Supplementary Information

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

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Supplemental Methods

***Mtb* CsoR plasmid construction, mutagenesis, protein expression and purification** Wild-type *Mtb* CsoR-pET3a plasmids were constructed as described previously.[1] Amino acid substitutions were introduced to the plasmid by Quickchange mutagenesis and confirmed by DNA sequencing. CsoR and mutants were expressed and purified using similar procedures as described before.[1] pET3a plasmids containing wild type or mutant CsoRs were transformed into *E. coli* BL21(DE3) and grown in LB media containing 100 mg/L ampicillin until OD600 reached 0.6-0.8. 0.4 mM IPTG was then added and cells were grown for additional 2 h before harvesting by low speed centrifugation. Cell pellets were suspended in 100 mL Buffer A (25 mM MES, 2 mM DTT, 1 mM EDTA, pH 5.8) and lysed by sonication. The lysate was centrifuged and 0.15% (v/v) polyethyleneimine (PEI) at pH5.8 was added to the supernatant to precipitate the nucleic acid. CsoR was then recovered by resuspending the PEI pellet in Buffer A containing 0.5 M NaCl. After centrifugation, the supernatant containing CsoR was subjected to $(NH_4)_2SO_4$ precipitation and the pellet was then resuspended in Buffer A and dialyzed against Buffer A containing 0.05 M NaCl. The sample was then purified by SP Fast Flow, Superdex-200 size exclusion and Q Fast Flow chromatography using similar conditions as previously decribed[1]. The resultant proteins were pooled and concentrated to less then 10 mL and dialyzed against Buffer S (10 mM HEPES, 0.2 M NaCl, pH 7.0) in an anaerobic Vacuum Atmospheres glovebox. The purity of the final products was estimated by visualization of Coomassie-stained 18% Tris-glycine SDS-PAGE gels to be ≥90%. Protein concentration was determined by either amino acid analysis carried out by the Texas A&M University Protein Chemistry Laboratory (PCL) or using a ε_{280}=1600 $M^{-1}cm^{-1}$. Free thiols were determined by the DTNB assay to be more then 90% of expected value. Less than 1% copper was detected by atomic absorption spectroscopy in all purified protein samples.

Unnatural amino acid incorporation by native chemical ligation C-terminal peptides of Mtb CsoR (residues 58-106, Leu58 substituted with Cys) were synthesized on a solid support resin (MBHA) using a CS Bio 4886 peptide synthesizer with incorporation of either 3-*N*-methylhistidine (MeH) with Boc-His(τ-Me)-OH (Bachem product no. A-2560; note that the Bachem refers to this as 1-*N*-methylhistidine) or thiazole (Thz) with Boc-3-(4-thiazolyl)-L-alanine (Chem-Impex, product no. 07378) at residue 61. *In situ* neutralization/BOC chemistry was used as described.[2] Deprotected peptide resins were cleaved from the support using HF/p-cresol, 95:5, at 0°C for one hour. Following ether extraction, the crude peptide was solvated in a 5% acetic acid solution and purified over a Vydac C18 RP-HPLC column using 0.1% TFA/acetonitrile as the eluting buffer. Analytical RP-HPLC and ESI-MS were used to verify 58-106 analogs to have the correct mass and acceptable purity. Desired fractions were then pooled and lyophilized to yield the dry 58-106 fragments.

The DNA sequence encoding the N-terminal peptide (residues 1-57) was cloned into the pTXB1 vector (New England Biolabs) between *Nde*I and *Spe*I restriction sites in frame to a C-terminal intein fusion domain to express as an intein fusion protein. T he fusion protein was expressed using *E. coli* BL21(DE3) as described above and cells were lysed in Buffer B (25 mM Tris, 0.5 mM NaCl, 2 mM TCEP, pH 8.0). Sodium 2-mercaptoethanesulfonate (MESNA) was added to the lysis supernatant which contains CsoR 1-57-intein to reach a concentration of 100 mM and stirred at 4 ^oC for 24 h to cleave the intein. The resultant CsoR 1-57 thioester was then purified

using an SP column and concentrated to a final volume of 2 mL to about 2 mM concentration in Buffer B with 5 mM MESNA. 1 mL of CsoR 1-57 containing the C-terminal thioester was used to dissolve 12 mg (about 1.8 mM in 1 mL) of the appropriate C-terminal peptide 58-106. 7 M urea, 10 mM TCEP and 100 mM MESNA were added to the ligation reaction and pH was adjusted to 7.5 using 1 M NaOH. The reaction was allowed to proceed at room temperature overnight. The ligated CsoR was then purified by a μRP (GE Healthcare) reverse phase column and refolded into Buffer S containing 2 mM DTT by stepwise increasing the pH. The final preparation was dialyzed in the anaerobic glovebox against Buffer S. The purity of the protein was estimated to be >90% by SDS-PAGE gel and the anticipated number of reduced Cys residues (3 expected) was confirmed by a standard DTNB assay.[3] The integrity of the resultant proteins was confirmed by MALDI-TOF mass spectrometry (H61MeH CsoR: 11311.0 D expected for 2-106/L58C, 11311.0 D observed; H61Thz CsoR: 11314.9 D expected for 2-106/L58C, 11313.7 D observed) as well as trypsin digestion followed by LC-MS/MS sequencing as shown in Figure S6 and described below.

In-gel trypsin digestion and LC-MS/MS Proteins were digested in-gel using a standard procedure[4]. Briefly, each excised gel spot was cut into small (less than 1 mm in each dimension) pieces, placed in a 0.6-mL microfuge tube, and covered with 100 μL of 25 mM ammonium bicarbonate in 50% acetonitrile (EMD Chemicals, Darmstadt, Germany) and sonicated for 10 min at room temperature to destain the proteins. The solution was removed from the tube and discarded. This destaining step was performed four times in all. G el pieces were vacuum-centrifuged for 15 min at 45° C to dryness. Any disulfide bonds were reduced by the addition of 100 μL of 10 mM DTT with incubation at 56° C for 60 min. After removing and discarding the solution, 100 μL of 55 mM iodoacetamide (IAM, Sigma-Aldrich) was added and the sample incubated in the dark at room temperature for 45 min to alkylate reduced cysteines. After removing and discarding the solution, 100 μL of 25 mM ammonium bicarbonate in 50% acetonitrile was added and the sample vortexed for 10 min. The solution was removed and discarded. Gels were again vacuum-centrifuged for 45 min at 45 °C to dryness. 60 μL of 12.5 mg/mL trypsin solution (Sigma-Aldrich, proteomics grade) in 25 mM ammonium bicarbonate was added to dried gel pieces. The gel pieces were rehydrated at room temperature for 5 min, then incubated at 37 ºC for 18 h. Following digestion, the liquid was removed from gel pieces and transferred to a new 0.6-mL microfuge tube. Two 40-μL aliquots of 5% formic acid in 50% acetonitrile were added to the gel pieces and vortexed for 10 min, then removed and added to the tube containing the digest solution. Trypsin digests (approximately 100 μL) were vacuum-centrifuged at 45° C to dryness (1.5 h), reconstituted in 10 μL of solvent A (see below) and transferred to autosampler vials for LC-MS/MS analysis.

Five μL of protein digest was loaded onto a 15 mm x 100 mm i.d. trapping column packed with 5 μm, 200 Å Magic C18AQ packing material (Microm BioResourses Inc., Auburn, CA). Peptides were eluted through a 150 mm x 75 mm i.d. analytical column with the same packing material, except 100 Å pore size, using a 30-minute gradient from 97% to 60% solvent A, 97:3:0.1 water/acetonitrile/formic acid (Solvent B is 0.1% formic acid in acetonitrile) at 250 nL/min using an Eksigent (Dublin, CA) nano-2DLC. From the end of the column, ions were electrosprayed directly into a ThermoFinnigan (San Jose, CA) LCQ Deca XP ion-trap mass spectrometer which recorded mass spectra and data-dependent tandem mass spectra of the peptide ions. In one experiment, and inclusion list with m/z values 896.6, 1195.0, and 1792.0

corresponding to the 4+, 3+, and 2+ ions of the peptide of interest. Tandem mass spectra were searched against the known amino acid sequence of CsoR without enzyme specificity (a "no enzyme" search) using a licensed copy of Mascot[5] for peptide identification. In addition to the automated database search results for the CsoR sequence, manual investigation of the data was used to identify candidate MS/MS spectra and assign identifications of the fragment ions for confirmation of the modified residues in CsoR.

BCS competition In a typical experiment, 10 to 15 μM CsoRs (monomer concentration) were mixed with 50, 250 and 500 μM BCS (or BCA for H61A CsoR) in buffer S anaerobically. 20 μM Cu(I) was then added to the mixture to a final volume of 250 μL. The mixtures were incubated in room temperature (~22 ^{o}C) for 3 h before the absorption spectrum of each sample was taken. $Cu^{I}(BCS)_2$ concentration in these samples was determined by comparing the absorbance at 483 nm with 20 μM $Cu^{I}(BCS)_2$ complex in the same buffer. The CsoR-Cu(I) affinity constant which assumes identical and independent sites on the CsoR oligomer (K_{Cu}) was then calculated using the following equation:[6,7]

$$K_{Cu} = \frac{\beta_2[\mathrm{Cu^{I}CsoR}][\mathrm{BCS}]^2}{[\mathrm{Cu^{I}(BCS)_2}][\mathrm{apoCsoR}]}$$

Here $\beta_2=10^{19.8}$ M^{-2} (or $10^{17.2}$ M^{-2} for BCA[8]) is the overall formation constant of $Cu^{I}(BCS)_2$ complex.[7] The average and standard deviation of the binding constants at three different BCS concentrations is reported in Table 1. For wild-type CsoR, K_{Cu} was further determined by titrating Cu(I) into a mixture of 19 μM CsoR and 60 μM BCS anaerobically in buffer S. The binding isotherm was fitted by Dynafit[9] using a simple competition model as shown in Figure S2.

Fluorescence anisotropy A 50 bp 5'-fluorescein (FL) labeled DNA (5'-FL-CGACTCCTTGG **GTAGCCCACCCCCAGTGGGGTGGGATAC**CATGAACGGGTG-3') containing the 28bp inverted repeat in bold letters was used. The double strand DNA was made by mixing it with 1.1 mol equiv of the unlabeled complement strand. The mixture was heated at 95 ^{o}C for 10 min and then cooled to room temperature. Formation of double strand DNA was further confirmed by native TBE polyacrylamide gel electrophoresis. To verify the stoichiometry, 10 μM unlabeled DNA was mixed with different concentrations of *Mtb* CsoR monomer up to 100 μM in buffer S with 2 mM DTT in room temperature. 100 μL of each mixture was loaded onto a Tricon Superdex 200 column (GE healthcare) on an Äkta-10 purifier. 80 μM CsoR monomer is able to saturate 10 μM DNA as shown in Figure S3, consistent with a model involving the binding of two tetramers to one DNA, similar to that established for *Bsu* CsoR.[10] A typical anisotropy experiment was carried out with 10 nM double strand DNA in buffer S with 2 mM DTT present as described.[10] Anisotropy was monitored by exciting the fluorescein at 490nm and the emission was monitored by using a 515 nm filter. With apo- or Cu(I)-bound CsoRs added, an average anisotropy of 5 measurements was reported for each addition. The resulting data were fitted to a stepwise model involving two non-dissociable tetramers binding to one DNA assuming a linear change in anisotropy with fractional saturation of the DNA.[9,10] The macroscopic binding constants as reported in Table S1 were calculated by $A_2=K_1K_2$, where K_1 and K_2 were the stepwise binding constants obtained from the fitting. The coupling free energy of each CsoR was calculated by $\Delta G_c = -RT\ln(A_2^{Cu}/A_2^{apo})$, where A_2^{apo} and A_2^{Cu} were the macroscopic DNA binding constants for apo- and Cu(I)-bound CsoRs, respectively.

Cu(I) X-ray absorption spectroscopy XAS samples, data collection and analysis were carried out as described before.[1,10] Essentially, wild-type as well as mutant CsoR samples were mixed with 0.8 monomer mol equiv of Cu(I) in 10 mM HEPES, 0.20 M NaCl, 30% (v/v) glycerol, pH 7.0, in an anaerobic environment and concentrated to ≈0.5~1.0 mM final protein concentration. Samples were loaded into standard XAS cuvettes or 5-well polycarbonate XAS cuvettes and immediately frozen in liquid N_2. XAS data were collected at Stanford Synchrotron Radiation Laboratory (SSRL) on beamline 9-3 or at the Canadian Light Source (CLS) on the HXMA beamline. EXAFS analysis was performed with EXAFSPAK software, using *ab initio* phase and amplitude functions computed with FEFF v7.2, according to standard procedures.[1,10]

Table S1 DNA binding affinities for various *Mtb* CsoRs[a]

Mtb CsoR		DNA binding affinity[b] A_2 (x10^{14} M^{-2})	r_0	r_∞
Wild-type	apo	3.7 (±1.0)	0.0975	0.1100
	Cu(I)	0.0079 (±0.0023)	0.1040	0.1170
WT 1-106	apo	17 (±7.8)	0.1100	0.1250
	Cu(I)	0.10 (±0.03)	0.1135	0.1285
L58C 1-106	apo	2.6 (±1.4)	0.1197	0.1290
	Cu(I)	0.015 (±0.01)	0.1160	0.1270
H61MeH	apo	0.67 (±0.23)	0.1170	0.1310
	Cu(I)	0.7 (±0.3)	0.1175	0.1300
H61Thz	apo	3.1 (±1.0)	0.1056	0.1210
	Cu(I)	1.4 (±0.4)	0.1092	0.1240
H61A	apo	n.d.[c]		
	Cu(I)	n.d.		
E81A	apo	14.7 (±5.7)	0.1000	0.1150
	Cu(I)	5.3 (±2.4)	0.0980	0.1143
E81Q	apo	26.6 (±10.0)	0.1085	0.1205
	Cu(I)	1.0 (±0.2)	0.1030	0.1145
E81D	apo	4.3 (±1.8)	0.1060	0.1190
	Cu(I)	0.36 (±0.2)	0.1050	0.1200
E81N	apo	2.8 (±1.1)	0.1075	0.1205
	Cu(I)	0.19 (±0.1)	0.1040	0.1170
Y35F	apo	14.3 (±3.6)	0.1000	0.1160
	Cu(I)	0.24 (±0.07)	0.1075	0.1230
Y35F/E81Q	apo	10 (±2.9)	0.1080	0.1240
	Cu(I)	1.7 (±0.8)	0.1117	0.1240

[a]Conditions: 10 mM HEPES, 0.2 M NaCl, pH 7.0, 25 ºC, with 2 mM DTT present only in the DNA binding experiments. [b]Determined using fluorescence anisotropy-based titrations like those shown in Figure S5. [e]n.d., not detected, $A_2 \leq 10^{10}$ M^{-1} under these conditions.

Table S2 XAS curve-fitting parameters for parent and ligated CsoR 1-106 derivatives[a]

Sample (k range) $\Delta k^3\chi$	Fit	Shell	R_{as} (Å)	σ_{as}^2 (Å^2)	ΔE_0 (eV)	f'[b]
WT CsoR 1-106	1	Cu-S_2	2.21	0.0017	-9.191	0.070
		Cu-N_1	1.96	0.0016	[-9.191][c]	
		Cu-C_1	[2.95]	[0.0032]	[-9.191]	
		Cu-C_1	[3.00]	[0.0033]	[-9.191]	
		Cu-N_1	[4.13]	[0.0020]	[-9.191]	
		Cu-C_1	[4.17]	[0.0020]	[-9.191]	
L58C 1-106	1	Cu-S_2	2.22	0.0022	-8.654	0.067
		Cu-N_1	2.03	0.0016	[-8.654]	
		Cu-C_1	[3.01]	[0.0032]	[-8.654]	
		Cu-C_1	[3.06]	[0.0033]	[-8.654]	
		Cu-N_1	[4.19]	[0.0020]	[-8.654]	
		Cu-C_1	[4.23]	[0.0020]	[-8.654]	
H61MeH	1	Cu-S_2	2.25	0.0014	-2.850	0.89
		Cu-N_1	2.04	0.0016	[-2.850]	
		Cu-C_1	[3.02]	[0.0032]	[-2.850]	
		Cu-C_1	[3.07]	[0.0033]	[-2.850]	
		Cu-N_1	[4.20]	[0.0020]	[-2.850]	
		Cu-C_1	[4.24]	[0.0020]	[-2.850]	

[a] Shell is the chemical unit defined for the multiple scattering calculation. Subscripts denote the number of scatterers per metal. R_{as} is the metal-scatterer distance. σ_{as}^2 is a mean square deviation in R_{as}. ΔE_0 is the shift in E_0 for the theoretical scattering functions.

[b] f' is a normalized error (chi-squared):

$$f' = \frac{\left\{\sum_i \left[k^3\left(\chi_i^{obs} - \chi_i^{calc}\right)\right]^2 / N\right\}^{1/2}}{\left[\left(k^3\chi^{obs}\right)_{max} - \left(k^3\chi^{obs}\right)_{min}\right]}$$

[c] Numbers in square brackets were constrained to be either a multiple of the above value (σ_{as}^2) or to maintain a constant difference from the above value (R_{as}, ΔE_0).

Table S3 XAS curve-fitting parameters for conventional site-directed mutants of CsoR[a]

CsoR	Cu—S			Cu—N/O			
	N	*R*	σ^2	*N*	*R*	σ^2	*F*
WT	2	2.209(3)	0.0027(1)	1	2.113(7)	0.0028(1)	0.235
Y35F	2	2.199(4)	0.0018(2)	1	2.114(1)	0.0018(2)	0.287
E81A	2	2.206(2)	0.0020(1)	1	2.110(2)	0.0021(1)	0.322
E81Q	2	2.212(3)	0.0033(2)	1	2.113(3)	0.0034(2)	0.384
Y35F-E81Q	2	2.213(7)	0.0045(3)	1	2.099(21)	0.0047(3)	0.478

[a]Coordination numbers, *N*, interatomic distances *R* (Å) and Debye-Waller factors σ^2 (Å^2). Values in parentheses are the estimated standard deviations (precisions) obtained from the diagonal elements of the covariance matrix. The accuracies will be much greater than these values, and are generally accepted to be ±0.02 Å for bond-lengths and ±20% for coordination numbers and Debye-Waller factors. The fit-error function *F* is defined as $F = \sqrt{\sum k^6 \left(\chi_{calc} - \chi_{expt}\right)^2 / \sum \chi_{expt}^2}$, where the summations are over all data points included in the refinement.

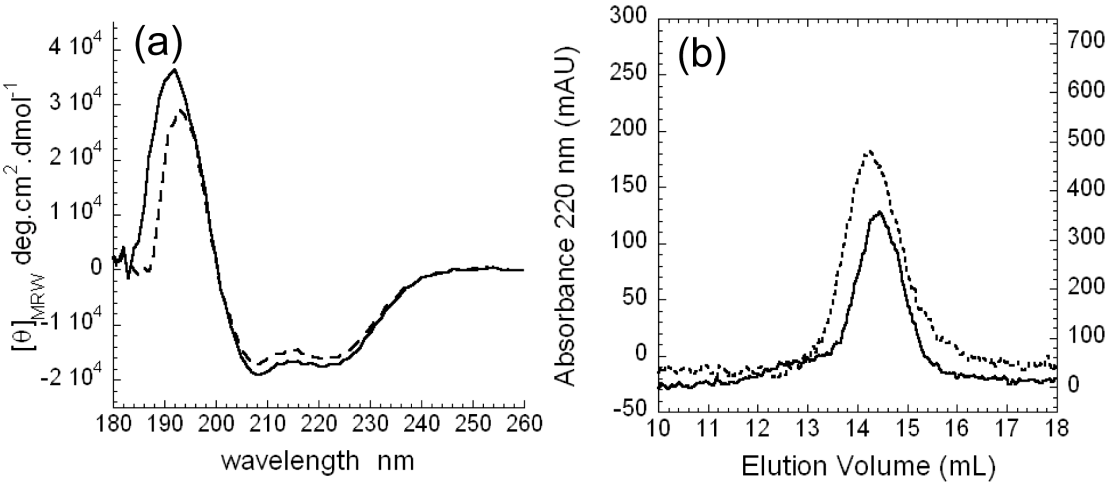

Figure S1 (a) Far UV-CD spectra plotted as mean residue ellipticity, $[\Theta]_{MRW}$ of C36A (*solid* curve) and H61A (*dashed* curve) CsoRs. Apo C36A CsoR binds the *cso* operator with an affinity identical to that wild-type CsoR, A_2=3.1 (±0.9) x 10^{14} M^{-2} while H61A CsoR is inactive in this assay, $A_2 \leq 10^{10}$ M^{-2} (Table S1). (b) Chromatrography of H61A CsoR (*dashed* line) and WT CsoR (*solid* line) on a Superdex 200 column. These data reveal that H61A CsoR adopts a well-folded α-helical tetramer. Conditions: 10 mM sodium phosphate, 0.2 M NaF, pH 7.0, 25 ºC.

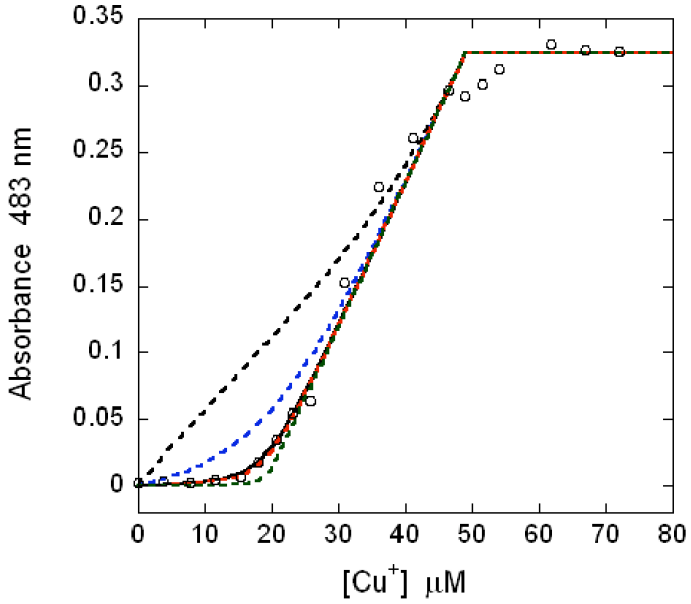

Figure S2 Wild-type CsoR-BCS competition titration. 19 μM CsoR (monomer) and 60 μM BCS was mixed with different concentrations of CuCl in an anaerobic chamber (10 mM HEPES, 0.2 M NaCl, 25 °C). Log K_{Cu} is fitted by a simple competition model to be 17.9 ± 0.4 as shown by the black solid line, consistent with the affinity determined using a different experimental strategy as described in Supplementary Methods. The dash lines represent simulations with log K_{Cu}=16 (*black*), 17 (*blue*), 18 (*red*) and 19 (*green*) under the same conditions.

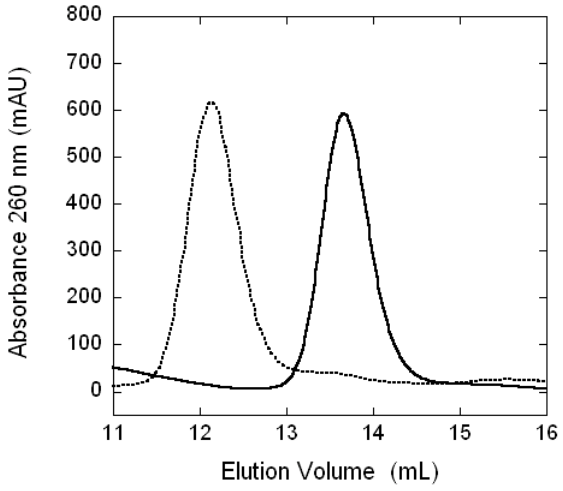

Figure S3 Two CsoR tetramers (8 monomers) bind to one 50 bp CsoO-containing DNA. Elution profile obtained with 10 μM DNA only (*solid line*) and 10 μM DNA mixed with 80 μM CsoR monomer from a Superdex 200 column as monitored by absorption at 260nm. Analogous observation was found in *Bsu* CsoR.[10] Conditions: 10 mM HEPES, 0.2 M NaCl, 2 mM DTT, pH 7.0, ambient temperature.

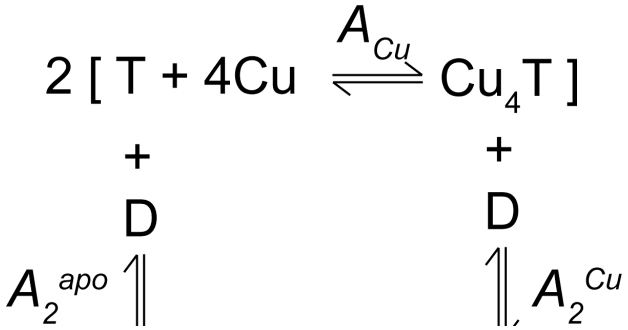

Figure S4 Coupled equilibria of *Mtb* CsoR tetramer (T)-DNA (D) complexes as a function of Cu. $A_{Cu}= K_{Cu}^4$, where K_{Cu} is the microscopic binding constant to each protomer as reported in Table 1, main text. A_{Cu} represents the macroscopic binding constant of four Cu(I) ions bound to a CsoR tetramer. A_2^{apo} and A_2^{Cu} represent the macroscopic binding constants of two CsoR tetramers binding to one DNA in absence or presence of Cu(I), respectively.

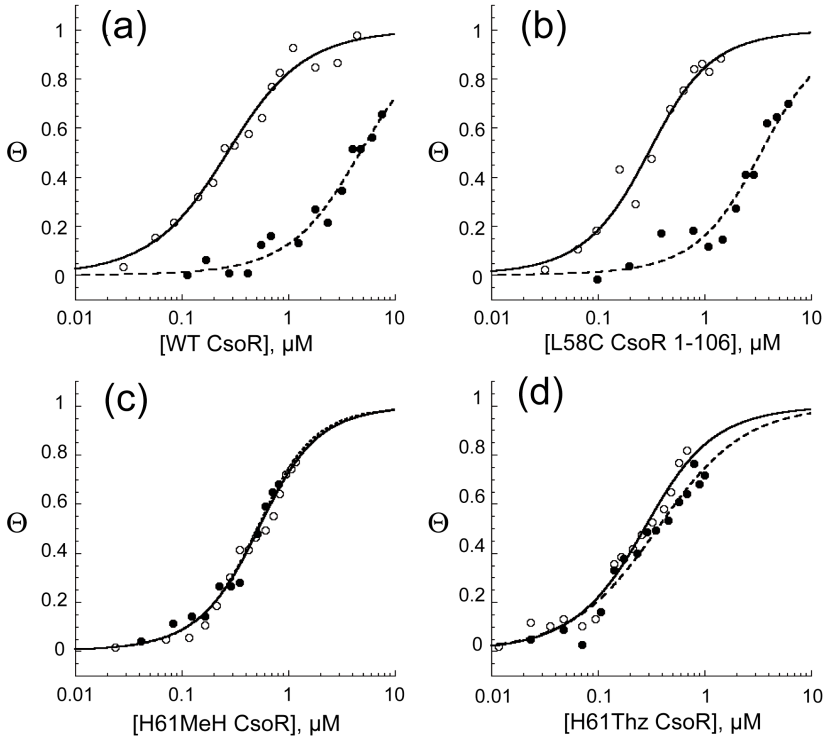

Figure S5. Normalized fluorescence anisotropy-based DNA binding isotherms for (a) WT, (b) parent L58C, (c) H61MeH and (d) H61Thz 1-106 CsoRs (expressed in CsoR monomer concentration) acquired in the absence (open symbols) and presence (filled symbols) of saturating Cu(I). Note that H61MeH and H61Thz CsoRs also contain the L58C substitution. Continuous lines through the data represent the best fit using a stepwise two tetramer DNA binding model with the fitting parameters given in Table S1. Conditions: 10 mM HEPES, 0.2 M NaCl, 2 mM DTT, pH 7.0, 25 ^{o}C.

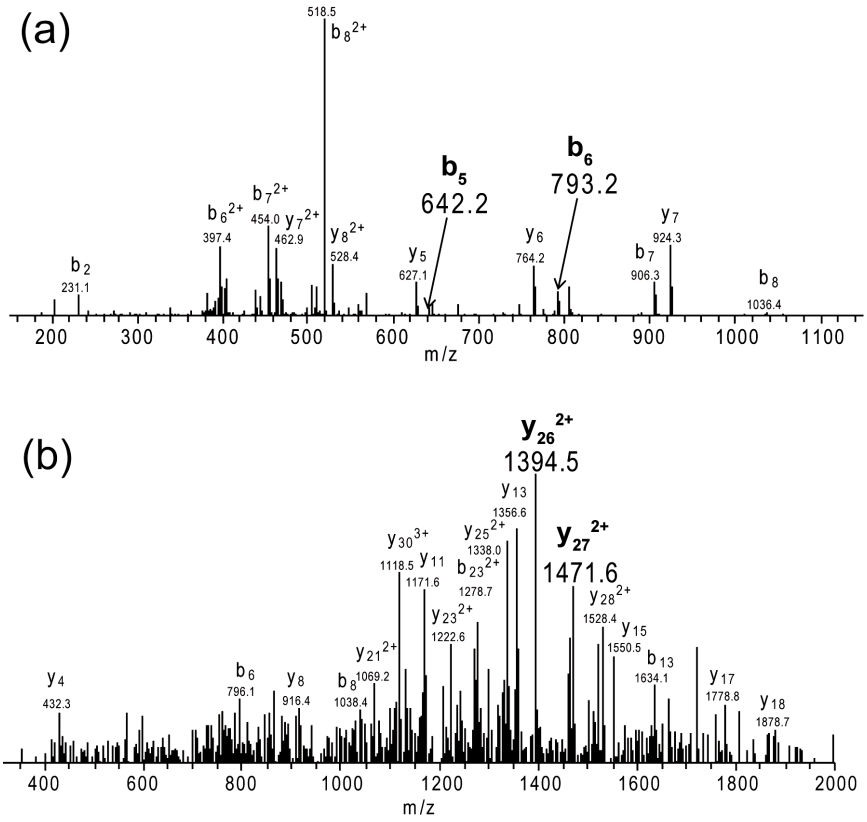

Figure S6 MS/MS results of the ligated CsoRs. (a) Tandem mass spectrum of doubly-charged peptide ^{56}VMCHN(MeH)LET^{64} from precursor ion m/z 578.1 with several identified fragment ions labeled. Fragment ions b_5 and b_6 confirm the location of the methyl group on His61. The underlined C corresponds to the L58C substitution which also defines the ligation junction. (b) Tandem mass spectrum of triply-charged peptide ^{56}VMCHN(Thz)LETCFSTAVLDGHGQA AIEELIDAVK^{87} from precursor ion m/z 1195.4 with several identified fragment ions labeled. Fragment ions y_{26} and y_{27} confirm the location of the thiazole substitution in place of His61.

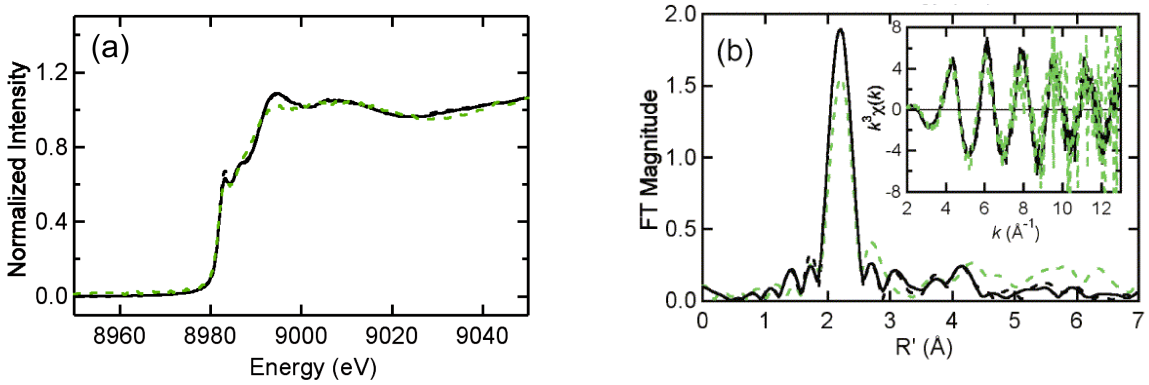

Figure S7 Cu X-ray absorption edge (a), EXAFS (b**,** *inset*), and Fourier transforms (b; k^3 weighted, k=2-13 Å^{-1}) for Cu(I)-bound WT 1-106 (*dashed black*), L58C 1-106 (*solid black*) and H61Thz (*dashed green*) CsoRs. The spectra for WT 1-106 and L58C 1-106 CsoRs are the same as those in Figure 2, main text, for comparison.

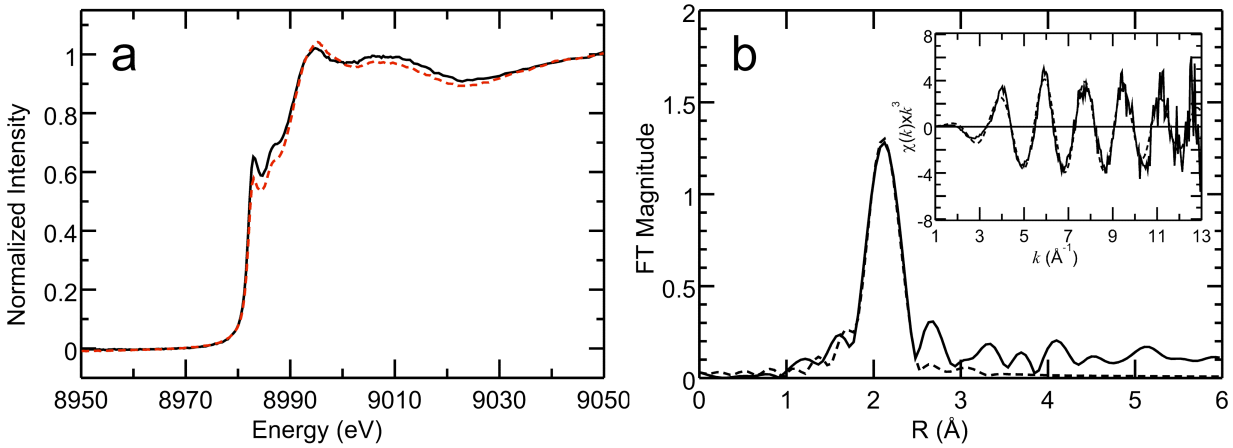

Figure S8 X-ray absorption spectroscopy (XAS) of Cu(I)-bound Y35F/E81Q CsoR. (a) Copper K-edge X-ray absorption near-edge spectra of Y35F/E81Q (*solid line*) and wild-type (*red broken line*) CsoRs. (b) EXAFS Fourier transforms (Cu-S phase-corrected) and EXAFS spectra (*inset*) with best fits (*broken lines*). Parameters that derive from the fitted curves are complied in Table S3.

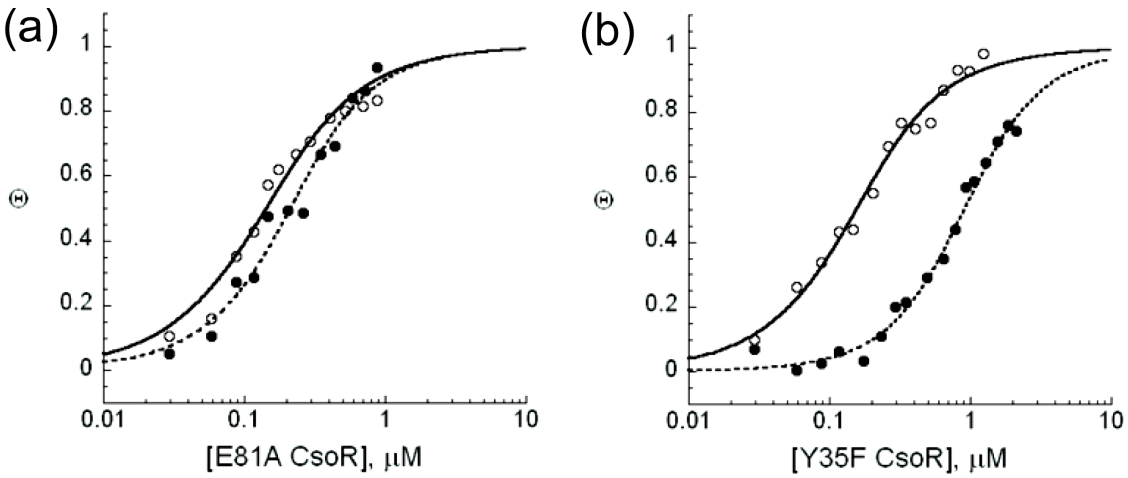

Figure S9 Normalized fluorescence anisotropy-based DNA binding isotherms of E81A (a) and Y35F (b) CsoRs (expressed in CsoR monomer concentration) acquired in the absence (●) and presence (○) of stoichiometric Cu(I). Curves represent the best fit using a stepwise two tetramer DNA binding model with the fitting parameters given in Table S1. Conditions: 10 mM HEPES, 0.2 M NaCl, 2 mM DTT, pH 7.0, 25 ^{o}C.

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