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Tryptophan Cu(I)– π interaction fine-tunes the metal binding properties of the bacterial metallochaperone CusF

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Abstract

The periplasmic metallochaperone CusF coordinates Cu(I) and Ag(I) through a unique site consisting of a Met₂His motif as well as a Cu(I)– π interaction between a nearby tryptophan, W44, and the metal ion. Through mutational analyses we investigate here the role that W44 in CusF plays in metal coordination. Nuclear magnetic resonance spectra show that the specificity of CusF for Cu(I) and Ag(I) is not altered by mutation of W44. X-ray absorption studies reveal that W44 protects the bound Cu(I) from oxidation as well as from adventitious ligands. Competition assays demonstrate that W44 does not significantly contribute to the affinity of CusF for metal, but that substitution of W44 by methionine, which forms a fourth Cu(I) ligand, substantially increases the affinity. These studies indicate that W44 is important in maintaining a moderate-affinity and solvent-shielded three-coordinate environment for Cu(I), which has implications for the function of CusF as a metallochaperone.

Keywords

Copper; Metallochaperone; Copper coordination

Introduction

The essential yet toxic nature of copper requires cells to have effective homeostasis mechanisms to ensure the supply of copper to metalloproteins while preventing cytotoxicity caused by excess copper. The CusCFBA system in *Escherichia coli* is responsible for detoxification of copper from the periplasm of this Gram-negative organism. CusCBA is expected to form a channel spanning the periplasmic space, as has been reported for homologous multidrug export systems [1,2]. Yet, while some similarities are expected between these homologous systems, the Cus system harbors a fourth component that is unique to monovalent metal resistance systems, CusF. In addition, the multidrug export systems have very broad substrate specificity [3], while the Cus system has been shown to be highly specific for the monovalent metal ions Cu(I) and Ag(I) [4–6].

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Our group has recently shown that CusF functions as a metallochaperone to the CusCBA system [7]. This role of CusF was evidenced by isothermal titration calorimetry data, which showed a metal-dependent complex formation of CusF and CusB. Metal transfer was shown to be direct and specific between these proteins. Furthermore, X-ray absorption spectroscopy (XAS) showed that metal transfer between the two proteins is rapid and reversible and is likely to be accomplished through ligand exchange reactions as seen between other metallochaperones and their target proteins [8–11]. Similar values for the dissociation constants of CusF and CusB for Ag(I), as well as the three-coordinate thioether coordination of Cu(I) in CusB, support the conclusion of CusF filling the role of a metallochaperone [4, 12].

Novel metal coordination chemistry for CusF has been reported, with CusF binding Cu(I) and Ag(I) via a Met₂His motif [6,13]. The metal, however, is tetragonally displaced toward the tryptophan indole ring of W44, which caps the fourth coordination site of Ag(I)/Cu(I). While the distances to the two closest carbon atoms of W44, CZ3 and CE3, are too long for W44 to contribute as a full ligand, it has been suggested that the tryptophan provides a Cu(I)– π interaction that stabilizes the metal coordination and possibly protects the metal from oxidation and coordination by adventitious ligands [6,13]. This type of interaction through Cu(I)– π bonding has not been previously observed in metalloprotein structures.

In CusF homologs, the position of the tryptophan is conserved as the position of a potential metal coordinating ligand, either a tryptophan or a methionine. This observation suggests that metal interactions by this residue may be functionally important, though it is unknown what role each of these residues may play and how they affect the Cu(I)/Ag(I) binding site. It has been reported that the affinity of the CusF W44M mutant is approximately 2 orders of magnitude tighter than that for the wild-type protein, though the numeric values for K_D were not reported [13]. Further investigation is needed to determine the effect of the W44 position on metal binding specificity, as well as its role in protecting the bound metal from the oxidizing environment of the periplasm.

Copper sites are found to match the functional requirements of the proteins in which they are situated. The unusual copper site in CusF is most likely necessary to fine-tune the Cu(I) coordination so that it is poised to allow for transfer of the metal to its target protein CusB, while protecting the metal from oxidation, unwanted metal loss, or coordination through adventitious ligands, which could interfere with metal transfer. In the work reported here, binding site mutants of CusF were generated to investigate the role of W44. CusF_{1–88}–W44M and CusF_{1–88}–W44A constructs were analyzed via atomic absorption spectroscopy (XAS) and nuclear magnetic resonance (NMR) experiments. The affinities of CusF for Cu(I) were measured via a competitive binding assay. Through these experiments we have found that W44 plays a specific role in CusF. The Cu(I)– π interaction of W44 with the metal ion limits the affinity of the metal ion for the protein, most likely to allow for efficient transfer to CusB. The indole side chain which caps the Cu(I) binding site provides protection of Cu(I) from oxidation, as well as from adventitious ligands. The Cu(I)– π interaction may therefore function as an anchor to hold the protective cap in place until the correct target protein is encountered.

Materials and methods

Site-directed mutagenesis

Site-directed mutagenesis was performed to alter individual amino acid residues in full-length CusF (hereafter referred to as CusF_{1–88}) and in a truncated CusF construct starting at residue 10 (hereafter referred to as CusF_{10–88}). The plasmids used as a template, pASK-IBA3 (IBA, Göttingen, Germany) containing the gene encoding CusF_{1–88} and pPR-IBA1 (IBA, Göttingen, Germany) containing the gene encoding CusF_{10–88}, were isolated from a *dam*⁺ strain of *E.*

coli. The primer pairs used to introduce the point mutation were antiparallel and overlapping. Amplification of the plasmids was achieved with the enzyme *Pfu* Ultra. To digest the *dam*-methylated template plasmid, PCR products were treated with *Dpn*I, then purified and transformed into *E. coli*. With use of this method, CusF₁₋₈₈-W44A and CusF₁₋₈₈-W44M in pASK-IBA3 as well as CusF₁₀₋₈₈-W44A and CusF₁₀₋₈₈-W44M in pPR-IBA1 were generated, and were verified by DNA sequence analysis.

Protein purification

Escherichia coli BL21/ΔDE3 cells containing the plasmid of interest were grown in Luria-Bertani medium or in M9 minimal medium containing [¹⁵N]ammonium chloride as the sole nitrogen sources to prepare isotopically labeled protein. CusF mutants were expressed and purified as previously described for the wild-type protein [4,6,14] and subsequently incubated with 10 mM EDTA to remove any divalent trace metals. Protein samples were dialyzed into the appropriate buffer, concentrated, and stored at 253 K until needed.

Preparation of NMR samples of CusF mutants

For the NMR samples, CusF₁₋₈₈-W44A, CusF₁₋₈₈-W44M, or CusF₁₀₋₈₈-W44M were dialyzed in 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES), pH 7.5 (buffer A). Samples of CusF₁₋₈₈-W44M were prepared as apoprotein, with 1 molar equivalent of Ag(I) (added as AgNO₃), with 1 or 2 molar equivalents of Cu(II) [added as Cu(SO₄)₄], or with 2 molar equivalents of Ni(II), Co(II), or Zn(II) (added as the chloride salts). The final concentration of the protein samples was 0.65 mM. Samples of CusF₁₋₈₈-W44A were prepared as the apoprotein, with 1 molar equivalent of Ag(I) (added as AgNO₃), or with 1 or 2 molar equivalents of Cu(II) (added as CuCl₂). The final concentration of these samples was 0.6 mM. CusF₁₀₋₈₈-W44M (0.9 mM final concentration) was prepared as the apoprotein, with up to 4 molar equivalents of Cu(SO₄)₄, or with 1 molar equivalent of Cu(I) (prepared as described previously for the wild-type protein [14]). For each sample, the pH was monitored to ensure no significant pH changes occurred upon addition of metals.

Spectra were collected at 298 K with a 600 MHz Varian Inova instrument equipped with a four-channel pulsed-field-gradient triple-resonance cold probe using pulse sequences from Varian Biopack. ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) [15] spectra of all samples were collected. Spectra were processed with NMRPIPE [16] and analyzed with NMRView [17].

XAS sample preparation, data collection, and analysis

All samples were prepared by methods similar to those used for the wild-type protein as previously described [6]. The final concentrations of CusF₁₋₈₈-W44M-Cu(I) and CusF₁₋₈₈-W44A-Cu(I) were 270 and 160 μM, respectively. Cu K-edge (8,980 eV) extended X-ray absorption fine structure (EXAFS) data for these CusF mutants were collected at the Stanford Synchrotron Radiation Laboratory on beamline 9-3. The configuration of the beamline, data collection parameters, and methods of data reduction and analysis were as previously described [6].

Crystallization and crystal structure determination of CusF₁₀₋₈₈-W44A-Cu(II)

Purified CusF₁₀₋₈₈-W44A was dialyzed against 20 mM HEPES (pH 7.5), and concentrated to 51 mg/mL. CuCl₂ was added to a twofold molar excess to apo-CusF₁₀₋₈₈-W44A. Crystals were obtained by the hanging-drop vapor diffusion method. Drops were set up by mixing 2 μL protein solution with 2 mL reservoir solution [0.1 M tris(hydroxymethyl)aminomethane-HCl pH 8.5, 27.5% PEG 4000, 0.2 M sodium acetate, 2 mM CuCl₂] and equilibrated against 1 mL reservoir solution at room temperature. Crystals grew in clusters of plates with

dimensions of 0.6 mm × 0.6 mm × 0.2 mm. The crystals were orthorhombic and the space group was $P2_1P2_1P2_1$ with one CusF₁₀₋₈₈-W44A-Cu(II) molecule per asymmetric unit.

After transfer to 35% PEG 4000 enriched with 2 mM CuCl₂ to prevent loss of the Cu(II) from the crystal, crystals were flash-frozen in liquid nitrogen. Data were measured at 100 K at the Stanford Synchrotron Radiation Laboratory beamline 9-2 with a wavelength of 0.97946 Å, processed with CrystalClear (D*TREK) [18,19], and scaled with SCALA in CCP4 [20]. MOLREP [21] in CCP4 [20] was run to perform molecular replacement using the CusF₁₀₋₈₈-Ag(I) coordinates, Protein Data Bank code 2QCP [6]. The structure was refined with anisotropic temperature factors using REFMAC5 [20] with inter-spersed manual rebuilding using COOT [22]. Data measurement and refinement statistics are given in Table S1. The coordinates and structure factors have been deposited in the Protein Data Bank under accession code 3E6Z.

Competitive binding assay

The protocol for this assay was adapted from Yatsunyk and Rosenzweig [11]. The conditions for the assay were identical to those previously reported; thus, K_1 , K_2 , and β_2 for the bicinchoninic acid (BCA)-Cu(I) complex, BCA₂-Cu(I), determined under these conditions can be used to calculate the apparent dissociation constants [11].

The protein samples were dialyzed into 50 mM HEPES, pH 7.5, 200 mM NaCl (buffer B) and concentrated to 1.17, 0.59, and 0.28 mM for wild-type CusF₁₋₈₈, CusF₁₋₈₈-W44A, and CusF₁₋₈₈-W44M, respectively. Protein samples were thoroughly degassed before placement in the anaerobic chamber. All necessary solutions were prepared under anaerobic conditions. Different amounts of apoprotein were titrated into a solution (solution A) containing 10 μM Cu(I), 100 μM to 1 mM BCA [BCA-to-Cu ratios of 10:1–100:1 were used to ensure all Cu(I) was initially chelated by BCA], and 0.2 mM ascorbate in buffer B. One milliliter of solution A was used to perform the assay and was put into a septum-sealed cuvette before removal from the anaerobic chamber. For the baseline, the absorbance of solution A minus 10 μM Cu(I) was measured. Equilibration was achieved after 10 min and absorption spectra between 300 and 850 nm were collected with a Cary 50 UV–vis spectrophotometer at room temperature. The transfer of Cu(I) from BCA to protein was determined by the change in the characteristic absorbance for BCA₂-Cu(I) at 562 nm. The titration was performed until no further changes occurred at 562 nm.

The data obtained were corrected for absorption at 800 nm and for dilution as protein was added. As discussed previously [11], if the dissociation constant, K_D , is calculated with the β_2 value, the protein's affinity may be overstated. Thus, we report K_D values calculated with K_1 as well as β_2 to give values for K_D in terms of the minimum and maximum values. The total copper concentration of the initial titration solution was determined by inductively coupled plasma optical emission spectroscopy and the protein concentrations were determined with the BCA protein assay (Pierce) with a minimum of three measurements giving values within no more than 5% of each other. All experiments were performed in triplicate. The dissociation constants, K_D , were determined by nonlinear fitting of the absorbance data at 562 nm using MATLAB (The MathWorks, Natick, MA, USA).

Results

Investigation into the role of W44

W44 in CusF contributes a Cu(I)- π interaction to the coordination of Cu(I) and Ag(I) [6,13], yet the explicit function of this residue has not yet been revealed. Possible roles of W44 include protection of the binding site from solvent access, ensuring specificity for Cu(I)/Ag(I),

stabilization of the oxidation state of the bound metal ion, fine-tuning the affinity for Cu(I)/Ag(I) to enable transfer of the metal ion to CusB, and recognition of the correct target protein during metal transfer. To investigate which if any of these effects W44 has on metal coordination in CusF, the alanine and methionine mutants at this position in CusF were further characterized and compared with the wild-type protein.

Alteration of W44 does not affect the overall structure of CusF or metal specificity

NMR HSQC spectra were collected to determine the effects of W44 mutations on CusF structure and metal specificity. The similarities of the spectra to the spectrum of the wild-type protein indicate that CusF₁₋₈₈-W44A, CusF₁₋₈₈-W44M, and CusF₁₀₋₈₈-W44M exhibit the native fold and are stable proteins. All three constructs are able to bind Ag(I), which serves as a Cu(I) analog under aerobic conditions, at the previously described Met₂His Cu(I)/Ag(I) binding site as evidenced by chemical shift changes similar to those observed for wild-type CusF₁₋₈₈-Ag(I) (Fig. S1). On the basis of previously reported spectra of Cu(I)- and Ag(I)-bound wild-type CusF [4,14], Cu(I) is expected to bind the CusF₁₋₈₈-W44A and CusF₁₋₈₈-W44M variants in a similar fashion. No global linewidth changes were observed in the spectra of the CusF-W44M or CusF-W44A variants, suggesting the proteins are monomeric under all conditions. Addition of Zn(II), Co(II), and Ni(II) to CusF₁₋₈₈-W44M only showed broadening of the N-terminal residues (Fig. S2 and data not shown), which is likely a result of the identity of the first five amino acids of CusF, NEHHH. An N-terminal XXH sequence, where X is any residue, has been termed an "ATCUN motif," and is expected to have a weak affinity for divalent metals such as Cu(II) and Ni(II) [23]. No shifting or broadening of residues at the Met₂His Cu(I)/Ag(I) binding site was observed in any of the spectra upon addition of these divalent metals.

The addition of Cu(SO)₄ to CusF₁₋₈₈-W44A resulted in disappearance of the peaks corresponding to the N-terminal region, indicating binding of paramagnetic Cu(II) at the ATCUN motif (Fig. S3). The spectra of CusF₁₋₈₈-W44M showed a complex effect upon the addition of Cu(SO)₄ (Fig. 1a). At an equimolar ratio of copper and protein, peaks at the N-terminus disappeared, indicating binding of Cu(II) to the ATCUN motif. Additionally, peaks from residues near the Met-His metal binding site also disappeared. These peaks could have disappeared because of the binding of paramagnetic Cu(II) to the Met-His metal binding site, or because of a mixed population of metal-free and metal-bound protein. To distinguish between effects at the two potential metal binding sites, a shorter construct, CusF₁₀₋₈₈-W44M, which does not have the ATCUN motif, was analyzed. CusF₁₀₋₈₈-W44M was titrated with 1, 1.5, 2, 2.5, 3, 3.5, and 4 molar equivalents of Cu(SO)₄ and a ¹H-¹⁵N HSQC spectrum was obtained after each addition. Broadening of some peaks corresponding to residues near the Met-His metal binding site were observed in the spectra during the early points in the titration. Further additions of Cu(SO)₄ (2 molar equivalents or greater) resulted in spectra that are identical to the spectrum of CusF₁₀₋₈₈-W44M bound to Cu(I) (Fig. 1b). These spectra indicate that even under aerobic conditions where Cu(II) predominates, CusF-W44M is able to bind Cu(I) from solution.

Metal environment of CusF-W44M and CusF-W44A examined by EXAFS

To determine the coordination of Cu(I) in CusF₁₋₈₈-W44A and CusF₁₋₈₈-W44M, EXAFS data were collected and analyzed (Fig. 2). The W44A variant exhibits a spectrum similar to that reported previously for wild-type CusF [6], and the best simulation achieved is for one histidine and two S(Met) scatterers at the distances given in Table 1. The CusF₁₋₈₈-W44M data differ from the CusF₁₋₈₈-W44A data in that the intensity of the Cu-S shell has increased substantially, and now fits well to three rather than two S(Met) scatterers. This observation is fully consistent with coordination by the methionine residue, which replaced W44. This species is now formally four-coordinate, and both Cu-N and Cu-S distances are longer than those

reported previously for the three-coordinate wild-type protein (Table 1), as would be expected. An increase in coordination number is also suggested by the absorption edge profile, where the 8,983-eV feature characteristic of a three-coordinate environment has collapsed to a shoulder.

Comparison of both edge and EXAFS-derived distances of the W44M and W44A variants, however, shows remarkable similarities. The Cu–N(His) and Cu–S(Met) bond lengths in the W44A variant resemble those of the W44M derivative more closely than those of the wild-type protein, suggesting that the alanine variant is a pseudo-four-coordinate species. While no additional ligand is apparent from the EXAFS fitting protocol, the data may suggest additional interaction of the Cu(I) site with some labile donor, possibly solvent. It is also apparent that the alanine variant may be more susceptible to oxidation, where the apparent shift in the absorption edge to higher energy coupled with the small but significant decrease in intensity at 8,983 eV may signal a minor population of oxidized copper sites. Thus, the W44 Cu(I)– π interaction or a formal fourth ligand contributed by a methionine protects the Cu(I) from oxidation. The labile donor that may occur in the alanine-substituted protein cannot afford this protection.

Structure of CusF–W44A–Cu(II) variant

The EXAFS studies suggested that the Cu(I) site in CusF–W44A is more susceptible to oxidation than the Cu(I) sites of wild-type CusF or the CusF–W44M variant. Additionally, the XAS data suggest a potential labile fourth ligand. To create a structural model for the metal site, crystallization conditions were sought for CusF–W44A in the presence of either Cu(I) or Cu(II). However, crystals were only obtained for CusF–W44A in the presence of Cu(II). In this case, Cu(II) binding is likely a result of the high concentrations of Cu(II) in the crystallization conditions, since NMR spectra indicate that CusF–W44A does not detectably bind Cu(II) at the Met₂His site even at a twofold molar excess concentration (Fig. S3). The copper absorption edge confirmed that the oxidation state in the crystal is Cu(II). Though the packing of CusF–W44A molecules in the crystal is different from that seen for the wild-type protein, the overall structure of the CusF–W44A variant is very similar to the previously determined wild-type CusF structure [6,13]. In CusF–W44A, Cu(II) is bound at the Met₂His site formed by residues H36, M47, and M49, with a fourth ligand contributed by the N-terminal methionine of a neighboring molecule (Fig. 3). The presence of this methionine at the metal site is likely due to the serendipitous arrangement of the CusF–W44A molecules within the crystal, since there is no evidence for dimerization from the NMR data.

Competitive binding assays reveal increased affinity of CusF_{1–88}–W44M for Cu(I)

To understand how the identity of the residue at position 44 influences the protein's metal binding affinity, a competition assay was performed on wild-type CusF_{1–88}, CusF_{1–88}–W44M, and CusF_{1–88}–W44A. BCA, which has a high affinity for Cu(I), was used as a competitor for Cu(I). The BCA assay can be used to determine dissociation constants beyond the detection limit of isothermal titration calorimetry, which was used previously to estimate the Cu(I) affinity for wild-type CusF [4]. Upon titration of protein, a stepwise decrease in the absorption at 562 nm, which is characteristic for BCA₂–Cu(I), was observed, indicating Cu(I) coordination by all three CusF constructs (Fig. 4). To avoid overstating the affinity for Cu(I), the values for the dissociation constant K_D were calculated using K_1 as well as β_2 (which is the product of K_1 and K_2) to establish the minimum value (highest possible affinity) and the maximum value (lowest possible affinity) of K_D for each protein (Table 2). The affinities of wild-type CusF and CusF_{1–88}–W44A are very similar; however, the W44M variant has an affinity 1–3 orders of magnitude greater.

Discussion

To elucidate the role of the unusual Cu(I)– π interaction at the metal binding site of CusF, we examined two specific mutations, W44M and W44A, of CusF that abolish this interaction. We tested these variants with respect to metal specificity, coordination, susceptibility to oxidation, and affinity. We found that the absence of the tryptophan does not broaden the specificity of the metal site to include divalent metals, and therefore W44 is unlikely to be a key determinant of substrate selection in CusF. While higher coordination numbers are generally preferred for Cu(II) as compared with Cu(I), simply increasing the number of potential ligands (as in the W44M and W44A variants) does not lead to the ability to bind divalent metals.

The amino acid identity of CusF at position 44 influences the coordination environment of Cu(I). XAS and crystallography data have shown that the wild-type protein has a largely three-coordinate Cu(I) environment contributed by two methionine residues, M47 and M49, a histidine residue, H36, and a Cu(I)– π interaction through the CE3 and CZ3 atoms of W44 [6, 13]. Alteration of W44 to a methionine changes the coordination of Cu(I) to a clearly four-coordinate Met₃His Cu(I) environment. The contribution of this third methionine most likely arises from an intramolecular interaction, since no changes in NMR linewidths which would indicate dimerization were observed. The alteration of W44 to an alanine residue results in a pseudo-four-coordinate species, with a possible fourth ligand being a labile donor such as a solvent molecule. Support for potential access by a fourth ligand is seen in the CusF–W44A–Cu(II) crystal structure, where a fourth ligand from another molecule contributes to the metal site. The XAS data of the W44A variant of CusF shows some oxidation of Cu(I), which suggests that one role for the tryptophan Cu(I)– π interaction in vivo may be to shield the Cu(I) site from solvent access in the oxidizing environment of the bacterial periplasm.

Protection from formation of four-coordinate adducts with additional ligands may also be important for proper CusF function. We have recently shown that CusF specifically interacts with and transfers metal to the periplasmic protein CusB, and therefore functions as a periplasmic chaperone [7]. In the Cus system as well as in other chaperone–target systems, such as the Atx1–Ccc2 complex [10], transfer of metal between proteins is thought to occur through intermediate higher-coordinate species with ligands contributed by both proteins. Therefore, for an individual protein, an initial low-coordination number is important to enable higher-coordination environments upon protein complex formation. Through W44, the coordination environment is limited for CusF alone. Though W44 may have to move to allow access to the metal in the CusF/CusB complex, there may be a lower energetic barrier to breaking the potentially weak Cu(I)– π interaction contributed by W44 than to breaking a formally bonded species.

The affinity of CusF for Cu(I) can be altered through mutation of W44. The introduction of a methionine, which is a good potential Cu(I) ligand, at position 44 results in significantly tighter Cu(I) binding. Our NMR results show that Cu(I) binding has sufficiently increased such that CusF–W44M is capable of binding Cu(I) from an aerobic solution where the predominant species is Cu(II). We rationalize this result by considering that even under aerobic conditions, there is a small amount of Cu(I) present. If this Cu(I) is removed from solution by binding to CusF–W44M, the equilibrium ratio of Cu(II) to Cu(I) will be reestablished through oxidation of some electron donor to give Cu(I). In an aqueous buffer solution many possible electron donors are present, including water itself. Though a protein residue could potentially be oxidized, it seems unlikely in this case. CusF contains no cysteines, which are the most likely residues to be oxidized, and the NMR spectra show no chemical shift differences between the samples where copper is added as Cu(I) or Cu(II).

In contrast, removal of the W44 side chain by mutation to an alanine results in a protein which has an affinity for Cu(I) similar to that of the wild-type protein. The Cu(I)- π interaction does not increase the affinity of the binding site for Cu(I), but may simply anchor the capping tryptophan at the binding site, thus preventing solvent accessibility and intrusion of adventitious ligands into the binding site. This in turn prevents a four-coordinate geometry, which could potentially result in the inability to transfer metal ions to CusB.

CopC is the only periplasmic protein for which the affinity for Cu(I) has been measured and is therefore the only protein for which a direct comparison of the affinity with the CusF Cu(I) affinity can be made. CopC has two distinct copper binding sites; one site binds Cu(I), the other Cu(II) [24]. Each of the sites only binds copper in the respective oxidation state. The dissociation constant for the Cu(I) site has been reported to lie in the range 10^{-7} – 10^{-13} M [25]. While the lower limit of the affinity of wild-type CusF_{1–88} for Cu(I) is 2 orders of magnitude tighter than that for CopC, the upper limit for the affinity is near the upper limit reported for CopC. The CusF W44M mutant further increases the affinity by another 1–3 orders of magnitude. The high affinity of CusF for Cu(I) suggests that the Cus system may be able to detoxify even very low levels of Cu(I) in the periplasm.

In conclusion, we have shown that W44 plays a very specific role in CusF. The Cu(I)- π interaction between W44 and the metal ion allows the protein to fine-tune its affinity for Cu (I). In addition, W44 acts as a cap to solvent-shield the bound Cu(I) located near the surface of the protein, therefore protecting Cu(I) from oxidation as well as from adventitious ligand exchange reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BCA	Bicinchoninic acid
EXAFS	Extended X-ray absorption fine structure
HEPES	<i>N</i> -(2-Hydroxyethyl)piperazine- <i>N'</i> -ethanesulfonic acid
HSQC	Heteronuclear single quantum coherence
NMR	Nuclear magnetic resonance
XAS	X-ray absorption spectroscopy

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Fig. 1. Copper binding to CusF₁₋₈₈W44M. ^1H - ^{15}N correlation spectra of **a** apo-CusF₁₋₈₈W44M (black) and CusF₁₋₈₈W44M with 1 molar equivalent of Cu(SO)₄ (red), **b** CusF₁₀₋₈₈W44M-Cu(I) (black) and CusF₁₀₋₈₈W44M after the addition of 2.5 molar equivalents of Cu(SO)₄ (blue). **c** Ribbon diagram of wild-type apo-CusF (Protein Data Bank code 1ZEQ [14]). Residues forming the metal site are shown in stick representation and W44 is labeled

Fig. 2. Extended X-ray absorption fine structure (EXAFS) data for CusF-W44M and W44A. Experimental (*black*) and simulated (*red*) Fourier transforms and EXAFS (*inset*) for **a** CusF₁₋₈₈-W44A-Cu(I) and **b** CusF₁₋₈₈-W44M-Cu(I). **c** X-ray absorption edge intensity for CusF₁₋₈₈-W44A-Cu(I) (*black*) and CusF₁₋₈₈-W44M-Cu(I) (*red*). The edge intensity of 8,983 eV is diagnostic of three-coordinate geometry

Fig. 3. Crystallographic model of Cu(II) coordination by CusF₁₀₋₈₈-W44A. Cu(II) is shown as an *orange sphere*. The residues shown in *green* are from one CusF molecule and the methionine residue shown in *cyan* (M9) is from the N-terminal residue of a neighboring molecule in the crystal

Fig. 4. Representative data set for CusF₁₋₈₈-W44A Cu(I)-binding competition assay. **a** Absorption decreases as the concentration of protein added increases. **b** Absorption change at 562 nm as a function of protein concentration

Table 1
 Fits obtained to the extended X-ray absorption fine structure of CusF mutants by curve-fitting using the program EXCURVE 9.2

	F^a	C-N(His) ^d			Cu-S			Cu-C/N/O			$-E_0$
		N ₀ , ^b	R (Å) ^c	DW (Å ²)	N ₀ , ^b	R (Å) ^c	DW (Å ²)	N ₀ , ^b	R (Å)	DW (Å ²)	
CusF ₁₋₈₈ -W44A-Cu(I)	0.635	1	2.02	0.008	2	2.32	0.009				4.47
CusF ₁₋₈₈ -W44M-Cu(I)	0.410	1	2.05	0.006	3	2.31	0.008				5.34
CusF ₁₋₈₈ ^e	0.341	1	1.954	0.004	2	2.261	0.012	1C	2.55	0.56	1.19

DW Debye-Waller factor

^a F is a least-squares fitting parameter defined as $F^2 = \frac{1}{N} \sum_{i=1}^N k_i^6 (\text{data} - \text{model})^2$

^b Coordination numbers are generally considered accurate to ±25%

^c In any one fit, the statistical error in bond lengths is ±0.005 Å; however, when errors due to imperfect background subtraction, phase-shift calculations, and noise in the data are compounded, the actual error is probably closer to ±0.02 Å

^d Fits modeled histidine coordination by an imidazole ring, which included single and multiple scattering contributions from the second-shell (C2/C5) and third-shell (C3/N4) atoms, respectively. The Cu-N-C_x angles were as follows: Cu-N-C2 126°, Cu-N-C3 -126°, Cu-N-N4 163°, Cu-N-C5 -163°

^e From [6]

Table 2

Affinity of CusF constructs for Cu(I)

Protein	K_D (M) (fitted with K_1 to provide a lower limit of affinity)	K_D (M) (fitted with β_2 to provide an upper limit of affinity)
Wild-type CusF ₁₋₈₈	$8.7 (\pm 0.8) \times 10^{-9}$	$4.6 (\pm 0.4) \times 10^{-12}$
CusF ₁₋₈₈ -W44A	$4.8 (\pm 0.9) \times 10^{-9}$	$2.7 (\pm 0.5) \times 10^{-12}$
CusF ₁₋₈₈ -W44M	$1.0 (\pm 0.0) \times 10^{-10}$	$4.8 (\pm 0.1) \times 10^{-15}$