Unusual Cu(I)/Ag(I) coordination of *Escherichia coli* CusF as revealed by atomic resolution crystallography and X-ray absorption spectroscopy

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(RECEIVED May 24, 2007; FINAL REVISION June 25, 2007; ACCEPTED June 26, 2007)

Abstract

Elevated levels of copper or silver ions in the environment are an immediate threat to many organisms. *Escherichia coli* is able to resist the toxic effects of these ions through strictly limiting intracellular levels of Cu(I) and Ag(I). The CusCFBA system is one system in *E. coli* responsible for copper/silver tolerance. A key component of this system is the periplasmic copper/silver-binding protein, CusF. Here the X-ray structure and XAS data on the CusF–Ag(I) and CusF–Cu(I) complexes, respectively, are reported. In the CusF–Ag(I) structure, Ag(I) is coordinated by two methionines and a histidine, with a nearby tryptophan capping the metal site. EXAFS measurements on the CusF–Cu(I) complex show a similar environment for Cu(I). The arrangement of ligands effectively sequesters the metal from its periplasmic environment and thus may play a role in protecting the cell from the toxic ion.

Keywords: metal tolerance; copper; silver; metal coordination; crystallography; X-ray absorption spectroscopy

Supplemental material: see www.proteinscience.org

The intracellular concentrations of metals must be carefully regulated to avoid toxic effects. One way in which *Escherichia coli* responds to elevated levels of copper or silver in its environment is through the up-regulation of the *cusCFBA* operon (Munson et al. 2000; Franke et al. 2001). Three of the proteins encoded by this operon,

CusCBA, are expected to form an efflux complex spanning the periplasm, similar to the well-characterized multidrug transporters (Franke et al. 2003). However, the fourth component of this system, CusF, is unique to copper/silver transport systems and is essential for the Cus system to achieve its maximal function (Franke et al. 2003). CusF shows high affinity for both Cu(I) and Ag(I), which have similar properties; however, it does not appreciably bind Cu(II) (Kittleson et al. 2006). Homologs of this small periplasmic metal-binding protein are present in all putative copper/silver tolerance systems, yet its function within these systems has not yet been described. CusF may act as a metallochaperone and be involved in metal tolerance through selection of metal substrates or it may regulate the efflux complex through protein–protein interactions. To further describe its role

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Abbreviations: rmsd, root mean square deviation; XAS, X-ray absorption spectroscopy DW: Debye-Waller.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.073021307>.

in metal tolerance, we sought fundamental chemical and structural details on the metal coordination geometry and overall structure of the metal-bound state of CusF.

Results and Discussion

The structure of CusF_{10–88}–Ag(I) was determined to 1.0 Å resolution using X-ray crystallography. This construct, which lacks the N-terminal nine residues but has the natural C terminus, was used to facilitate crystallization, as NMR studies have shown the N-terminal region is flexible in solution and has no involvement in metal binding (Loftin et al. 2005). The final crystallographic statistics are excellent (Supplemental Table 1). Overall, the structure of CusF in the Ag(I)-bound state is very similar to the previously reported structure of apo-CusF (pdb code 1ZEQ) (Loftin et al. 2005), with a global

backbone rmsd of 0.49 Å for the CusF_{10–88}–Ag(I) and apo-CusF_{6–88} structures (Fig. 1A). Thus, the β-barrel structure of CusF is retained and can accommodate the metal ion with only minimal local changes.

Strong electron density for a single silver ion was observed and is consistent with full occupancy of the Ag(I) site (Fig. 1B). Residues H36, M47, and M49 take part in the coordination of Ag(I), as previously proposed (Loftin et al. 2005). While only one conformation was found for the H36 side chain, the electron density shows two distinct side-chain conformations for M47 and M49. These conformers can be grouped into two sets of M47 and M49, belonging to major and minor conformations, populated to 70% and 30%, respectively (Fig. 1B). Neither of these pairs of conformers are similar to the single conformation of the methionines in the apo-CusF structure. Though this indicates there is some plasticity in

Figure 1. Crystal structures of Ag(I)-bound CusF. (A) Backbone overlay of apo-CusF (blue) and CusF-Ag(I) (green) structure showing the Ag(I)-coordinating residues. (B) Stereo image of the 1.0 Å $2F_o - F_c$ electron density map (contoured at 1.5σ) of the binding-site region. Phases were calculated using all atoms except those from H36, M47, and M49 and the Ag(I) ion. Residues H36, W44, and both side-chain conformations of M47 and M49 (cyan-minor conformation, green-major conformation) are shown. The figure was generated using PyMOL (DeLano 2002) and Adobe Photoshop.

the geometry of Ag(I) binding, overall, the binding-site region is well-ordered and has similar temperature factors as the rest of the structure. The greater flexibility of the methionine residues may be attributed in part to the fact that M47 and M49 are the two most solvent-exposed residues of the binding site.

The distances from the silver ion to the coordinating side-chain atoms of H36, M47, and M49 (Table 1) are similar to bond lengths reported for silver bound to small molecule complexes (Davies et al. 1999; Chen et al. 2003) and protein ligands (Gitschier et al. 1998; Changela et al. 2003). However, CusF shows some differences in Ag(I) coordination compared with the few other biologically relevant protein–Ag(I) structures deposited in the Protein Data Bank. For example, CueR, a copper-induced transcriptional regulator, binds Ag(I) as well as Cu(I) in a linear coordination via two cysteine residues (Changela et al. 2003). Cysteines are also used by many other metalloproteins to coordinate Cu(I). However, in the oxidizing environment of the periplasm, where CusF is located, methionines are much more commonly used as Cu(I) ligands than cysteines (Jiang et al. 2005; Zhang et al. 2006). His(Met)_x coordination is appearing as a common feature of Cu(I) and Ag(I)-binding proteins in oxidizing environments (Arnesano et al. 2002, 2003; Peariso et al. 2003; Banci et al. 2005; Zhang et al. 2006).

An unusual feature of the metal site of CusF is the close proximity of W44 to the Ag(I) ion. The indole ring of the W44 side chain is positioned above the metal ion and is slightly tilted. The CE3 and CZ3 atoms of the tryptophan are at distances of 2.82 Å and 2.96 Å from the Ag(I) ion. While the tilted tryptophan ring could potentially form an η^2 – π interaction with the Ag(I) ion, this type of interaction has not been reported to date for a protein–

Ag(I) interaction. A search of the Cambridge Structural Database (Allen 2002) for small molecule–Ag(I) complexes shows that the distances from the CusF tryptophan atoms to Ag(I) are longer than the 2.4–2.6 Å distances commonly observed for Ag(I)–aromatic interactions. However, for two small molecule–Ag(I) complexes, longer bonds lengths of 2.716 ± 0.005 Å and 2.806 ± 0.005 Å (Wang et al. 2005) and 2.47 ± 0.06 Å and 2.92 ± 0.07 Å (Turner and Amma 1966) have been reported. Though the Ag(I)–W44 distances in the CusF–Ag(I) structure are too long to make a compelling case for W44 serving as a fourth ligand, it is notable that among CusF homologs the residue at this position is conserved as a potential metal ligand, either a tryptophan or methionine. It is difficult to evaluate whether crystal packing could play a role in the close position of W44; however, there is no apparent residue from a symmetry-related molecule that can be pinpointed as having an effect. In addition, the absorption spectrum of CusF–Ag(I) is redshifted compared with apo–CusF, indicating that in solution the tryptophan is close enough to perturb the absorption of the W residue, perhaps via metal-to-ligand charge transfer from the Ag(I) to the indole group (Shimazaki et al. 1999; Osako et al. 2004) (see Supplemental Fig. 1).

The overall arrangement at the metal site of the CusF–Ag(I) structure shows a distorted trigonal planar geometry. The Ag(I) protrudes slightly out of the plane of the H36, M47, and M49 ligands toward the W44 side chain by 0.45 Å and 0.72 Å for the major and minor conformations, respectively (Table 1). This deviation from coplanarity could be due to interaction with the tryptophan; however, it is also possible that geometrical restrictions of the ligands result in a deviation from the ideal geometry. The distortion from ideal geometry is reflected additionally in the bond angles for N–Ag–S and

Table 1. Bond lengths and angles in the silver site of CusF

	Major Conformation	Minor Conformation
Bond lengths (Å)		
Ag–N(H36)	2.23(±0.01)	2.23(±0.01)
Ag–S(M47)	2.45(±0.01)	2.68(±0.03)
Ag–S(M49)	2.57(±0.01)	2.44(±0.02)
Ag–CE3/CZ3(W44)	2.82(±0.01)/2.96(±0.02)	2.82(±0.01)/2.96(±0.02)
Bond angles (deg)		
N(H36)–Ag–S(M47)	115.6(±0.05)	98.7(±0.06)
N(H36)–Ag–S(M49)	128.6(±0.04)	140.1(±0.07)
S(M47)–Ag–S(M49)	105.3(±0.03)	88.4(±0.07)
N(H36)–Ag–CE3/CZ3(W44)	91.2(±0.05)/101.8(±0.06)	91.2(±0.05)/101.8(±0.06)
S(M47)–Ag–CE3/CZ3(W44)	116.6(±0.04)/129.6(±0.05)	95.3(±0.05)/117.4(±0.06)
S(M49)–Ag–CE3/CZ3(W44)	97.2(±0.04)/71.2(±0.04)	127.0(±0.07)/108.8(±0.08)
Geometric distances, (Å)		
Ag–NSS ^a	0.45	0.72

^aThe distance from the silver ion to the plane formed by N(H36), S(M47), and S(M49). The positive value indicates that the silver ion is displaced from the NSS plane toward W44.

S-Ag-S, which differ from the 120° expected for trigonal geometry (Table 1).

CusF is also able to bind Cu(I), yet so far, crystals of Cu(I)-loaded CusF have not been obtained. Although the properties of Cu(I) and Ag(I) are similar, their coordination may not be identical. X-ray absorption provides an alternative strategy for determining the local environment of metal centers in proteins and does not require diffractable crystals. Therefore, to investigate the differences and similarities of Ag(I) and Cu(I) binding in CusF, we probed the CusF–Cu(I) site with XAS.

XAS data were collected on two independent samples of Cu(I)-loaded CusF and gave identical results within experimental error (Table 2, samples 1 and 2). The absorption edge region of the spectrum shows a weak feature at 8983.0 eV with intensity equal to 0.58 of the normalized edge height (Fig. 2). The position and intensity of this peak is characteristic of Cu(I) bound to the protein in a three-coordinate environment (Pickering et al. 1993; Ralle et al. 2003). Figure 2 shows the Fourier transform and EXAFS for CusF–Cu(I). The spectrum consists of intense oscillations extending beyond $k = 12.8 \text{ \AA}^{-1}$, the energy cutoff used to avoid background errors due to small amounts of contaminating Zn(II). The first shell of the phase-corrected FT maximizes at $\sim 2.3 \text{ \AA}$ (characteristic of Cu[I]-thioether or thiolate coordination), but also exhibits a broadening with a slight shoulder on the low-R side of the peak, suggestive of a low Z (O/N) scatterer coordinated to Cu(I).

Since the Ag(I) structure identified H36, M47, and M49 as coordinating ligands, a model structure derived from one His and two Met residues was used as a starting point for spectral simulations of the EXAFS data. Multiple scattering from the outer shells of C and

N atoms of the imidazole ring was incorporated into the fitting routine. However, this initial fit (Table 2, Fits 1/A and 2/C) did not fully reproduce the width of the FT on the high-R side of the main peak, which (given the excellent signal to noise ratio of the data) suggested the possible presence of a fourth scatterer. The best fit was obtained with the inclusion of a nonhistidine C/O/N scatterer (Table 2, Fits 1/B and 2/D). The absorption edge profile implies that the interaction of this ligand cannot be strong enough to perturb the structure significantly toward 4 coordinate, and the long distance and high DW factors also indicate a weak interaction. It is possible that this contribution is from the W44 indole ring.

Other fits to the data were attempted using different combinations of three first shell scatterers (Table 2, Fits 2/E–G); however, the data are best fit by coordination of two S, one N(histidine), and one C/N/O(nonhistidine) scatterer (Table 2, Fits 1/B and 2/D). For all of the Cu(I) ligands, the distances are $\sim 0.3 \text{ \AA}$ shorter than the corresponding Ag(I) distances determined from crystallography, as expected from the larger size of the Ag(I) ion. The EXAFS results thus fully compliment the crystallographic description of the CusF–Ag(I) structure.

The role of CusF in metal tolerance is not clear. One postulated role of CusF is as a metallochaperone; however, there are several features of metal-bound CusF that differ from those of characterized metallochaperones. In the CusF–Ag(I) crystal, Ag(I) is fully coordinated by ligands from a single CusF monomer. In the crystals of some metal trafficking proteins, such as CopC (Zhang et al. 2006), Hah1 (Wernimont et al. 2000), and ScAtx1 (Banci et al. 2004), dimerization and metal sharing between proteins has been observed, even though the

Table 2. Fits obtained to the EXAFS of CusF by curve-fitting using the program EXCURVE 9.2

Sample/Fit	F^b	Cu-S			Cu-N(His) ^a			Cu-C/N/O			$-E_0$
		No ^c	R ($\text{\AA}$) ^d	DW ($\text{\AA}^2$)	No ^c	R ($\text{\AA}$) ^d	DW ($\text{\AA}^2$)	No ^c	R ($\text{\AA}$) ^d	DW ($\text{\AA}^2$)	
1/A	0.406	2	2.253	0.013	1	1.945	0.005				0.42
1/B	0.341	2	2.261	0.012	1	1.954	0.004	1 C	2.55	0.056	1.19
2/C	0.529	2	2.259	0.010	1	1.968	0.006				1.52
2/D	0.432	2	2.262	0.011	1	1.970	0.006	1 C	2.54	0.015	1.24
2/E	0.439	2	2.262	0.011	1	1.970	0.006	1 O	2.47	0.031	1.24
2/F	0.484	2	2.259	0.012	2	1.984	0.021	1 O	2.47	0.031	0.74
2/G	0.662	1	2.259	0.005	2	1.980	0.014	1 O	2.47	0.031	0.60
2/H	0.682	1	2.256	0.005	3	1.980	0.022	1 O	2.47	0.031	0.02

^aFits 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°.

^b F is a least-squares fitting parameter defined as $F^2 = \frac{1}{N} \sum_{i=1}^N k^6 (Data - Model)^2$.

^cCoordination numbers are generally considered accurate to $\pm 25\%$.

^dIn any one fit, the statistical error in bond lengths is $\pm 0.005 \text{ \AA}$. 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 $\pm 0.02 \text{ \AA}$.

Figure 2. X-ray absorption spectra of Cu(I)-loaded CusF. (A) Absorption edge with 8983 eV edge feature typical of 3-coordination with only minor perturbation from a fourth ligand; (B) experimental (black) vs. simulated (red) Fourier transform and EXAFS (inset) using parameters listed in Table 2, Fit 1/B.

proteins in solution are monomers. Furthermore, these metallochaperones seem to use the same homodimer interface observed in the crystal for heterodimer formation with the target molecule as seen in Cu(I)HAH1-WLN2/WLN4 (Achila et al. 2006) and Atx1-Ccc2 (Banci et al. 2006). CusF shows no evidence of dimerization in either the apo- or metal-bound state in solution (Loftin et al. 2005) or in the crystal. The metal-binding site of each molecule in the crystal lattice is distant from any other metal site, and no nearby ligands from other molecules approach the metal site. In CusF, W44 may act as a capping residue that blocks the close approach of a ligand from another molecule.

Numerous metallochaperones have two- or three-coordinate metal sites or are four-coordinate as a result of protein-protein interactions and metal sharing between two molecules (Wernimont et al. 2000; Ralle et al. 2003; Banci et al. 2006; Zhang et al. 2006). For these metallochaperones, the metals are poised for transfer from one species to another in a process in which ligands would be provided by both molecules resulting in an intermediate three- or four-coordinate complex (Arnesano et al. 2001).

In CusF, three ligands from one molecule coordinate the metal, and access for a fourth ligand is blocked by the tryptophan residue that caps the metal site. A structural rearrangement would be necessary to allow a ligand from an acceptor protein to interact. Therefore, if CusF indeed acts as a chaperone, a gating mechanism for metal transfer would seem to be necessary, induced by the protein-protein-donor-acceptor interaction.

Materials and Methods

Preparation of CusF_{10–88} for crystallization

The gene encoding CusF_{10–88} with an initiating N-terminal methionine was cloned into pPR-IBA1 (IBA) by standard molecular genetics techniques using PCR for gene amplification. This construct was designed to facilitate crystallization by eliminating the nine N-terminal residues that are known to be unstructured in solution and to play no role in metal binding (Loftin et al. 2005). *E. coli* BL21-DE3 cells containing the plasmid encoding CusF_{10–88} were grown in LB medium. Overexpression and purification was achieved as described previously (Kittleson et al. 2006). The pure protein was treated with 10 mM EDTA, dialyzed against 20 mM HEPES (pH 7.5), and concentrated to 20 mg/mL.

Crystallization of CusF_{10–88}-Ag(I)

AgNO₃ was added to the apo-CusF to yield a twofold molar excess of silver to CusF_{10–88}.

Crystals were obtained by the hanging-drop vapor diffusion method. Drops were setup by mixing 10 μL of protein solution with 10 μL of reservoir solution (100 mM sodium acetate trihydrate [pH 4.6], 2.3 M ammonium sulfate, and 2 mM silver nitrate) and equilibrated against 1-mL reservoir solution at room temperature. Crystals grew as clusters of long rods, with dimensions up to 0.4 mm × 0.4 mm × 0.8 mm. The crystals are orthorhombic, the space group is P2₁2₁2₁ with one CusF_{10–88}-Ag(I) molecule per asymmetric unit.

Crystal structure determination of CusF_{10–88}-Ag(I)

Crystals were flash-frozen in liquid nitrogen after successive transfer to crystallization solutions enriched to 2.6 M, 3.1 M, and 3.3 M ammonium sulfate. Data were measured at 100 K at Stanford Synchrotron Radiation laboratory beamline 9–2 with a wavelength of 0.97946 Å, processed with CrystalClear (D*TREK) (Leslie 1992; Pflugrath 1999) and scaled with SCALA in CCP4 (Bailey 1994). MOLREP (Vagin and Teplyakov 1997) in CCP4 (Bailey 1994) was run to perform molecular replacement using the apo-CusF_{6–88} coordinates, pdb code 1ZEQ (Loftin et al. 2005). The structure was refined using REFMAC5 (Bailey 1994) with interspersed manual rebuilding using COOT (Emsley and Cowtan 2004). A Ramachandran plot generated with PROCHECK (Laskowski et al. 1993) shows that the final model exhibits good geometry with 97.1% of the residues in the most favored regions and 2.9% in additional allowed regions. Block-diagonal least squares refinement was applied to the final models using SHELX-97 (Sheldrick and Schneider 1997) with Ag(I) and residues 36–49 refined without restraint, but all other atoms fixed

to generate standard errors for distances and angles in the groups attached to the silver ion. Data measurement and refinement statistics are given in Supplemental Table 1. The coordinates and structure factors have been deposited in the PDB with accession code 2QCP.

Preparation of CusF₁₋₈₈-Cu(I) for XAS

E. coli BL21-DE3 cells containing the pASK-IBA3 (IBA) plasmid with the gene encoding CusF residues 1–88 were grown in LB medium. Overexpression and purification was achieved as described previously (Kittleson et al. 2006). The pure protein was treated with 10 mM EDTA and dialyzed against 20 mM MOPS (pH 7.0). The protein sample was degassed and placed into a Coy anaerobic chamber. All solutions were prepared in the anaerobic chamber to ensure that they are oxygen free. A stock solution of 0.5 M ascorbate buffered with 20 mM MOPS (pH 7.0) was added to the protein sample to a final concentration of 50 mM. CuCl₂ was added to the protein solution to 80% of the determined CusF₁₀₋₈₈ concentration to avoid the disproportionation of excess Cu(I) into Cu(0) and Cu(II). The sample was dialyzed against 20 mM MOPS (pH 7.0) and 10 mM ascorbate. A total of 25% (v/v) of ethylene glycol was added to the sample, which was then transferred into EXAFS cells and flash frozen in liquid nitrogen before removal from the anaerobic chamber. Samples were stored in liquid nitrogen until data collection. The final concentration of CusF₁₋₈₈-Cu(I) was 0.5 mM.

Collection and analysis of XAS data

Cu K-edge (8.9 keV) extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES) data were collected at the Stanford Synchrotron Radiation Laboratory operating at 3 GeV with currents between 100 and 50 mA. All samples were measured on beamline 9–3 using a Si[220] monochromator and a Rh-coated mirror upstream of the monochromator with a 13 KeV energy cutoff to reject harmonics. A second Rh mirror downstream of the monochromator was used to focus the beam. Data were collected in fluorescence mode using a high-count-rate Canberra 30-element Ge array detector with maximum count rates below 120 kHz. A 6- μ Z-1 Ni oxide filter and Soller slit assembly were placed in front of the detector to reduce the elastic scatter peak. Six scans of a sample containing only sample buffer were collected, averaged, and subtracted from the averaged data for the protein samples to remove Z-1 K β fluorescence and produce a flat pre-edge baseline. The samples (80 μ L) were measured as aqueous glasses (>20% ethylene glycol) at 10 K. Energy calibration was achieved by reference to the first inflection point of a copper foil (8980.3 eV) placed between the second and third ionization chamber. Data reduction and background subtraction were performed using the program modules of EXAFSPAK (George 1990). Data from each detector channel were inspected for glitches or dropouts before inclusion in the final average. Spectral simulation was carried out using the program EXCURVE 9.2 (Gurman et al. 1984, 1986; Binsted and Hasnain 1996; Binsted et al. 1998) as previously described (Blackburn et al. 2000).

EXAFS data were simulated using a mixed-shell model consisting of imidazole and methionine coordination. The imidazole ring geometry was constrained to ideal values of the internal bond lengths and angles, while the first-shell distance (R) and Debye-Waller factor for the Cu-N(imid) shell

(including the single and multiple scattering contributions for the imidazole rings), the Cu-S(met) shell, and E₀ were refined. In these preliminary refinements, the imidazole ring outer shell C and N atoms were constrained to move relative to the first shell Cu-N distance so as to maintain the idealized ring geometry. Later in the refinement, this constraint was lifted, and the outer shells of the imidazole rings were allowed to float within 10% of their original idealized positions. In practice, final outer shell coordinates for acceptable fits deviated by less than the permitted amount from the idealized position. Parameters refined in the fit included shell occupancy N, Cu-scatterer distance R, and Debye-Waller factor ($2\sigma^2$) for each shell, and E₀ the threshold energy for photoelectron ionization that was constrained to be the same for all shells of scatterers.

Electronic supplemental material

Supplemental Table 1: Crystallographic data collection and refinement statistics. Supplemental Figure 1: Absorbance spectra of apo-CusF₁₀₋₈₈ and CusF₁₀₋₈₈-Ag(I).

Acknowledgments

We thank Andrew Bauman and Amanda Barry for assistance with collection of the XAS data, Sue Roberts for assistance with the collection and processing of the crystallographic data, and Ken Karlin and F. Ann Walker for helpful discussions. We gratefully acknowledge the use of facilities at the Stanford Synchrotron Radiation Laboratory, which is supported by the National Institutes of Health Biomedical Research Technology Program, Division of Research Resources, and by the U.S. Department of Energy, Basic Energy Sciences (BES), and Office of Biological and Environmental Research (OBER). This work was supported by grants from the National Institutes of Health GM54803 to N.J.B. and GM079192 to M.M.M.

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