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Highly Sensitive and Selective Gold(I) Recognition by a Metalloregulator in *Ralstonia metallidurans*

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All living organisms face challenges in acquiring essential metal ions for normal cellular function, controlling cellular concentrations of various metal ions, and detoxifying toxic metals. In bacteria, control of cellular metal ion concentration is often achieved by metal-responsive transcriptional regulators. The MerR family of proteins are a group of commonly encountered bacterial metallo-regulators.[1,2] Among them, CueR is a major regulator of copper homeostasis in *E. coli* and other bacteria.[2] In the presence of excess copper(I), CueR activates transcription of *cueO* and *copA* to reduce the level of cellular copper. In addition to copper(I), CueR can also detect and respond to silver(I) and gold(I) *in vivo*. In contrast, another MerR family regulator, GolS from *Salmonella typhimurium*, responds mainly to gold(I).[3] A domain-swapping experiment suggested that this specificity appears to be largely determined by the C-terminal metal-binding loop,[3] although no biochemical characterization of the protein has been reported.

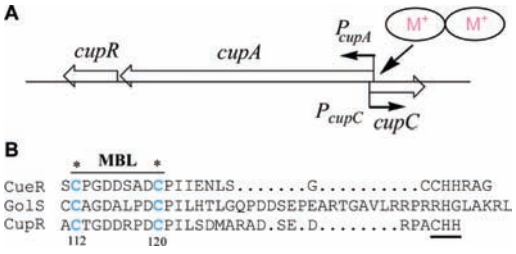

Figure 1. (A) Genetic organization of the *cup* gene cluster on *R. metallidurans* CH34 chromosome one. CupR dimer is represented by ellipses. The metal ion M^+ is either gold(I) or copper(I). (B) Alignment of the metal binding loop (MBL) and the C-terminal region of CueR (*E. coli*), GolS (*S. typhimurium*), and CupR (*R. metallidurans*). The conserved residues Cys112 and Cys120 involved in metal binding are highlighted. The C-terminal "CHH" motif in CupR is underlined.

A recent study found that *Ralstonia metallidurans*, a β-proteobacterium highly resistant to metals, is able to precipitate gold from $AuCl_4^-$ solution and is likely involved in the formation of grains of secondary gold in soils.[4] We were attracted to this very interesting subject of gold response. A survey of the *Ralstonia metallidurans* CH34 genome revealed that a *cupR* regulator (Rmet_3523) was annotated to regulate the three genes *cupC* (a heavy metal transport-detoxification protein), *cupA* (a heavy metal translocating p-type ATPase), and *cupR* itself (Figure 1A). Binding of CupR to the dyad symmetric *merR* type operator sequence between the −35 and −10 box of the *cupA* promoter was confirmed by a gel mobility shift assay with a K_d of 40 nM obtained (Figure S2).

Northern hybridization was used to assay the induction level of the three genes by different metal ions. Moderate concentrations of Zn(II), Cd(II), Hg(II), Fe(III), Co(II), Ni(II), Tl(I), or Pb(II) ions did not alter the transcription levels of these genes (Figures 2A and S3). When $CuSO_4$ or $HAuCl_4$ was added into the media, transcription of all three genes increased (Figure 2B−D). However, the expression pattern exhibited a significant difference. While the induction by copper could be detected at a 20 μM concentration, an increase in the $CuSO_4$ concentration from 50 to 100 μM failed to raise the transcription level further, suggesting the existence of a copper specific regulatory system distinct from CupR. On the other hand, transcription levels of these three genes rose with the increasing concentration of gold. At 100 μM, $HAuCl_4$ has a significant stronger effect on transcriptional activation than $CuSO_4$.

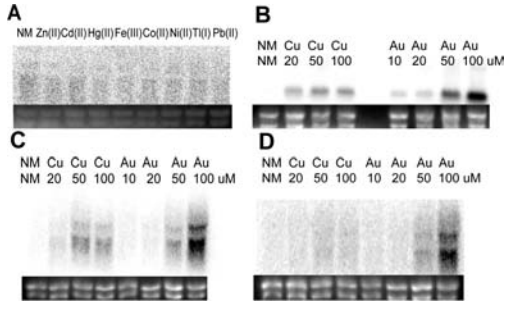

Figure 2. Metal-mediated transcriptional activation of *cup*. (A) No induction of *cupR* in the presence of Zn(II) (100 μM), Cd(II) (20 μM), Hg(II) (20 μM), Fe(III) (100 μM), Co(II) (100 μM), Ni(II) (100 μM), Tl(I) (20 μM), or Pb(II) (20 μM). NM: no metal. Induction of *cupC* (B), *cupA* (C), and *cupR* (D) in the presence of various concentrations of $CuSO_4$ or $HAuCl_4$. Both gold(III) and copper(II) are reduced to the monocation forms in the bacterial cytoplasma.

The stronger response of CupR to gold than to copper indicates that this regulator possesses the selectivity to sense gold stress (see ref 5 for more information about gold toxicity).[5] Using the

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charge transfer band between copper(I) and sulfur ligand to monitor *in vitro* copper(I) binding (Figure S4A),[6] we found that the conserved cysteines in the metal binding loop, Cys112 and Cys120, are important for metal binding by CupR. When the "CHH" motif at the C-terminus (Cys137 to His139) was deleted (CupR137del mutant), the truncated mutant protein still binds copper(I) and gold(I) (Figure S4B).

When copper(I)-CupR was titrated with bcs (bathocuproine disulfonate sodium salt),[7] the first equivalent of copper(I) could be readily titrated out, while the second equivalent required higher concentrations of bcs. Thus two dissociation constants, 7.7×10^{-17} and 6.3×10^{-19} M, were obtained for copper(I) binding to CupR (Tables 1 and S1). When the copper(I)-CupR137del complex was subjected to this titration, the two sites showed a similar binding to copper(I) with a K_d of $\sim 2.1 \times 10^{-18}$ M (Tables 1 and S1). These measured copper(I) affinities for both wild-type CupR and CupR137del are lower than that reported for CueR (Table. 1). Next, we performed metal competition experiments between CupR and *E. coli* CueR. We found that the same equivalent of apo CueR can compete for copper(I) from copper(I)-CupR (Figure S5A), while, in a reverse reaction, copper(I) transfer from copper(I)-CueR to apo CupR was not observed (Figure S5B). Consistent with the data, when recombinant CupR was purified from *E. coli*, copper was not detected by ICP-MS while CueR purifies with bound copper.

Table 1. K_d Values of CupR, CupR137del, and *E. coli* CueR[8] to Copper(I) and Gold(I)

protein	CupR	CupR137del	CueR
copper(I)	7.7×10^{-17} M, 6.3×10^{-19} M	2.1×10^{-18} M	2×10^{-21} M
gold(I)	7.2×10^{-35} M	4.0×10^{-32} M	2×10^{-35} M

The dissociation constant of gold(I)-CupR was determined by equilibrating the protein in buffered solutions containing KCN and $K[Au(CN)_2]$ and quantifying gold(I)-bound CupR from the far-UV absorption. The gold content in each sample was also quantified by ICP-MS (Table S2), which matches well with the UV measurement. The K_d for gold(I) binding to CupR was estimated to be around 7.2×10^{-35} M (Figure S6A). The affinity of gold(I) to CupR137del was also measured, and a K_d of 4.0×10^{-32} M was obtained (Figure S6B). This number is significantly lower than that of the wild-type CupR (Table 1). We also found that only one cysteine in the apo wild-type protein can react with fluoresin-maleimide.[11] Binding of copper(I) or gold(I) partially protects this cysteine from alkylation (Table S3). Thus, the C-terminal residues of CupR play important roles in maintaining the protein's high affinity to gold(I). The wild-type CupR appears to employ a strategy of lowering its affinity to copper(I) (relative to CueR) while maintaining a high affinity to gold(I), thus gaining selectivity toward gold(I) as well as avoiding interference from the cellular copper pool.

We next employed X-ray absorption spectroscopy (XAS) to gain structural information about the metal-binding environment in CupR. The copper(I)-CupR at copper K-edge data are shown in Figure 3A, along with the data from $[Cu(EDTB)](ClO_4)_2$, $[Cu(L_1\text{-pr})](BF_4)$, and $[Cu(py)_4](ClO_4)$, a two-, three-, and four-coordinate copper(I) model complex, respectively.[9,10] The rising edge of CupR exhibits a characteristic feature of copper(I) complexes at ~8984 eV. The intensity of this feature is dependent on the metal 4*p* orbital ligand field splitting. From the comparison, it is clear that the CupR protein exhibits an edge structure resembling that of a three-coordinate copper(I) complex and, importantly, does not exhibit the intense feature at ~8984 eV typical of the $1s \rightarrow 4p_z$ feature as those linear two-coordinate complexes. This feature is different from the *E. coli* copper(I)-CueR characterized previously.[11]

The CupR EXAFS data and the Fourier transform are shown in Figure 3B. All fits were restricted to a coordination number of three in accordance with the results from XAS edge fitting. The data are best fit with a three-coordinate site, including two sulfur-based ligands at 2.12 Å and a third, longer interaction, quite possibly a sulfur ligand at 2.26 Å (Figure 3C, Table S4, and Supporting Information). When we examined the copper(I)-CupR137del mutant, a different edge spectrum was obtained, and the EXAFS data are best fit with two sulfur based ligands at 2.13 Å (Figures 3D and S8, Table S5). Details about the data fitting are discussed in the Supporting Information. The gold L-edge X-ray absorption data lack the edge feature, and we were

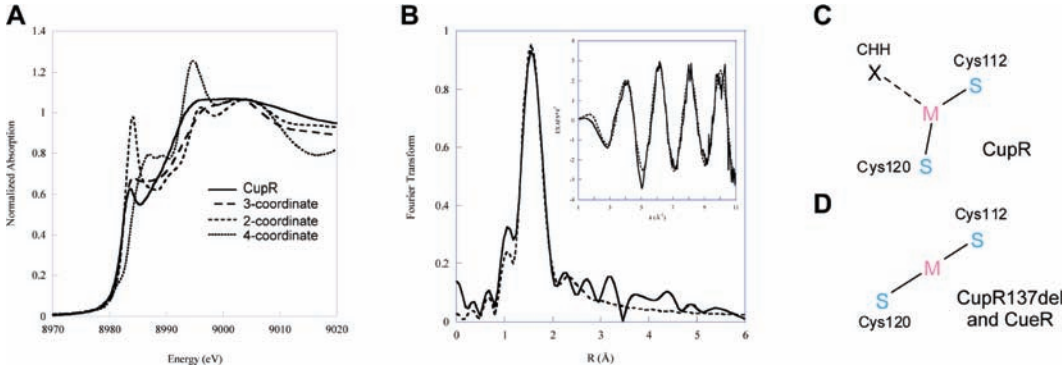

Figure 3. (A) Copper K-edge XANES data from CupR and the two-, three-, and four-coordinate model complexes. The 1s→4p feature at ~8984 eV is indicative of the ligand field and therefore of copper(I) coordination geometry. (B) Fourier transform of the EXAFS data (solid) and best fit (dashed) consisting of two sulfur ligands at 2.12 Å and one sulfur ligand at 2.26 Å (or a long nitrogen/oxygen ligand). Inset: EXAFS data (solid) and fit to the data (dashed) (no phase correction is included). (C) Proposed metal coordination geometry in copper(I)-CupR. (D) Metal coordination geometry in copper(I)-CupR137del (this work) and copper(I)-CueR.[8,11]

not able to reveal the metal center structure in the gold(I)-CupR protein. However, the biochemistry and X-ray absorption data strongly indicate the binding of the third ligand from the CHH motif to copper(I) or gold(I).

Although copper functions as an essential trace metal for most living organisms, copper in excess amounts is toxic to the cell. In *E. coli*, a MerR family transcription regulator CueR binds and regulates cellular available copper(I) with an estimated K_d of $\sim 10^{-21}$ M.[8] CueR recognizes copper(I) ion using two conserved residues Cys112 and Cys120 in a linear, two-coordinate geometry.[8,11] In *M. tuberculosis*, CsoR uses two Cys and one His to bind copper(I) in a trigonal geometry.[12]

Ralstonia metallidurans strain CH34 is a nonspore forming bacillus adapted to survive various transition and heavy metal stresses.[13] The genetic organization of the *cup* operon is different from that of *cueR*, *copA*, and *cueO* in *E. coli* but similar to the gold-responsive cluster *gol* in *S. typhimurium*.[3] We show here that the main function of the *cup* operon appears to be defending against gold stress. Being more thiophilic than copper(I), gold(I) at elevated concentrations could strongly interfere with normal biological functions of copper(I). Evolving a transcriptional regulator specific to gold(I) is important to defending against gold stress while still maintaining a healthy copper(I) level. CupR achieves this function by possessing a high affinity to gold(I) while possessing a moderate affinity to copper(I).

As expected, the two conserved Cys residues, Cys112 and Cys120, play key roles in both copper(I) and gold(I) binding. Interestingly, the "CHH" motif at the C-terminus also plays a role. Deletion of this motif leads to a several hundred-fold decrease of the protein's affinity to gold(I), while the affinity toward copper(I) stays relatively unchanged. Thus, this motif is important for gold(I) recognition. Copper K-edge X-ray absorption spectroscopy was employed to study the local structure of the copper(I) center in the copper(I)-CupR complex. It is notable that the XANES features are distinctively different from the *E. coli* copper(I)-CueR complex but resemble well characterized three-coordinate copper(I) compounds.[9] The EXAFS data are best fit to a copper center composed of two short sulfur ligands and one long ligand, a coordination geometry compatible for gold(I) but unfavorable for copper(I).[14] Supporting this assignment, the CupR137del mutant with deletion of the C-terminal "CHH" motif binds copper(I) in a typical two-coordinate geometry. Thus, the gold(I) preference arises from a metal environment that is different from *E. coli* CueR. Future experiments will focus on elucidating the structure of the distinct gold(I) recognition mode in CupR.

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Supporting Information Available: Including suppoting methods, Tables S1−S5, and Figures S1−S8. This material is available free of charge via the Internet at http://pubs.acs.org.

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