The PcoC Copper Resistance Protein Coordinates Cu(I) via Novel S-Methionine Interactions

Katrina Peariso,[‡] David L. Huffman,[†] James E. Penner-Hahn,*,[‡] and Thomas V. O'Halloran*,[†,§]

Department of Chemistry, and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208, and Department of Chemistry, The University of Michigan, Ann Arbor, Michigan 48109
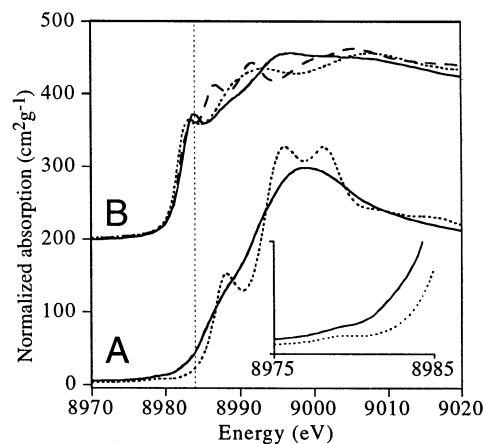
Received October 14, 2002 ; E-mail: jeph@umich.edu; t-ohalloran@northwestern.edu

Copper is an essential element, and several pathways have recently been shown to allocate this ion to key enzymatic sites inside the cell. These include the copper trafficking metallochaperone proteins, whose chemistry is dominated by low coordination number Cu(I) dithiolate ligation.[1] When excessive copper is encountered, bacteria produce a variety of proteins that limit copper toxicity. Here we show that a copper resistance protein from *E. coli* utilizes a biologically unprecedented Cu(I) thioether ligation that is distinct from that seen in well-understood copper proteins, but that is well suited to the metal-transfer chemistry of copper resistance and trafficking.

The *pco* proteins encoded by an *E. coli* resistance plasmid enhance survival under conditions of extreme copper stress.[2−4] Two of these are known to bind copper: a multicopper oxidase, PcoA, and a protein of unknown function, PcoC.[5] In one proposal, PcoC functions as a periplasmic Cu(I) scavenging protein that docks with PcoA to facilitate oxidation of copper to the less toxic Cu(II) form.[5] Alternatively, Cu(I) binding to PcoC may prevent either its disproportionation or its reaction with molecular oxygen to give superoxide. While neither the chemistry nor the mechanism of PcoC is well understood, genetic data reveal that it plays an essential role in a tightly coupled copper detoxification pathway.[6−11] PcoC has no cysteines, but does have an unusual methionine-rich sequence motif which is also found in PcoA[5] and the CTR family of copper transport proteins.[12] PcoC has three histidines at positions 1, 49, and 92 in the 103aa primary sequence of the mature protein. His49 is located within the Met-rich domain, MTGMKGMSSHSPM.

PcoC readily binds 1 equiv of Cu(II) per protein monomer at pH 7.5, but does not bind Cd(II) and binds Hg(II) only at high pH.[13] PcoC also binds 1 equiv of Ag(I),[14] a mimic of Cu(I), suggesting that it can function as a Cu(I) binding protein. Cu(I)-PcoC is stable with respect to disproportionation at millimolar concentrations for long periods at 4 C. To establish how PcoC stabilizes Cu(I), the reduced protein was prepared under anaerobic conditions,[15] and its coordination environment was assessed by X-ray absorption spectroscopy.[16,17]

Figure 1 shows the XANES spectra of both Cu(II) (Figure 1A) and Cu(I) (Figure 1B) forms of PcoC. There is a large shift to higher energy on going from Cu(I) to Cu(II), consistent with that seen in model complexes. The extremely weak 1s → 3d transition demonstrates that the Cu(II) is very nearly centrosymmetric. The intensity of the 1s → 4p transition in Cu(I)-PcoC is similar to that for three-coordinate Cu(I) model complexes.[18] However, the higher energy resonances observed in the XANES region are shifted relative to those in the trigonal Cu-S models, suggesting that the

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[†] Department of Chemistry, Northwestern University.
[‡] Department of Chemistry, University of Michigan.
[§] Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University.

Figure 1. (A) XANES spectra for Cu(II)-PcoC (solid line) and Cu(II)-tetraimidazole (dotted line). Inset shows the 1s → 3d region. (B) Cu(I)-PcoC (solid line) with $[(C_6H_5)_4P]Cu(SPh)_3$ (dotted line) and $[Cu(ethylthiourea)_3]_2SO_4$ (dashed line). Vertical dashed line at 8984 eV.
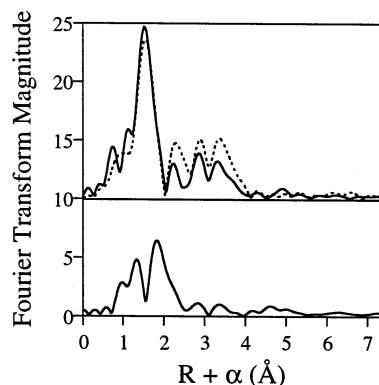
Figure 2. Fourier transform of k^3-weighted EXAFS ($k = 2−13$ Å^{-1}) data for Cu(II)-PcoC (top, solid line) and Cu(I)-PcoC (bottom). Data for Cu(II)tetraimidazole (dotted line, top) are shown for comparison.

Cu environment of Cu(I)-PcoC is distinct from that of either model complex. This is further corroborated by the EXAFS data.

The EXAFS data for Cu(II)-PcoC give rise to a single peak at $R + \alpha = 1.5$ Å, suggesting that in the oxidized state Cu is coordinated only to low-Z ligands (Figure 2). The data are best fit as a single shell of N/O ligands at 1.96 Å (Table 1, Figure S1). The outer-shell scattering ($R + \alpha = 2−4$ Å) agrees well with that seen for Cu(II)tetraimidazole, suggesting that the Cu is ligated to histidine imidazole ligands. The outer-shell scattering in Cu(II)-PcoC is less intense than that in Cu(II)tetraimidazole, suggesting that there are fewer than four histidine ligands in PcoC. This is confirmed by quantitative fitting of the outer-shell scattering. The best fit (Table S1, Figure S2) is with two histidines and two N/O

Table 1. Cu-PcoC EXAFS Fit Results

	CN[a]	R[b]	σ²[c]	CN[a]	R[b]	σ²[c]	F[d]
Cu(I)	2S	2.30	8.8	1N	1.91	3.0	0.042
	1S	2.31	3.1	2N	1.94	10.7	0.041
Cu(II)	4N/O	1.96	3.3				0.21

[a] Integer coordination number. [b] Bond length in Å. [c] Debye−Waller factor × 10³ in Å². [d] Mean-square-deviation between data and fit.

ligands, although ligation by a third histidine is difficult to exclude on the basis of EXAFS amplitudes alone. A $Cu(His)_2(N/O)_2$ site is consistent with recent EPR and ENDOR data.[5]

The EXAFS data for Cu(I)-PcoC are dramatically different from those for the Cu(II) protein. The first shell peak is split into two peaks at $R + \alpha = 1.4$ and 1.9 Å, and the outer-shell scattering is significantly damped. The Cu(I)-PcoC data can only be fit by including both Cu−(N/O) and Cu−(S/Cl) scatterers. Because chloride was rigorously excluded from the EXAFS samples, the heavier atom is assigned as S. As expected from the XANES, fits assuming a total coordination number of four are significantly worse than those with a coordination number of three. However, the 2S + 1N fits and 1S + 2N fits are nearly indistinguishable. We favor the 2S + 1N fit on the basis of the fact that it gives more reasonable Debye−Waller factors (Table 1). This would be consistent with the large decrease in outer-shell imidazole scattering. The distance and Debye−Waller factor for the sulfur shell are both somewhat larger than those typical for Cu(I)-thiolates. However, these sulfurs presumably come from methionine ligands, because there are no cysteines in PcoC, and no exogenous thiols were added. The longer bond length and higher Debye−Waller factor for the S shell are consistent with thioether ligation, and the calculated bond valence sum[19] is ~1.1. The weak outer-shell scattering in the Cu-(I)-PcoC Fourier transform is consistent with retention of only one of the His ligands that binds Cu(II).

While the structures of Cu(I)- and Cu(II)-PcoC are not known, recent structural studies reveal that apo-PcoC crystallizes as a weakly associating dimer, with a well-defined β-barrel fold.[20] This is a common fold and is typical of blue-copper proteins which bind both Cu(I) and Cu(II). In contrast to the electron-transfer proteins, which bind Cu(I) and Cu(II) in nearly identical geometries, PcoC accommodates Cu(I) and Cu(II) in very different sites. Cu(II) is bound by at least two histidines and two other N- or O-ligand donors, while Cu(I) is bound in a three-coordinate site, most likely with two thioethers (i.e., S-Met) and one N/O donor. This ligation is unprecedented among structurally characterized proteins and may account for the unusually low affinity of Hg(II) and Cd(II) for the Cu(I) site. The coordination chemistry of PcoC is distinct from that seen in known copper enzymes. Although methionine displaces a water when Cu is reduced in peptidylglycine monooxygenase,[21] this occurs without loss of histidine ligands. The low coordination number of the Cu(I) site in PcoC is well suited for metal-transfer reactions between proteins, such as those mediated by the prototypical metallochaperone Atx1, because the PcoC-Cu(I) site has a low coordination number and is expected to be kinetically labile.[1] This new copper-thioether chemistry provides a basis for understanding the chemistry of the methionine-rich domains in high-affinity copper uptake transporters in humans and the metal detoxifying multicopper oxidases in bacteria.

Note Added in Proof. Recent reports describe preparation of Cu-PcoC with metal:protein = 0.9 (Lee, S. M.; Grass, G.; Rensing, C.; Barrett, S. R.; Yates, C. J. D.; Stoyanov, J. V.; Brown, N. L. *Biochem. Biophys. Res. Comm.* **2002**, *295*, 616−620) and of Cu(II)-CopC, a *P. syringae* homologue of PcoC, with metal:protein = 1, (Arnesano, F.; Banci, L.; Bertini, I.; Thompsett, A. R. *Structure* **2002**, *10*, 1337−1347). The latter includes an NMR structure of apo-CopC.

Supporting Information Available: EXAFS data, first shell fits, and multiple scattering fits (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) PcoC was purified as previously described,[5] and then exchanged repeatedly into 50 mM HEPES/Na, pH 7.5 (to remove chloride), followed by addition of 2 equiv of Cu(II)SO₄. Repeated dilution/concentration (Amicon Centricon-3) in this buffer gave final protein concentration = 4.0 mM; metal:protein = 0.9. Sample was combined with glycerol to 30%, loaded in a Lucite cuvette, and frozen in liquid N₂. Hg(II) could not be incorporated using this protocol, but was incorporated using 50 mM sodium borate, 50 mM NaCl, pH 9, followed by exchange into 50 mM Tris/Na, 50 mM NaCl, pH 7.5, metal:protein = 0.8. Cd(II) was not incorporated under either condition.
(14) One equivalent of AgNO₃ was added to PcoC in 10 mM HEPES/Na, pH 7.5, 250 mM NaCl, followed by repeated dilution/concentration into 100 mM sodium phosphate, pH 7.2. Final metal:protein = 0.8.
(15) Cu(II)PcoC in 50 mM HEPES/Na, pH 7.5, was reduced with excess sodium ascorbate in a N₂ filled glovebox. The purple color[5] ($\epsilon_{576} = 100$ M⁻¹ cm⁻¹) immediately bleached. The sample was extensively dialyzed to remove the reducing agent, combined with glycerol to 30%, loaded into a Lucite cuvette, and frozen in liquid N₂. The protein concentration 1.8 mM; metal:protein = 0.9.
(16) X-ray absorption data were measured at SSRL beamline 7-3 (details are given in Supporting Information). X-ray absorption near edge structure (XANES) data were normalized by fitting the data below and above the edge to the McMaster X-ray absorption cross-sections. EXAFS data reduction used a first-order polynomial in the preedge and a two-region cubic spline through the EXAFS. Data were converted to k-space using $E_0 = 9000$ eV. Fits to both Fourier filtered ($R = 0.9$−3.0 Å) and unfiltered data gave equivalent structural parameters. EXAFS data were fit using amplitude and phase functions calculated with FEFF v8.10[17] for Cu−O at 2.00 Å, and Cu−S at 2.25 and 2.40 Å. A scale factor of 0.89 and ΔE_0 of 13.0 were calibrated by fitting EXAFS data for crystallographically characterized complexes. Coordination numbers were fixed at integer values, and R and σ^2 were freely variable parameters.
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