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# Unnatural amino acid substitution as a probe of the allosteric coupling pathway in a mycobacterial Cu(I) sensor

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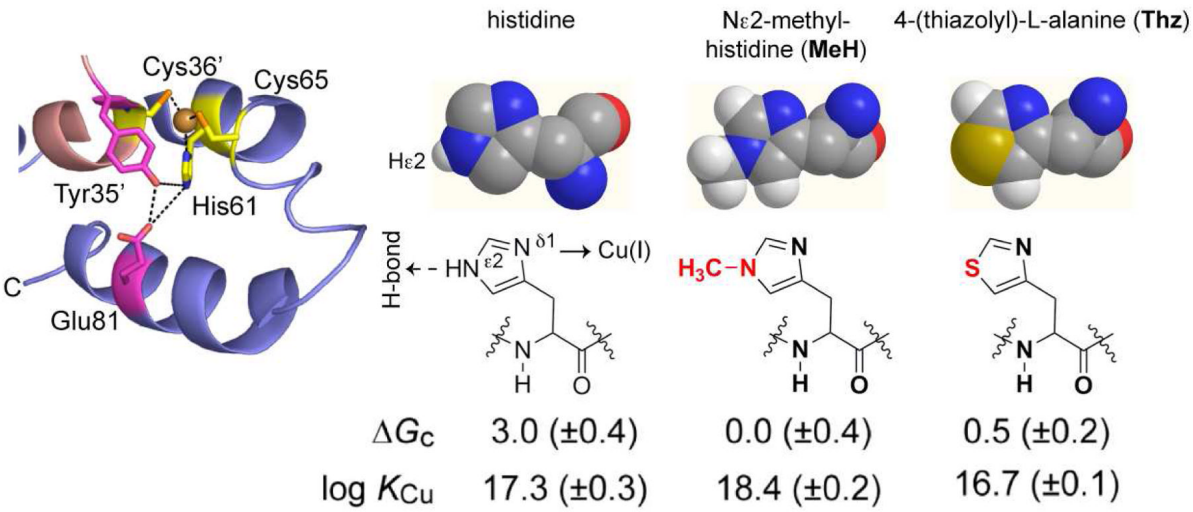
## Abstract




The Cu(I) sensor *Mycobacterium tuberculosis* CsoR is a founding member of a new metalloregulatory protein family. Here we show that two "atom" substitutions of the Nε2 face of a Cu(I) coordinating histisine-61 allosterically uncouple Cu(I) and DNA binding, with no effect on Cu (I) binding affinity and coordination structure. A model analogous to the allosteric switch mechanism in *Staphylococcus aureus* CzrA, a zinc sensor protein with a completely different fold, is proposed.

Elucidation of structural and dynamical pathways of communication or coupling between ligand binding sites, *i.e.*, classical allostery, is of topical interest and fundamental importance in biological regulation,[1–3] but remains a challenge to detect and quantify.[4–6] Metalloregulatory proteins are specialized allosteric proteins that control the intracellular availability of essential transition metal ions by binding a specific metal, which in turn,

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**Supporting Information Available:** Supplementary Methods, Tables (S1–S3) and Figures (S1–S9) are available free of charge via the Internet at http://pubs.acs.org.

activates or inhibits operator DNA binding, thus programming a defined transcriptional output.[7,8] In this study, we target the "second coordination shell" of the Cu(I) sensor *M. tuberculosis* CsoR and successfully abrogate allosteric coupling of Cu(I) and DNA binding.

*Mtb* CsoR is a Cu(I) regulatory protein distantly related to the Ni(II)/Co(II) sensor *E. coli* RcnR,[10] in which Cu(I)-binding allosterically decreases operator DNA binding affinity. This leads to transcriptional derepression of the copper-sensing operon (*cso*) in response to Cu(I) stress.[9] The Cu(I) ion in CsoR occupies a pocket at the protomer interface, where the Sγ atoms of Cys36' and Cys65, and the Nδ1 atom of His61 form a trigonal coordination complex (Figure 1).[9] The structure further reveals that the Nε2 face of His61 points toward the side chains of two conserved "second coordination shell" residues, Tyr35 and Glu81 (Figure 1). This structure suggests that Tyr35, Glu81 and His61 form a hydrogen bonding network that stabilizes the allosterically inhibited Cu(I) bound state.[9,11] Substitution of the Cu(I) ligand His61 with Ala changes the Cu(I) coordination number from three to two, resulting in a linear *bis*-thiolato Cu (I) complex.[9] This substitution also greatly reduces the *cso* operator binding affinity by ≥10,000-fold to undetectable levels, despite adopting a folded, α-helical tetrameric assembly state (Figure S1). Thus, His61 is a multifunctional residue, the distinct physicochemical roles of which cannot be directly probed by conventional mutagenesis.[9] We therefore employed native chemical ligation to perform an “atom” substitution of His61 by replacement with Nε2-methyl-histidine (MeH) or 4-(thiazolyl)-L-alanine (Thz) in order to directly test this allosteric coupling model (Figure 1). Both substitutions are expected to preserve the Cu(I) coordination and DNA binding properties of His61 while blocking the ability of the opposite face of His61 to donate hydrogen bonds to Tyr35' and/or Glu81.

The Cu(I) binding affinity (log $K_{Cu}$) of wild-type *Mtb* CsoR was determined to be 18.0 (±0.2) from both a conventional titration of Cu(I) into a mixture of CsoR and BCS (Figure S2), as well as an assay that employs varying BCS concentrations, rather than Cu(I) concentrations (Table 1 and Supplementary Methods).[9,12,13] The coupling free energy ($\Delta G_c$) was obtained from the ratio of the macroscopic CsoR DNA binding affinities in the presence ($A_2^{Cu}$) and absence ($A_2^{apo}$) of bound Cu(I) measured by fluorescence anisotropy[7] (Supplementary Methods and Figures S3–S5). Cu(I) binding to wild-type CsoR significantly decreases the *cso* operator DNA binding affinity, resulting in a $\Delta G_c$ of +3.6 (±0.2) kcal/mol (pH 7.0, 0.2 M NaCl, 2 mM DTT, 25 °C) (Table 1), with no effect on the assembly state of the CsoR tetramer.[13]

A semisynthetic route using an intein fusion strategy was used to introduce MeH and Thz analogs of His61 (Figure 1 and Supplementary Methods). To increase the efficiency of such a strategy, L58C CsoR 1–106 was chosen as the parent molecule to incorporate both H61MeH and H61Thz substitutions by chemical ligation, since it showed no significant differences from wild-type CsoR in Cu(I) binding affinity (Table 1), Cu(I) coordination geometry (Figure 2 and Table S2), DNA binding affinity and negative allosteric regulation by Cu(I) (Table 1 and Figure S5). The integrity of the resulting H61MeH and H61Thz *Mtb* CsoRs was confirmed by trypsin digestion and tandem LC-MS/MS sequencing (Figure S6).

Both H61MeH and H61Thz *Mtb* CsoRs bind Cu(I) with an affinity comparable to the parent L58C CsoR 1–106 (log $K_{Cu}$=17.3) (Table 1) and 100-1000-fold higher affinity than that of H61A CsoR, which forms a digonal $S_2$ complex.[9] This suggests that H61MeH and H61Thz CsoRs form trigonal coordination complexes with Cu(I), a result confirmed by the Cu(I) near-edge region of the x-ray absorption spectrum (Figure 2, Table S2 and Figure S7). Quantitative analysis of the EXAFS spectrum of H61MeH CsoR reveals a coordination geometry that is identical to wild-type CsoR (Table S2).[9] Both H61MeH and H61Thz CsoRs bind the *cso* operator with near wild-type affinity in their apo-forms which reveals the semisynthetic proteins are folded correctly and likely form stable tetramers (Figure S5 and Table S1).[13]

Strikingly, the DNA binding affinities of both H61MeH and H61Thz CsoRs are not strongly regulated by Cu(I), with $\Delta G_c$=0 (±0.4) and 0.5 (±0.2) kcal mol$^{-1}$, respectively (Table 1). These findings clearly establish that the Nε2 face of His61 is specifically required to link thermodynamically and functionally Cu(I) binding to DNA binding, with no significant influence on the magnitude of the Cu(I) binding affinity or coordination geometry.

To further test this structural linkage model (Figure 1), we targeted the other "second coordination shell" residues, Tyr35 and Glu81, using conventional site-directed mutagenesis. Y35F and all E81 mutant CsoRs retained wild-type Cu(I) binding affinity and trigonal $S_2N$ Cu (I) coordination geometries by x-ray absorption spectroscopy (Table 1, Table S3 and Figure S8). Y35F CsoR is characterized by a 33% decrease in $\Delta G_c$, while *cso* operator DNA binding by E81A CsoR is nearly refractory to Cu(I) regulation ($\Delta G_c$= 0.6 kcal mol$^{-1}$) (Table 1 and Figure S9). More conservative substitutions of Glu81, *i.e.*, with Gln, Asp or Asn, also give rise to significant decreases in $\Delta G_c$, to about 50% of wild-type CsoR, while Cu(I) binding affinities remain unaffected (Table 1). Inspection of the magnitude of $\Delta G_c$ for a double mutant, Y35F/E81Q CsoR, reveals a near additivity of $\Delta G_c$ measured for the component single mutants (Table 1). Thus, these residues play important roles, but only weakly interact energetically with one another to drive allosteric negative regulation of CsoR by Cu(I). This suggests that hydrogen bonds from Tyr35 and Glu81 *to* His61, and not to each other (Figure 1), are most critical for metalloregulation in this system.

We propose that the heteroaromatic character of His61 contacts the DNA in a way that does not require the Nε2 atom of the imidazole ring. Cu(I) coordination to the Nδ1 atom is necessary but not sufficient to drive allosteric switching, but critically, initiates formation of a hydrogen-bonding network involving the Nε2 face of the same histidine, and the side chains of Y35 and E81, that ultimately leads to dissociation of the CsoR tetramer from the DNA. It is remarkable that opposite Nδ1 and Nε2 faces of a key metal coordinating histidine residue, albeit one far from the DNA binding site,[14] are proposed to play exactly analogous roles in the arsenic repressor (ArsR) family zinc sensor CzrA, despite the completely unrelated protein fold and different metal that is sensed.[14–16] This speaks to the evolutionary generality of a mechanism of allosteric regulation that exploits the unique physicochemical features of a key histidine side chain to orchestrate cellular metal homeostasis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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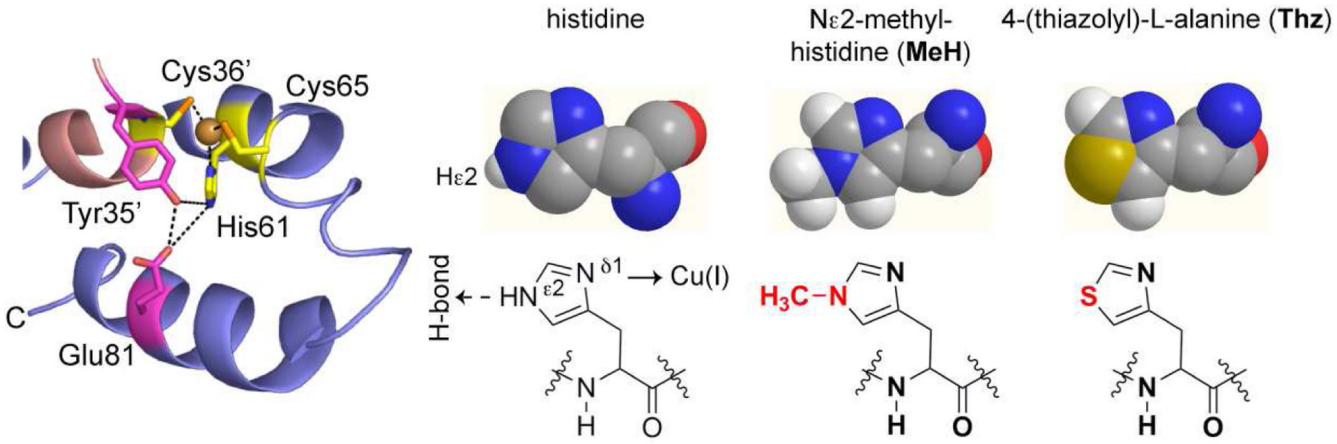


**Figure 1.**

Structure around the Cu(I) binding region of the *Mtb* CsoR dimer interface (protomers shaded *slate* and *salmon*)[9] and space filling and chemical structures of histidine, Nε2-methyl-histidine (MeH) and 4-(thiazolyl)-L-alanine (Thz) used in this study. Note the Nδ1 nitrogen of His61 forms a Cu(I)-N coordination bond while the Nε2 face is within hydrogen bonding distance of Y35' (*salmon* protomer) and E81.

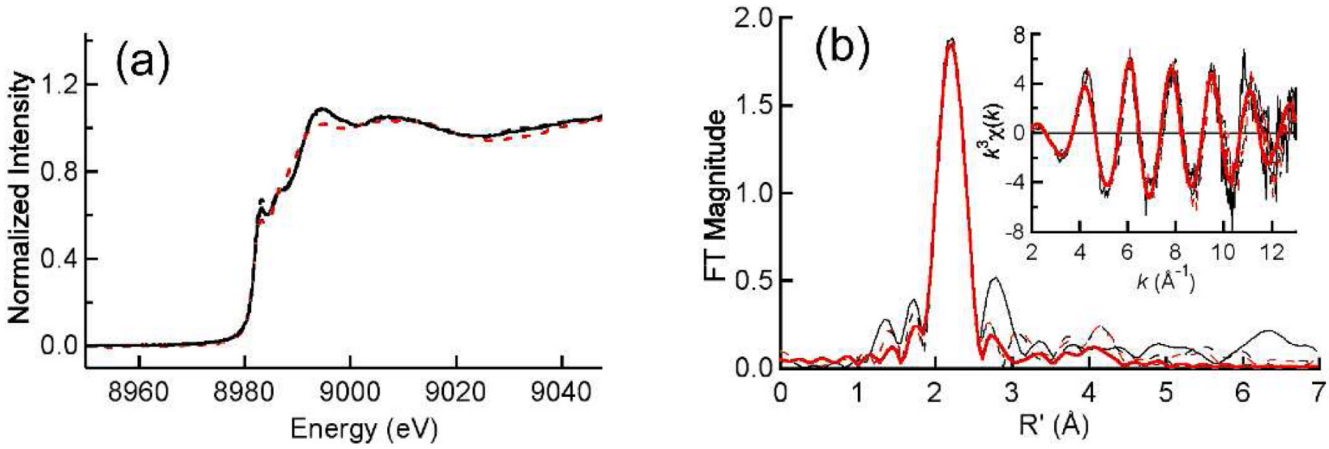


**Figure 2.**

Cu X-ray absorption edge (a), EXAFS (b, *inset*), and FT (b; $k$ = 2–13 Å$^{-1}$, $k^3$ weighting) for WT 1–106 (*black dashed*), L58C (*black solid*), and H61MeH (*red dashed*) CsoRs. The best fit of H61MeH CsoR based on a $CuS_2(imid)_1$ model is shown in *bold red* in (b). Fitting parameters are compiled in Table S2.

**Table 1**

Cu(I) affinities and allosteric coupling free energies for various *Mtb* CsoRsa[a]

| ***Mtb* CsoR** | **log $K_{Cu}$[b]** | **$\Delta G_c$ (kcal/mol)[d]** |
|---|---|---|
| Wild-type | 18.0 (±0.2) | 3.6 (±0.2) |
| WT 1–106 | 17.3 (±0.4) | 3.0 (±0.3) |
| L58C 1–106 | 17.3 (±0.3) | 3.0 (±0.4) |
| H61MeH | 18.4 (±0.2) | 0 (±0.4) |
| H61Thz | 16.7 (±0.1) | 0.5 (±0.2) |
| H61A | 14.5 (±0.3) | n.d.[e] |
| | 14.9 (±0.4)[c] | |
| E81A | 17.7 (±0.4) | 0.6 (±0.3) |
| E81Q | 17.6 (±0.5) | 1.9 (±0.3) |
| E81D | 17.8 (±0.4) | 1.5 (±0.3) |
| E81N | 17.3 (±0.4) | 1.6 (±0.4) |
| Y35F | 16.7 (±0.4) | 2.4 (±0.2) |
| Y35F/E81Q | 17.6 (±0.3) | 0.8 (±0.3)[f] |

[a] Conditions: 10mM HEPES, 0.2 M NaCl, pH 7.0, 25 °C, with 2 mM DTT present only in the DNA binding experiments.

[b] Determined by competition with the chromophoric complex $Cu^{I}(BCS)_2$ (log $\beta_2$=19.8) or

[c] $Cu^{I}(BCA)_2$ (log $\beta_2$=17.2) as described in Supplementary Methods.

[d] Determined using fluorescence anisotropy-based titrations like those shown in Figure S5, with detailed fitting parameters compiled in Table S1.

[e] n.d., not detected, $A_2 \leq 10^{10}\ M^{-1}$ under these conditions.

[f] $\delta = \Delta\Delta G_c^{Y35F/E81Q} - (\Delta\Delta G_c^{Y35F} + \Delta\Delta G_c^{E81Q}) = +0.1$ kcal/mol where $\Delta\Delta G_c^{mutant} = \Delta G_c^{mutant} - \Delta G_c^{WT}$.