-rich peptide is required for a fast Cu(I) transfer to the enzyme active site. This result indicates that interactions between the two copper binding motifs influence the kinetics of copper transfer from the N-terminal domain to the active site.

In conclusion, we suggest that the juxtaposition of two sequence motifs potentially involved in the binding of Cu(I) and Cu(II) in the N-terminal domain of *H. ducreyi* Cu,ZnSOD could have a double role: to facilitate copper acquisi-

tion in environments poor of this metal ion and to assist Cu(I) transfer from the high-affinity Met-rich motif to the enzyme active site.

ACKNOWLEDGMENT

We acknowledge the European Synchrotron Radiation Facility for provision of synchrotron radiation facilities and Jean-Jacques Menthonnex for assistance during the experiments. Experiments have been performed at beamline BM30B as part of the proposal number SC-1371.

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BI050925X