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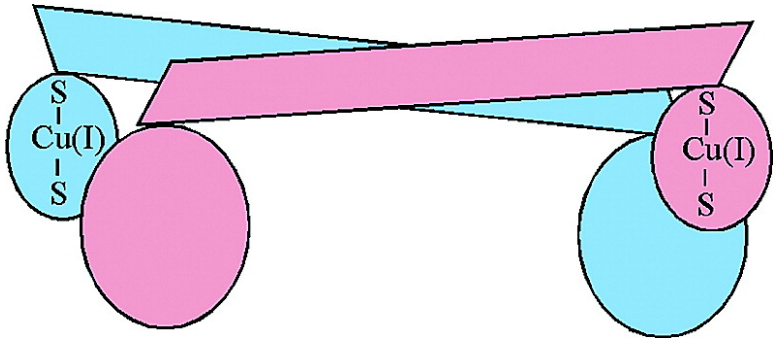

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An Atypical Linear Cu(I)−S_2 Center Constitutes the High-Affinity Metal-Sensing Site in the CueR Metalloregulatory Protein

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The CueR protein is a copper-responsive genetic switch that regulates transcription of genes encoding the primary copper-export system in *E. coli*, CopA and CueO.[1−4] CueR exhibits a zeptomolar (10^{-21} M) sensitivity to free Cu(I),[5] reminiscent of metal sensitivity in other MerR-family proteins such as Hg-MerR[6,7] and Zn-ZntR.[8−10] A recent crystal structure of CueR[5] reveals a linear CuS_2 geometry for one of the two copper binding sites in the CueR dimer; however, other possible sites were disordered, leaving open the questions of copper stoichiometry, geometry, and oxidation states in the key metal-activated state. In particular, this raises the possibility that the digonal Cu site could be an artifact of crystallization. Here, we present direct evidence that only two cysteine residues constitute the high-affinity coordination environment in CueR, that the copper is present as Cu(I), and that both sites in the CueR dimer have linear S−Cu(I)−S coordination in solution. This geometry is rare in protein coordination chemistry but is well suited for a receptor that must distinguish Cu(I) from other essential metal ions.

Unlike MerR and ZntR, CueR has four cysteines as potential metal-binding ligands, two of which are conserved across the metal-sensing MerR family.[5] To investigate the roles of these cysteines, three CueR proteins were constructed with either a point mutation at one of the conserved cysteines (Cys112Ser and Cys120Ser) or a double mutation in the C-terminal cysteine pairs (Cys129Ser/Cys130Ser). Dialysis against Cu(I) under N_2 showed that wild-type (WT) CueR can bind up to 3 equiv of copper per monomer; however, addition of a Cu(I)-competitor such as dithiothreitol (DTT) removed weakly bound metal ions and left only 1 equiv of bound copper.[11] This copper is likely to interact with C112 and C120, because mutation of either residue abolished tight Cu binding, but retained the ability to bind 2 equiv of Cu in the absence of DTT.[11] The C-terminal double mutant retains tight binding of only 1 equiv of copper in the presence of DTT, suggesting that the weakly bound copper ions are associated with C129 and C130.

The association of copper with the cysteines in solution was further confirmed by chemical modification of CueR with AMS, a reagent specific for reduced thiols. The mobility of the metal-free WT-CueR in SDS-PAGE shifted upon modification of four cysteine residues by AMS (Figure 1, lanes 1 and 2).[11] Copper binding blocked modification of most of the cysteines (lane 3), most likely due to Cu−S bond formation, because addition of high concentrations of a stringent Cu(I) competitor, bathocuproine sulfonate (BCS),[12] restored thiol modification by AMS (lane 4). The C129S/C130S mutant gave a similar mobility shift pattern for AMS-modification of the remaining two cysteines (lanes 5−8), demonstrating that mutation of C129/C130 does not interfere significantly with formation of the tight metal-binding site.

Direct evidence that the tight copper-binding site contains copper in the +1 oxidation state is seen in the XANES spectrum of

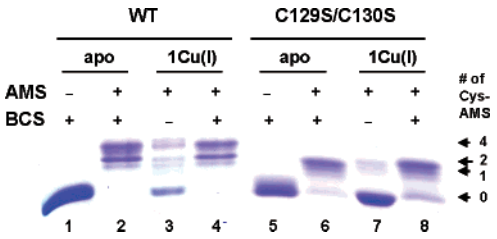

Figure 1. SDS-PAGE of thiol modified WT and double mutant CueR. [CueR]:[AMS]:[BCS] = 1:8:400. The number of AMS-modified cysteines corresponding to each protein band is shown on the right.

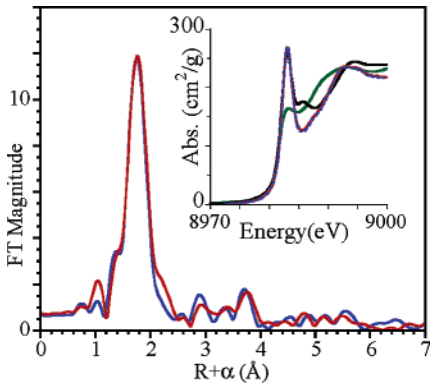

Figure 2. X-ray absorption spectra for WT (red) and C129S/C130S (blue) CueR with 1 equiv of Cu(I) per monomer. The Fourier transforms are dominated by an intense Cu−S peak with a weaker multiple-scattering peak at higher *R*. XANES spectra (inset) for both proteins are similar to digonal $[Cu(I)(SC_{10}H_{13})_2]^-$ (black) and distinct from trigonal $[Cu(I)(SPh)_3]^{2-}$ (green) (mutant shown as dashed line to enhance visibility of WT spectrum; model data from ref 13).

WT-CueR with 1 equiv of copper bound per monomer (Figure 2, inset). The intense 1s → 4p transition at 8983 eV[11] is diagnostic of Cu(I) in an approximately linear geometry[14] and is very similar to the spectrum for two-coordinate $[Cu(I)(SC_{10}H_{13})_2]^-$ but distinct from the spectrum for three-coordinate $[Cu(I)(SC_6H_5)_3]^{2-}$ (Figure 2, inset).[13]

The linear bis-cysteine ligation is confirmed by the EXAFS. The Fourier transform of the EXAFS (Figure 2) shows a prominent peak at $R + \alpha = 1.8$ Å that can be well modeled by a single Cu−S shell with two sulfurs at 2.14 Å from the Cu. This distance is typical of two-coordinate Cu(I)-thiolate complexes but is ∼0.1 Å shorter than those in trigonal complexes.[15] The small peak at ∼3.7 Å could, in principle, be due to a Cu−Cu interaction. However, if this were due to an unbridged Cu−Cu pair, the EXAFS would not be detectable at such a long distance; if it were due to a bridged pair, the apparent Cu−Cu distance would require an unprecedented linear thiolate bridge. Instead, we attribute the 3.7 Å peak to first-shell multiple scattering (MS),[16,17] involving Cu → S_1 → Cu → S_2 → Cu scattering. MS is extremely sensitive to geometry and is only

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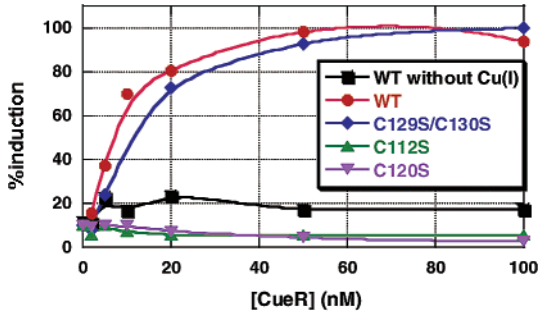

Figure 3. Runoff transcription assays of CueR and its mutants in response of CueR and Cu(I), showing that both C112 and C120 are required for Cu(I) sensing by CueR.

expected to make a significant contribution to the EXAFS for a nearly linear coordination environment. We find that first-shell MS can only reproduce the high-*R* features with a S−Cu−S angle of >175° (Table S2). Smaller angles give unreasonable distances and Debye−Waller factors for the MS pathways. There is, in addition, a weak feature at $R + \alpha = 2.9$ Å that can be modeled as Cu−C scattering at ca. 3.2 Å (Table S2). This is not as well defined as the Cu−S scattering, but nevertheless gives a reasonable Cu−S−C angle of 106−109°. This coordination geometry is in sharp contrast with those reported for MerR[18] and ZntR[5] in which metal ion binds to three cysteines across the dimer interface.

The coordination environment of the tightly bound metal site in CueR is independent of the nonconserved cysteines. Both the EXAFS and the XANES spectra of C129S/C130S-CueR with 1 equiv of Cu(I)/monomer are indistinguishable from those for WT-CueR (Figure 2). Addition of substoichiometric Cu(I) to either WT or the double mutant CueR has a similarly negligible effect on structure (Figures S1 and S2, Table S2). If the average Cu structure changed when one versus two Cu sites were occupied, or if the Cu structure was affected by C129 and C130, these differences would be readily detectable by X-ray absorption.[19] The absence of detectable change demonstrates that the digonal CuS_2 geometry is present for both Cu sites in the CueR dimer in solution.

Runoff transcription assays[11] (Figure 3) can be used to identify the transcriptional active site. Addition of Cu(I) increased transcription induction dramatically, and the transcript level reached saturation at ∼50 nM of WT-CueR. This formally corresponds to ∼60 copies of the protein in *E. coli* cells under normal growth conditions. These results are consistent with in vivo studies in which the *copA* gene transcription depends on both the CueR protein and the extracellular copper ions.[1−4] The induction profile for C129S/C130S-CueR is similar to that for WT-CueR, indicating that these cysteines are not essential for the metal-responsive transcriptional activation. In contrast, mutation of either C112 or C120 abolished completely the dose-responsive increase of induction, demonstrating that both C112 and C120 are required for high-affinity copper sensing by CueR under physiologically relevant conditions and that the tight-binding Cu site must be occupied for transcriptional activity.

In this work, we have demonstrated that the copper sensor CueR tightly binds 1 equiv of Cu(I) via a digonal S−Cu−S center in solution. Both C112 and C120 ligate to this functional copper center, while C129 and C130 do not. This coordination geometry corroborates the solid-state structure of CueR[5] but establishes for the first time that Cu(I) binding to C112/C120 alone affords the transcriptional active form of the protein. These data are consistent with in vivo mutagenesis studies.[20] Interestingly, a linear S−Cu(I)−S center in biology has so far only been demonstrated in the solvent-accessible site of metal trafficking proteins such as Cu-transporting ATPase and copper chaperones.[21−23] In these cases, the two-coordinate site is proposed to facilitate rapid Cu transfer via a three-coordinated intermediate formed between the chaperone and its target protein.[13,24,25] It is possible that the C-terminal fragment containing C129 and C130, which is disordered in the crystal structure of CueR,[5] can chauffeur copper between the external source and the buried sensing site. The fact that zeptomolar Cu(I) sensitivity of CueR arises from a surprisingly simple linear two-coordinate geometry leads us to suggest that the chelate effect and secondary ligand interactions play important roles in metal recognition.

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Supporting Information Available: Experimental procedures, Cu(I) dialysis results, plots of X-ray absorption data, and complete EXAFS fitting results (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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