Conserved Mechanism of Copper Binding and Transfer. A Comparison of the Copper Resistance Proteins PcoC from *Escherichia coli* and CopC from *Pseudomonas syringae*

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Supporting Information

Table S1 **PCR Primer Sequence with mutated bases in bold**

No.	Name	Oligomer Sequence
1.	PcoC-R-CopC	5'-GCTTTTTAATTCCGG**gtg**GGCTTGGGCCAATGC-3'
2.	PcoC-F-PcoC	5'-GCATTGGCCCAAGCC**cac**CCGGAATTAAAAAGC-3'
3.	PcoC-H1F-F	5'-GCATTGGCCCAAGCC**ttt**CCGGAATTAAAAAGC-3'
4.	PcoC-H1F-R	5'-GCTTTTTAATTCCGG**aaa**GGCTTGGGCCAATGC-3'
5.	PcoC-M40L-F	5'-GCAAAATTAACG**ctg**ACGGGTATGAAAG-3'
6.	PcoC-M40L-R	5'-CTTTCATACCCGT**cag**CGTTAATTTTGC-3'
7.	PcoC-H49F-F	5'-GGCATGTCATCA**ttt**TCTCCGATGCCG-3'
8.	PcoC-H49F-R	5'-CGGCATCGGAGA**aaa**TGATGACATGCC-3'
9.	PcoC-H92F-R	5'-GTGGTGGGATCC**tca**CTTCACTGTAAAGGTGTAA TTACCGGTAATAGGGAACGTATCTGAAGAAAC-3'

Monomeric and dimeric forms of *apo*-PcoC and –nA-PcoC.

nA-PcoC, as isolated after CM-52 cation-exchange chromatography, was confirmed to be a mixture of monomer (~ 90%) and dimer (~ 10%), as observed previously (Huffman, D. L.; Huyett, J.; Outten, F. W.; Doan, P. E.; Finney, L. A.; Hoffman, B. M.; O'Halloran, T. V. *Biochemistry* **2002** *41*, 10046-10055.). The two forms were indistinguishable by SDS-PAGE and ESI-MS but could be separated by gel filtration (Figure S1) or by Mono-S cation exchange chromatographies (Figure S2). While remaining unchanged upon incubation at 4 ^{o}C for one week, incubation at 20 and 37 ^{o}C overnight converted the dimeric protein to its monomeric form in 50 and 100 % yields, respectively (Figure S1). The nature of the interaction leading to dimer formation is not known. Addition of EDTA had no detectable effect on dimer conversion, excluding the possibility of a metal-bridged form.

As observed for nA-PcoC, a low percentage of dimeric species was detected. This component was separated and excluded from further investigation.

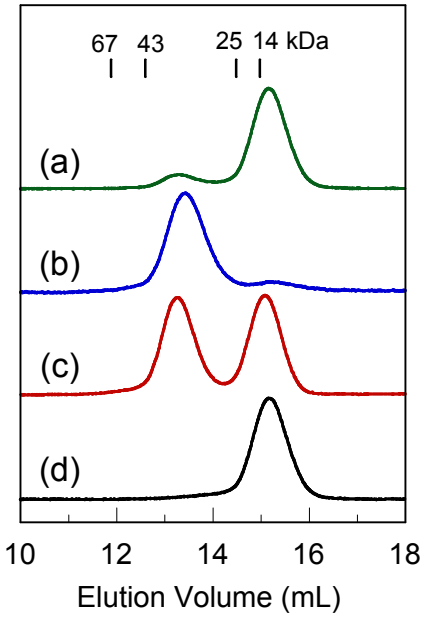


Figure S1. Elution profiles on a Superdex 75 FPLC gel filtration column (HR 10/30; Pharmacia): (a) nA-PcoC as isolated from the cation-exchange column CM-52; (b) dimeric fractions from sample (a). The elution profile remained unchanged after incubation at 4 ^{o}C for one week in the presence or absence of EDTA; (c) sample (b) incubated at 20 ^{o}C for 20 h; (d) sample (b) incubated at 37 ^{o}C for 20 h. The elution profiles of all metalated forms were indistinguishable from the elution profile (d).

Conditions: flow rate, 0.7 mL/min; buffer, KPi (25 mM; pH 7), NaCl (100 mM). The indicated molar masses are the elution positions under the same conditions of the standards albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) from Amersham Pharmacia.

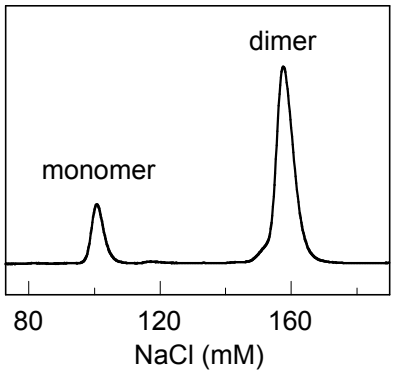


Figure S2 Elution profiles of a mixture of monomeric and dimeric forms of nA-PcoC on the Mono-S HR5/5 cation exchange column under the conditions of Figure 6.

Table S2 **ESI-MS Data of the Isolated PcoC Proteins**

Protein	Molar Mass (Da)	
	Calculated	**Found**
PcoC	10978.6	10979
nA-PcoC	11049.7	11049
PcoC H1F	10988.6	10988
PcoC M40L	10960.6	10960
PcoC H49F	10988.6	10988
PcoC H92F	10988.6	10988
PcoC H49F-M40L	10970.6	10970

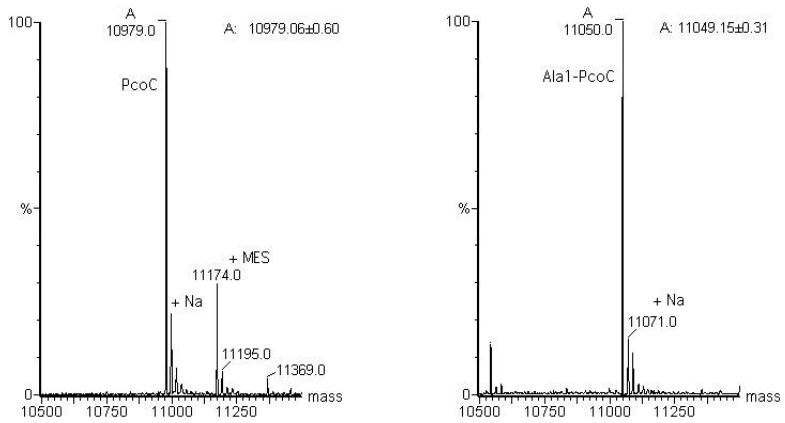
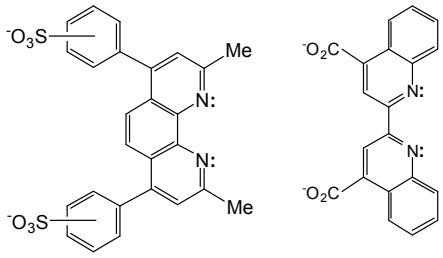


Figure S3 ESI-MS spectra of native PcoC (left) and nA-PcoC (right).



bcs bca

Figure S4 Structures of reagents bcs and bca.

Table S3 Estimation of Dissociation Constant K_D(□Cu^{II}) and K_D(↓Cu^{II}) by Competition with Ligand EGTA in 20 mM KPi (pH 7.0), 0.1 M NaCl.[a]

Protein	$[Cu^{II}]_{tot}$ (μM)	$\Delta F_X/\Delta F_1$[c]	$[Cu^{II}P]$ (μM)	$[Cu^{II}L]$ (μM)	$([P]_{tot}/[CuP]) - 1$	$([L]_{tot}/[CuL]) - 1$	K_D[d] (x10^{-14} M)
□□ [b]	2.31	0.20	1.01	1.30	3.96	2.83	5.6
	3.24	0.28	1.39	1.85	2.60	1.70	6.1
	4.16	0.36	1.81	2.36	1.77	1.12	6.3
	5.55	0.52	2.61	2.94	0.91	0.70	5.2
	6.94	0.68	3.40	3.53	0.47	0.41	4.5
↓□ [b]	2.60	0.17	0.84	1.76	4.94	1.84	6.7
	4.68	0.36	1.81	2.87	1.76	0.74	5.9
	6.76	0.58	2.90	3.86	0.72	0.29	6.2
	8.84	0.84	4.19	4.65	0.19	0.08	6.3

[a] $[apo\text{-PcoC}]_{total} = [EGTA]_{total} = 5.0$ μM in each solution;

[b] □□: wild type *apo*-PcoC; ↓□: M40L/H49F *apo*-PcoC;

[c] See experimental section for details;

[d] At pH 7.0 and 0.1 M ionic strength, $K_A(Cu^{II}\text{-egta}) = 4.0 \times 10^{13}$ M^{-1} (Martell, A. E.; Smith, R. M.; Eds *Critical Stability Constants, Vol. 1: Amino Acids*; Plenum: New York, 1974.); the average K_D values obtained from the slopes are 5.8×10^{-14} and 6.8×10^{-14} M^{-1} for □□ and ↓□, respectively.

Cu^I Dissociation Constants. We have previously estimated the affinity of CopC for Cu^+ using bathocupröine disulfonate (bcs; Figure S4) as competing ligand (Xiao, Z.; Loughlin, F.; George, G. N.; Howlett, G. J.; Wedd, A. G. *J. Am. Chem. Soc.* **2004**, *126*, 3081-3090). However, bcs has a much higher affinity for Cu^+ ($\beta_2 = 10^{19.8}$ M^{-2}) than does CopC. Consequently, the experiments could supply only a minimum value of $K_D(Cu^I\square) \geq 10^{-13}$ M. In the present work, bicinchonic acid (bca; Figure S4), a related ligand of lower affinity, was employed (see Materials and Methods). Its association constant β_2 is not known but is significantly smaller than that of bcs. It is possible to assess the relative Cu affinities for different proteins via estimation of ratios $K_D(Cu^I\square)/\beta_2$ via eqn 2.

When in excess, bca forms a stable 1:2 purple complex $[Cu^I(bca)_2]^{3-}$ (Xiao, Z.; Wedd, A. G. unpublished observations). A stepwise increase in total protein concentration induced a proportional decrease in the intensity of the two absorption bands at 358 and 562 nm characteristic of $[Cu^I(bca)_2]^{3-}$ (Figure S5). Numerical data are listed in Table S4. Over the range of Cu^I occupancies (0.05 – 0.5), estimates of ratio $K_D(Cu^I\square)/\beta_2$ calculated from eqn 2 for wild type PcoC and CopC were constant within experimental error. The ratio is not sensitive to protein concentration, consistent with minimal influence from Cu^I(PcoC)(bca) ternary complexes. The derived ratio $K_D(Cu^I\text{-CopC})/K_D(Cu^I\text{-PcoC}) \sim 2$, implying that PcoC has a slightly higher affinity for Cu^I.

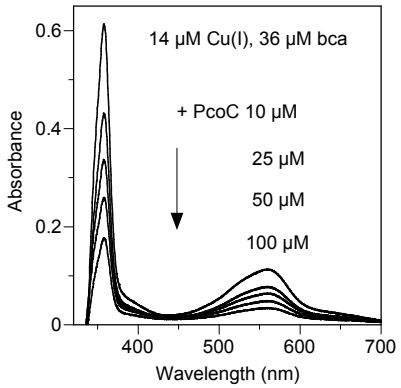


Figure S5. Decrease in intensity of the absorption spectrum of $[Cu^I(bca)_2]^{3-}$ with increasing concentration of wild type *apo*-PcoC □□ in MES buffer (20 mM; pH 6; NaCl, 100 mM) and asc (100 μM) under anaerobic conditions. From top: $[\square\square]_t$ = 0, 10, 25, 50, 100 μM, respectively.

Table S4 Estimation of $K_D(Cu^IP)$ for Wild Type PcoC and CopC Proteins and for Variant PcoC Proteins by Competition with bca for Cu^I.[a,b]

protein	$[bca]_{total}$ (μM)	$[P]_{total}$ (μM)	A_{358}	$[Cu(bca)_2]$ (μM)	[CuP] (μM)	$K_D(Cu^IP) \times (\beta_2)^{-1} \times 10^4$	relative to $K_D(Cu^IPcoC)$
none	36	0	0.62	14.5	0		
wt PcoC	36	10	0.43	10	4.5	4.8	1
(□□)	36	25	0.36	7.8	6.7	5.1	
	36	50	0.26	6.0	8.5	5.1	
	36	100	0.18	4.1	10.4	4.6	
	36	150	0.14	3.2	11.3	4.4	
wt CopC	36	25	0.39	9.1	5.4	10.3	~ 2
(□□)	36	50	0.32	7.5	7.0	10.7	
	36	100	0.24	5.5	9.0	9.0	
	36	150	0.21	4.8	9.7	10.0	
	36	200	0.18	4.1	10.4	9.8	
H1F	40	50	0.30	7.1	7.4	6.0	~1.2
(□X)	40	100	0.23	5.4	9.1	6.2	
nA-PcoC	40	50	0.29	6.7	7.8	5.0	~1
(□⇩)	40	100	0.21	5.0	9.5	5.2	
M40L	40	50	0.43	10.0	4.5	25	~5
(↓□)	40	100	0.36	8.4	6.1	24	
H49F	40	50	0.44	10.1	4.4	27	~6
(↓□)	40	100	0.38	8.8	5.7	29	
M40L/H49F	40	50	0.46	10.7	3.8	38	~8
(↓□)	40	100	0.40	9.2	5.3	35	

[a] MES (20 mM; pH 6), NaCl (100 mM) and asc (100 μM) under anaerobic conditions.

[b] Total Cu^I concentration in all equilibrium solutions was 14.5 μM.

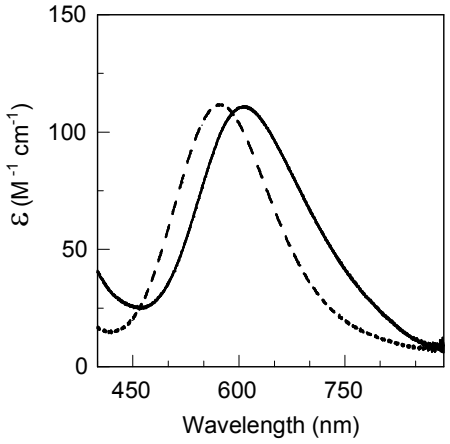


Figure S6. Solution spectra of Cu^{II}-nA-PcoC (dashed line) and Cu^{II}-PcoC (solid line) in 20 mM KPi (pH 7) and 100 mM NaCl.

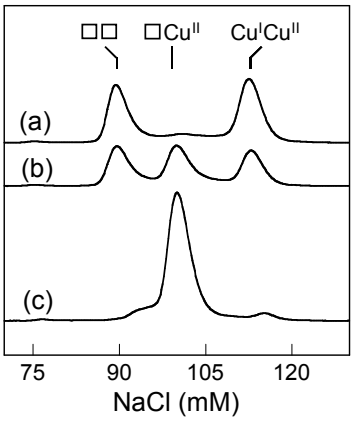


Figure S7. Oxidation of wild type PcoC protein Cu^{I}□ by air. Elution profiles of product proteins on a Mono-S HR5/5 cation exchange column under the conditions of Figure 3. The sample was injected:-

(a) immediately after preparation (see text);

(b) after exposure to air for 2 min;

(c) after exposure to air for 80 min;