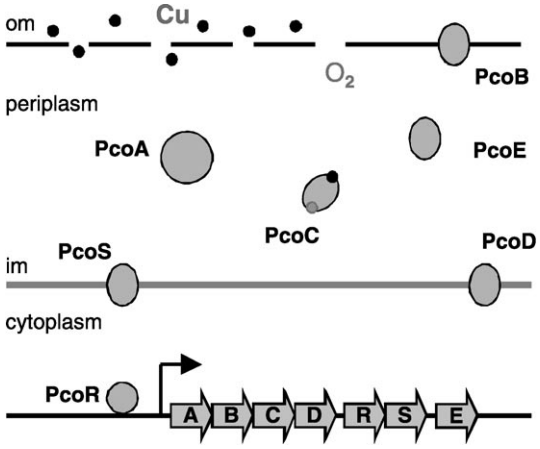
DOI: 10.1002/cbic.200800100

Copper Resistance in *E. coli*: The Multicopper Oxidase PcoA Catalyzes Oxidation of Copper(I) in $Cu^{I}Cu^{II}$-PcoC

Karrera Y. Djoko, Zhiguang Xiao,* and Anthony G. Wedd[a]

Fluctuations in nutrient copper levels in the micromolar range are controlled in Gram-negative bacteria by chromosomal tolerance operons.[1] Resistance to excess copper at the millimolar level is induced by additional plasmid-borne operons *pco* and *cop* in *Escherichia coli* and *Pseudomonas syringae*, respectively (Scheme 1).[1–3] The current model of copper resistance proposes that three soluble proteins (PcoA, PcoC, PcoE in *E. coli*) are expressed to the periplasm, that two copper pumps (PcoB, PcoD) are present in the outer and inner membranes, and that there is a copper sensing system (PcoS, PcoR).



Scheme 1. Partial model for copper resistance in *E. coli (pco)*. om: outer membrane; im: inner membrane.

PcoC and CopC are highly homologous β-barrels (~11 kDa) that bind both Cu^{I} and Cu^{II} at sites separated by about 30 Å.[4–6] These sites are tailored to bind their individual ions with high affinity ($K_D \sim 10^{-13}$ M): $Cu^{I}(His)(Met)_{2or3}$ (trigonal or tetrahedral), $Cu^{II}(His)_2(N\text{-term})(OH_2)$ (square planar).[5,6] Each shows little affinity for the other ion. All possible types of intermolecular copper transfer reactions have been demonstrated: oxidative transfer from the Cu^{I} site to the Cu^{II} site, reductive transfer from the Cu^{II} site to the Cu^{I} site, and non-redox transfers between Cu^{I} and Cu^{II} sites. This versatile chemistry is consistent with a role for PcoC as a copper carrier (chaperone) in the oxidizing periplasm, but which functions are employed remains unknown.

[a] *K. Y. Djoko, Dr. Z. Xiao, Prof. A. G. Wedd*
School of Chemistry and Bio21 Research Institute, University of Melbourne
Parkville, Victoria 3010 (Australia)
Fax: (+61) 3-9347-5180
E-mail: z.xiao@unimelb.edu.au

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

apo-PcoA (63.9 kDa) was over-expressed in *E. coli* and isolated in high purity in the presence of ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), and glycerol (Figure S1). The protein ran as a monomer on a Superdex-75 size exclusion column (Figure S1). Incubation with more than five equiv of Cu^{2+}_{aq} in the presence of glutathione and removal of unbound ions by gel filtration led to optimal development of an absorbance spectrum characteristic of a multicopper oxidase (type 1 center: 600 nm (ε, 4000 $M^{-1}cm^{-1}$); OH-bridged type 3 binuclear center: ~340 nm sh; A_{280}/A_{600}, 23.0; Figure S2).[7] Both type 1 and type 2 Cu^{II} centers were also detected in the frozen EPR spectrum (type 1 center: $g_{\perp}=2.07$, $g_{\parallel}=2.28$, $A_{\parallel}=8.5\times10^{-3}\,cm^{-1}$; type 2 center: $g_{\perp}=2.07$, $g_{\parallel}=2.30$, $A_{\parallel}=17\times10^{-3}\,cm^{-1}$; Figure S3). Isolated *holo*-PcoA contained 4.3 (0.4σ) equiv of copper and exhibited phenol oxidase activity with substrate 2,6-dimethoxyphenol (DMP) at pH 7 (Figure S4; 550 mM DMP per mM PcoA per min), a feature of most multicopper oxidases. Unexpectedly, this enzyme exhibited maximal activity in the absence of added Cu^{2+}_{aq}, whereas related multicopper oxidases require this ion in excess to induce maximal phenol oxidase activity.[8–9]

Reaction of *holo*-PcoA with *air-stable* $Cu^{I}Cu^{II}$-PcoC under catalytic conditions (1:50) in air-saturated MOPS buffer (pH 7) led to quantitative generation of product Cu^{II}-PcoC (represented as $\square Cu^{II}$, where □ is the empty Cu^{I} site; Figure 1); this is consistent with oxidation of bound Cu^{I}. The catalysis was suppressed dramatically in deoxygenated buffer (Figure S5), consistent with O_2 acting as the oxidant. The product protein $\square Cu^{II}$ has little affinity[6] for co-product Cu^{2+}_{aq}, which appeared to be released into solution. Clean regeneration of $Cu^{I}Cu^{II}$-PcoC occurred upon addition of reductant NH_2OH (that is, Figure 1D converted to Figure 1A). This is a robust catalyst: there was negligible loss of activity after four cycles of oxidation and reduction.

The product PcoC protein $\square Cu^{II}$ suppressed cuprous oxidase activity, consistent with a previous suggestion that PcoC interacts with PcoA.[10] Inclusion of increasing concentrations of $\square Cu^{II}$ in the initial reaction mixture in Figure 1 led to proportional decreases in the rate of the catalytic reaction, whereas addition of generic proteins such as lysozyme had no effect (Figure 2). These results, when coupled with the high affinity of PcoC for Cu^{I} ($K_D \sim 10^{-13}$ M), demonstrate that PcoA catalyzes the oxidation of Cu^{I} bound in $Cu^{I}Cu^{II}$-PcoC. They suggest that PcoA and PcoC cooperate to convert Cu^{I} into the less toxic Cu^{II} in the O_2-rich periplasm.

This system exhibits two new features: cuprous oxidase activity with a likely biological partner and maximal phenol oxidase activity in the absence of excess Cu^{2+}_{aq}. In seeking a model for this behavior, it was noted that PcoA has some homology (23%) with the tolerance enzyme CueO and, impor-

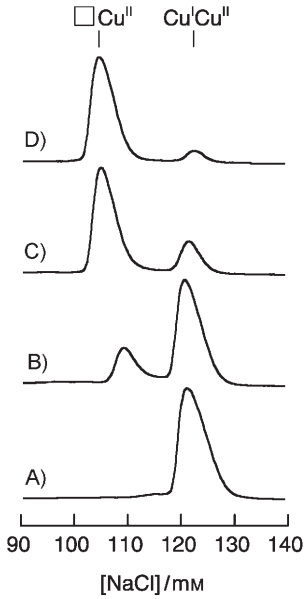


Figure 1. Oxidation of $Cu^{I}Cu^{II}$-PcoC (25 μM) catalyzed by *holo*-PcoA (0.5 μM) in air-saturated MOPS buffer (20 mM; pH 7; $NaNO_3$, 100 mM). Separation of products on a Mono-S cation exchange column at A) 0, B) 2, C) 30, and D) 60 min. Protein identities were confirmed by ESI-MS and metal analyses as described previously.[6]

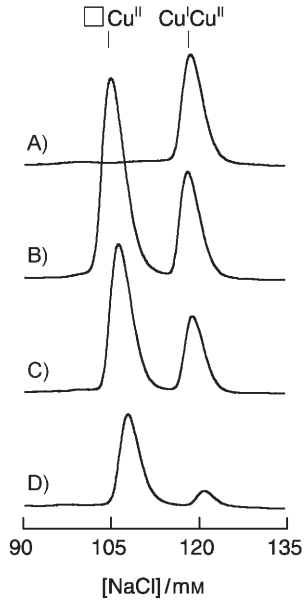


Figure 2. Suppression of *holo*-PcoA cuprous oxidase activity by the product protein Cu^{II}-PcoC: A) $Cu^{I}Cu^{II}$-PcoC (25 μM) in air-saturated MOPS buffer (20 mM; pH 7; $NaNO_3$, 100 mM) before reaction; B) 30 min after addition of *holo*-PcoA (0.5 μM) and Cu^{II}-PcoC (50 μM) into solution A; C) as B, but half of the added Cu^{II}-PcoC (25 μM); D) as B, but without added Cu^{II}-PcoC or with added lysozyme (50 μM). Equal volumes of reaction solution were used in each chromatographic analysis and the relative intensities of each elution profile were not normalized. Reaction progress is thus monitored by comparison of the relative intensities of the $Cu^{I}Cu^{II}$-PcoC peaks.

tantly, all the anticipated ligands for types 1, 2 and 3 copper are intact (Figure S6). The X-ray crystal structure of CueO reveals the molecular nature of the four copper sites associated with this multicopper oxidase machinery (Figure 3).[11] In addition, there is a fifth copper site $Cu(Met)_2(Asp)_2$ (labeled * in Figure 3) that would be expected to favor binding of Cu^{I} over

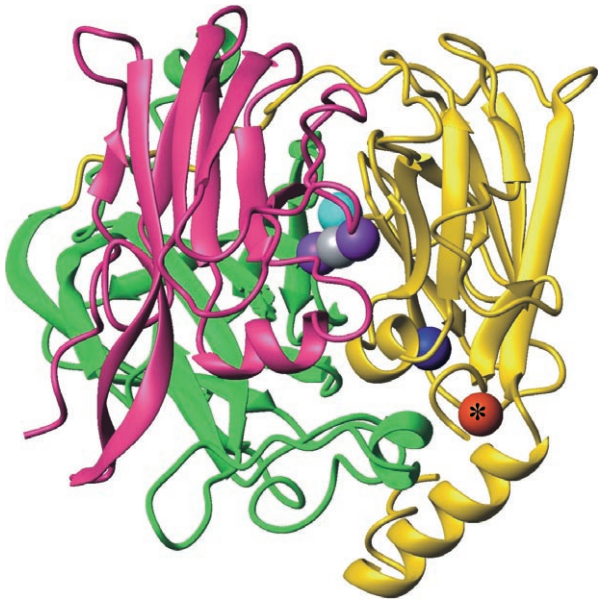
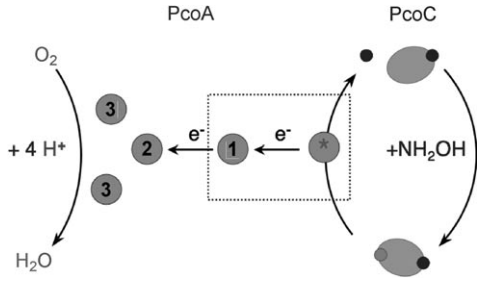


Figure 3. Ribbon model of the copper tolerance enzyme CueO highlighting three structural domains (D1: pink, D2: green, D3: gold) and different copper centers (1: blue, 2: teal, 3: purple, additional Cu*: red). The figure was generated from the coordinates from PDB ID: 1N68.

Cu^{II}. This Cu* site is located on a Met-rich α-helix, which physically blocks substrate access to the type 1 site but which is in electronic contact with that site through hydrogen-bonded ligands (as indicated by the box encompassing the type 1 and Cu* sites in Scheme 2). While three of the four ligands for the Cu* site are not conserved in PcoA, many potential ligands for Cu^{I} are available in this Met-rich region (Figure S6).



Scheme 2. Model for cuprous oxidase activity.

Scheme 2 provides a model for the cuprous oxidase activity of PcoA. The Met-rich Cu* site docks substrate $Cu^{I}Cu^{II}$-PcoC by direct interaction with the Met-rich Cu^{I} site of PcoC. Cu^{I} is oxidized and released into solution as Cu^{2+}_{aq}. Protein product

□Cu^{II} is a competitive inhibitor, restricting access of substrate $Cu^{I}Cu^{II}$-PcoC to the catalytic Cu* site. Notably, intermolecular interactions close to the empty Cu^{I} site of *apo*-PcoC lead to dimerization in the solid state.[12] In addition, efficient transfer of Cu^{I} between PcoC proteins occurs.[6] Similar interactions would appear to exist between PcoA and PcoC to facilitate effective oxidation of Cu^{I} bound in $Cu^{I}Cu^{II}$-PcoC.

We have recently expressed and isolated a related protein CopK from *Cupriavidus metallidurans*, which binds both Cu^{I} and Cu^{II} with similar affinities to PcoC.[13] Catalytic oxidation of air-stable $Cu^{I}Cu^{II}$-CopK under the same conditions as in Figure 1 was, however, more than 20 times slower (Figure S7), presumably as this protein is not optimized for interaction with PcoA from *E. coli*.

Scheme 2 also provides a model for the phenol oxidase activity: a copper atom remains in the Cu* site to act as a one-electron oxidant to produce the initial radical product. This correlates with the requirement of related enzymes for excess Cu^{2+}_{aq} in solution to induce maximal phenol oxidase activity.[10,11] This condition would appear to optimize occupancy of the catalytic Cu* site in these copper tolerance enzymes. Added copper, however, is not required for maximal phenol oxidase activity in the resistance enzyme PcoA, plausibly because the affinity for copper in the Cu* site is higher or the phenol substrate can access the type 1 Cu site *directly* with no need for a bound Cu* ion in this system.

Addition of Ag^{+}_{aq} or Hg^{2+}_{aq} to *holo*-PcoA led to a rapid loss of copper from the type 1 site (decrease in absorbance at 600 nm in the solution spectrum of Figure S2 and loss of type 1 signal in the frozen glass EPR spectrum of Figure S3) and inhibition of the phenol oxidase activity (Figure S8). However, the cuprous oxidase activity for $Cu^{I}Cu^{II}$-PcoC *was not* inhibited by Ag^{+}_{aq} under the same conditions. PcoC product protein □Cu^{II} has a high affinity for Ag^{I} and apparently competes efficiently with the PcoA sites for Ag^{+}_{aq} ion. In telling contrast, the cuprous oxidase activity is poisoned by Hg^{2+}_{aq} ions, consistent with the known low affinity of □Cu^{II} for Hg^{II}.[5,6]

These preliminary results provide sufficient molecular characterization to suggest that PcoC and PcoA interact to convert Cu^{I} into the less toxic Cu^{II} in the dioxygen-rich periplasm of *E. coli*. The two proteins may combine with the outer membrane protein PcoB (Scheme 1) to export copper from the periplasm. Both PcoA and PcoB must be expressed for optimal resistance.[1] PcoC may also provide nutrient copper to the inner membrane protein PcoD to allow assembly of *holo*-PcoA in the cytoplasm prior to export by the TAT pathway. Experiments are in train to explore these possibilities, as well as the potential of PcoA to act as a ferrous oxidase and as a siderophore oxidase.[10]

Experimental Section

Isolation of *apo*-PcoA and generation of *holo*-PcoA: The PcoA gene encoding mature protein without the leader sequence (first 32 residues) was amplified from plasmid pRJ1004 (ref. [2]) and cloned into vector pET20b(+) between the *Nde*I and *Hind*III sites. The protein was expressed in *E. coli* BL21(DE3) CodonPlus(+) cells after induction with isopropyl-β-D-thiogalactopyranoside and purified by sequential chromatography on DE-52 anion-exchange, Phenyl-Sepharose hydrophobic interaction, and Superdex 75 gel-filtration columns. EDTA (1 mM), DTT (1 mM), and glycerol (10–20%) were included in all chromatography buffers to facilitate purification and to maintain protein solubility. They were removed with a desalting column before copper loading and analysis. *holo*-PcoA was prepared by overnight incubation of *apo*-PcoA with excess Cu^{2+}_{aq} (10 equiv) in the presence of glutathione in MOPS buffer (20 mM; $NaNO_3$, 100 mM; pH 7), followed by removal of excess metal ion and glutathione on a desalting column. The concentration of *apo*-PcoA was estimated from the absorbance at 280 nm derived from PcoA primary sequence ($\varepsilon = 80\,800\ M^{-1}cm^{-1}$), and the *holo*-PcoA concentration was determined by the Bradford assay with *apo*-PcoA as a standard. Total copper content was determined with bathocuproine disulfonate (bcs) reagent in the presence of denaturant guanidine (6 M).[6]

Enzyme activity assay—phenol oxidase activity: The assay mixture consisted of *holo*-PcoA (0.25 μM) and dmp (2 mM) in air-saturated MOPS buffer (20 mM; $NaNO_3$, 100 mM; pH 7). Oxidation of dmp was followed spectrophotometrically at 469 nm ($\varepsilon = 14\,800\ M^{-1}cm^{-1}$).

Cuprous oxidase activity with $Cu^{I}Cu^{II}$-PcoC: The assay mixture consisted of *holo*-PcoA (0.5 μM) and $Cu^{I}Cu^{II}$-PcoC (25 μM) in air-saturated MOPS buffer (20 mM, pH 7; $NaNO_3$, 100 mM). Oxidation of $Cu^{I}Cu^{II}$-PcoC was followed by separation of the protein products on a Mono-S HR5/5 cation-exchange column (0.5 × 5 cm). Protein identities were confirmed by ESI-MS and metal analyses as described previously.[5,6]

For the control reaction under anaerobic conditions, the same assay mixture was prepared in a glove box (O_2 level < 10 ppm) with deoxygenated MOPS buffer. The reaction was quenched with excess Hg^{2+} before each analysis with the Mono-S column. Identical experiments were performed in air-saturated MOPS buffer to ensure that there was no artifact associated with Hg^{2+}.

Acknowledgements

We thank Dr. Richard Luke (La Trobe University) for access to the pRJ1004 plasmid, Lee Xin Chong for assistance with Figure S8, Dr. Simon Drew for assistance with EPR, and the Australian Research Council for support under Grant A29930204.

Keywords: bioinorganic chemistry · copper resistance protein · metalloenzymes · multicopper oxidase

[1] C. Rensing, G. Grass, *FEMS Microbiol. Rev.* **2003**, *27*, 197–213.
[2] T. J. Tetaz, R. K. J. Luke, *J. Bacteriol.* **1983**, *154*, 1263–1268.
[3] D. A. Cooksey, *FEMS Microbiol. Rev.* **1994**, *14*, 381–386.
[4] F. Arnesano, L. Banci, I. Bertini, S. Mangani, A. R. Thompsett, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3814–3819.
[5] L. Zhang, M. Koay, M. J. Maher, Z. Xiao, A. G. Wedd, *J. Am. Chem. Soc.* **2006**, *128*, 5834–5850.
[6] K. Y. Djoko, Z. Xiao, D. L. Huffman, A. G. Wedd, *Inorg. Chem.* **2007**, *46*, 4560–4568.
[7] E. I. Solomon, U. M. Sundaram, T. E. Machonkin, *Chem. Rev.* **1996**, *96*, 2563–2605.
[8] F. Solano, P. Lucas-Elío, D. López-Serrano, E. Fernández, A. Sanchez-Amat, *FEMS Microbiol. Lett.* **2001**, *204*(1), 175–181.
[9] A. T. Fernandes, C. M. Soares, M. M. Pereira, R. Huber, G. Grass, L. O. Martins, *FEBS J.* **2007**, *274*, 2683–2694.

[10] D. L. Huffman, J. Huyett, F. W. Outten, P. E. Doan, L. A. Finney, B. M. Hoffman, T. V. O'Halloran, *Biochemistry* **2002**, *41*, 10046–10055.
[11] S. A. Roberts, G. F. Wildner, G. Grass, A. Weichsel, A. Ambrus, C. Rensing, W. R. Montfort, *J. Biol. Chem.* **2003**, *278*, 31958–31963.
[12] A. K. Wernimont, D. L. Huffman, L. A. Finney, B. Demeler, T. V. O'Halloran, A. C. Rosenzweig, *J. Biol. Inorg. Chem.* **2003**, *8*, 185–194.
[13] L. X. Chong, Z. Xiao, unpublished observations.

Received: February 18, 2008
Published online on June 6, 2008

