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C-terminal Cysteines of CueR Act as Auxiliary Metal Site Ligands upon Hg$^{2+}$ Binding—A Mechanism To Prevent Transcriptional Activation by Divalent Metal Ions?

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Intracellular Cu binding, the activator conformation is stabilized by Escherichia coli CueR responds more to Zn or Au structure than to other function- with a high is controlled by the transcription haltion at pH 8.0, the metal bindings ite-structure of CueR (bottom). Conserved cysteines from both sites. We hypothesize that the C-ter-

CueR proteins from various bacteria contain two additional well conserved cysteines at the C-terminal, disordered segment of the protein (Figure 1).[2] Crystal structures of the activator

The CueR metalloregulatory protein controls cellular copper homeostasis by activating the transcription of cueO and copA genes in prokaryotes and some eukaryotes.[1] CueR responds to Cu²⁺, Ag²⁺ and Au⁺, but not to the divalent ions Hg²⁺ or Zn²⁺.[3] SC-XRD studies on Escherichia coli CueR and EXAFS in solution revealed that the inducer metal ions are coordinated by C112 and C120 residues in a linear, bis-cysteinate fashion.[2, 3] These two cysteines are essential to the protein function, as shown by mutation studies (C112S and/or C120S) both in vitro[3] and in vivo.[4]

CueR proteins from various bacteria contain two additional well conserved cysteines at the C-terminal, disordered segment of the protein (Figure 1).[2] Crystal structures of the activator

and the repressor forms of the DNA-bound CueR dimer suggest that a two-turn helix between the metal binding loop and the CCHH motif may have a key role in the protein function.[5] Upon Ag⁺ binding, the activator conformation is stabilized by the docking of the C-terminal helix (via residues I122, I123, L126) into an opened, hydrophobic pocket, formed by residues of the dimerization helix and the DNA-binding domain. This results in a small “scissoring” movement and bending of the DNA chain allowing the transcription to be carried out by the RNA polymerase. The allosteric role of the C-terminal helix was confirmed by constructing the Cu⁺-independent constitutive activator (T84V/N125L/C112S/C120S) and the constitutive repressor (truncation from I122) mutants of CueR.[5]

Several representative examples can be found in the literature where non-cognate metal ions bind to a metalloprotein with the same or even higher affinity than the inducer metal ion. However, despite the high affinity binding of non-cognate metal ions, they cannot trigger the functional structural change of the protein, because the coordination number or geometry differ.[6–9] Thus, studying the interaction of metallo-

Figure 1. Structure of CueR (E.coli) (PDB id.: 1Q05-modified) showing the potential metal binding sites (top). Sequence alignment of CueR proteins from various organisms (bottom). Conserved cysteine residues are highlighted in yellow.
However, according to model peptide studies and QM/MM calculations, Hg ions may be coordinated even more efficiently. Moreover, Hg is also able to bind to a C-terminal CCHH motif and therefore coordination of Hg ion by the CCHH motif is also highly probable.

With the present work we aim to explore the role of the C-terminal CCHH motif with a particular focus on the binding of Hg to CueR. To achieve this, we studied the Hg-interaction of E. coli CueR and its truncated variant, lacking seven C-terminal residues (including the CCHH motif), ΔC7-CueR. The integrity of this variant was confirmed by CD spectroscopy and electrophoretic mobility shift assay, see Figure S3.

A series of ESI-MS spectra were recorded with the two protein variants, see Figures 2, S4 and S5. The disappearance of the signals of the apo-form in the presence of 1.0 equivalent of Hg implies that Hg ions display high affinity to both proteins. The spectra obtained at twofold Hg-excess per protein clearly demonstrate the availability of two binding sites for Hg ions in the Wild-type (WT) CueR. These are most likely the metal ion binding loop formed by C112 and C120, and the C-terminal CCHH motif. Participation of the latter CCHH sequence motif in Hg binding is supported by the lack of signals corresponding to a Hg-CueR complex, even at twofold Hg-excess over the truncated protein. Both the Hg-CueR and Hg-CueR species are observed at 1.0 equivalent Hg, suggesting that there is no significant difference in the Hg-binding affinities of the two sites.

Hg-perturbed angular correlation (PAC) spectroscopy was used to elucidate the metal site structures and dynamics at the nanosecond timescale, see Figure 3 and Supporting Information Figure S6. At pH 6.0 and Hg-CueR of 0.2 and 1.0, the signals agree well with a HgS coordination geometry, that is, coordination of Hg by two cysteinates. This is also the case at Hg-CueR of 2.0, although a slightly larger linewidth is observed, in particular for the first peak at around 4 rad ns⁻¹. This line broadening presumably reflects the occupation of two HgS sites, and it can originate either from minor differences in structure of the two sites, or from metal site dynamics at the nanosecond time scale becoming more pronounced upon binding of the second Hg (Figure 3).

The spectrum recorded with 0.2 equivalent of Hg per CueR at pH 8.0 is more complex than at pH 6.0. Qualitatively, the first peak is shifted to slightly lower frequency and exhibits considerable broadening, and the second peak (ca. 2.8 rad ns⁻¹) is significantly attenuated, to the extent that it barely rises above the noise level. A reliable analysis of the data requires the inclusion of two nuclear quadrupole interactions (NQIs). One of these NQIs is very similar to that observed in the spectra at pH 6.0, most likely reflecting a HgS structure. The other NQI has a higher asymmetry parameter and a lower frequency, see Table S1, indicating a higher coordination number than 2. The lower frequency agrees well with an ideal trigonal planar HgS structure, but the relatively high asymmetry parameter rules out this possibility. However, in the simple angular overlap model (AOM), a T-shaped HgS coordination geometry gives the same frequency as a trigonal planar structure, but an asymmetry parameter of 1. Thus, a HgS structure in between trigonal planar and T-shaped, with the third ligand in a slightly longer Hg–S distance seems to be a plausible structural interpretation of the low frequency signal. It is also

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**Figure 2.** Deconvoluted native ESI-MS spectra of the WT and truncated CueR in the absence and presence of 0.2, 0.5, 1.0 and 2.0 equivalents of Hg ions. Individual samples contained 20 μm protein in a 10 mM NH₄HCO₃ buffer, 0.5 mM TCEP, pH 7.5.

**Figure 3.** Experimental (grey) and fitted (black) Hg PAC spectra of WT and truncated CueR in the presence of DNA with 0.2, 1.0 and 2.0 equivalents of Hg. Top left: WT at pH 6.0; top right: WT at pH 8.0 C₇–CueR = 12 μM, 0.5 equiv. DNA, and bottom: ΔC7-CueR at pH 8.0 C₇–CueR = 8.4 μM, 0.5 equiv. DNA.
possible that the PAC data reflect a trigonal planar HgS$_2$N structure, with a histidine coordinating, as this would give an asymmetry parameter different from zero. However, this seems less likely, given the thiophilicity of Hg$^{II}$, and the UV absorption data, vide infra. Finally, it is conceivable that the spectrum reflects intermediate (nanosecond) exchange between HgS$_2$ and HgS$_3$ structures. Notice that this entails a flip of principal axis of the electric field gradient tensor, which has $V_{zz}$ along the axis of HgS$_2$ but perpendicular to the HgS$_3$ plane, and therefore the asymmetry parameter will depend on the dynamics in a non-trivial manner. It cannot be excluded that the data recorded at 1.0 equivalent of Hg$^{II}$ also contain signals reflecting both of these species, but the reduced chi-square does not improve significantly upon including a second NQI. Consequently, we have only included the high frequency NQI (HgS$_3$) in the analysis. For the experiment with 2.0 equivalents of Hg$^{II}$ the signal may be satisfactorily fitted with just one (high frequency) NQI, presumably reflecting HgS$_2$ structure for both Hg$^{II}$ bound to CueR (Figure 3).

Most interestingly, the $^{199}$Hg PAC spectrum recorded at pH 8.0 with 0.2 equivalents Hg$^{II}$ for ΔC7-CueR exhibits a signal reflecting only HgS$_3$ structure (Figure 3). The fact that the ΔC7-CueR Hg$^{II}$ site exhibits a HgS$_3$ structure strongly supports the interpretation presented above for the WT CueR: if HgS$_3$ is formed by occupation of the functional site, a third thiolate is recruited from the CCHH motif, or vice versa, Hg$^{II}$ binds to the CCHH motif and recruits one of the cysteines from the functional binding site. With 2.0 equivalents of Hg$^{II}$ per ΔC7-CueR at pH 8.0, the signal changes as compared to experiments with ≤ 1 equivalent Hg$^{II}$, presumably because the functional metal site is filled, and the additional Hg$^{II}$ accommodates a coordination geometry other than linear HgS$_2$ due to weak or non-specific Hg$^{II}$ adducts. This agrees well with the ESI-MS data, where no Hg$_2$ΔC7-CueR was observed. Thus it is likely that the signal includes more than one NQI. Surprisingly, the signal shifts to slightly higher frequency, which is difficult to account for, except if a positive charge appears in the equatorial plane of HgS$_3$, vide infra.

To further characterize the metal site coordination geometries, we applied UV absorption spectroscopy (Figure 4). Hg$^{II}$-thiolate complexes possess characteristic charge transfer (CT) bands in the region of 230–300 nm. Moreover, features of the absorption spectrum reflect the coordination geometry of the complexes. Using Hg(SEt)$_2$ and [Et$_4$N][Hg(SBut)$_2$]$_2$ model compounds, the UV-absorption spectra of linearly and trigonal planar coordinated Hg$^{II}$, respectively, were characterized.[24] Linearly coordinated Hg$^{II}$-thiolate species display a transition at around 230 nm.[22] The increase of the coordination number shifts the absorption bands towards longer wavelengths.[23, 25] The spectrum of a trigonal Hg$^{II}$-thiolate complex has a characteristic absorption maximum at 245 nm with a distinct shoulder at around 290 nm.[22] Qualitatively, the absorption difference spectra at sub-equimolar Hg$^{II}$:WT CueR ratios exhibit a characteristic absorption at around 290 nm reflecting the presence of HgS$_3$ structure (Figure 4), in agreement with the PAC data, vide supra. The PAC data indicate 40% HgS$_2$ and 60% HgS$_3$ at 0.2 equivalents Hg$^{II}$. We used the recorded spectrum with 2.0 equivalents Hg$^{II}$ per WT CueR (Figure 4A) to determine the molar absorption of the HgS$_2$ species (green curve in Figure 4C). Next, we predicted the pure HgS$_2$ molar absorption spectrum (Figure 4C, purple curve) by assuming that the experimentally determined spectrum is given by 0.6 HgS$_2$ + 0.4 HgS$_3$. The UV absorption spectra derived in this manner for HgS$_2$ and HgS$_3$ agreement well with those reported in the literature,[23] strongly supporting the interpretation of the PAC data presented above. We present molar absorption data at selected wavelength values in Table 1. The UV absorption spectra recorded for ΔC7-CueR exclusively exhibit the signature of HgS$_3$. 

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**Figure 4.** UV absorption difference spectra of WT CueR (A) and ΔC7-CueR (B) titrated with Hg$^{II}$ ions (0.1–2.4 equivalents). Spectra recorded in the presence of 1.0 and 2.0 equivalents of Hg$^{II}$ are shown with dashed lines. pH 7.5, $c_{WT\text{CueR}} = 14\ \mu\text{M}$, $c_{ΔC7\text{CueR}} = 12\ \mu\text{M}$. (C) Estimated molar absorbance for the HgS$_2$ and HgS$_3$ species derived from the WT CueR UV absorption spectrum recorded with 2.0 equiv. Hg$^{II}$ and 0.2 equiv. Hg$^{II}$ combined with the relative population of the two species derived from $^{199}$Hg PAC data, see the text for details.
The increased availability of deprotonated cysteines is formed with inorganic ions bound to WT CueR at pH 8.0. Binding of structures similar geometrical rearrangement was observed when the two thiolates as bridging ligands. Interestingly, this agrees with the unexpectedly high frequency observed by PAC spectroscopy, which can be explained by the presence of a positive charge (the second Hg²⁺) in the HgS₂ structure, vide supra. The fact that the species with two Hg²⁺ bound per CueR monomer is not observed in ESI-MS implies that the binding of the second Hg²⁺ is relatively weak.

In Figure 5, we present model structures which agree with all the experimental data presented in this work. At pH 8.0 with 0.2 and 1.0 equivalent Hg⁴⁺, two species co-exist, most likely the linear HgS₂ and a HgS₃ structure with the equatorial Hg-S bond being longer than the other two. Such structures have also been observed in small, Hg⁴⁺ containing inorganic compounds. The increased availability of deprotonated cysteines with increasing pH agrees well with this change in speciation observed from pH 6.0 to pH 8.0, that is, a change from HgS₂ towards HgS₃ coordination mode, and a similar trend has been observed for de novo designed proteins by Iranzo et al. The additional thiolate is most likely recruited from the residual thiolate when Cu²⁺ is relatively weak.

Table 1. Spectroscopic properties of the HgS₂ and HgS₃ species compared to Hg³⁺/MerR and Hg/L16C complexes. The two entries for CueR are from this work, see Figure 4C.

| Sample                  | λ [nm] | ε [cm⁻¹ mm⁻¹] |
|-------------------------|--------|---------------|
| Hg-MerR²²              | 242    | 19.8          |
|                         | 260    | 14.6          |
|                         | 290    | 6.45          |
| 1:2 Hg/L16C²²          | 240    | 2.70          |
|                         | 247    | 16.8          |
|                         | 265    | 10.6          |
|                         | 295    | 5.00          |
| 1:3 Hg/L16C²²          | 240    | 12.6          |
|                         | 250    | 12.6          |
|                         | 290    | 5.82          |
| CueR (HgS₂)            | 230    | 3.52          |
| CueR (HgS₃)            |        |               |

Figure 5. Model structures of Hg⁴⁺ bound to WT CueR at pH 8.0. Binding of Hg⁴⁺ to CueR gives rise to an equilibrium between HgS₂ and HgS₃ when Hg⁴⁺:CueR < 2, and to pure HgS₃ coordination upon addition of 2 Hg⁴⁺ ions per protein monomer. This can only be accounted for if the CCHH C-terminal motif participates in the coordination of Hg⁴⁺, see the text for details.

It may seem intriguing that with 1.0 equivalent Hg⁴⁺ both the PAC and UV absorption spectra differ significantly from those recorded with 0.2 equivalent Hg⁴⁺. However, a simple probabilistic model qualitatively accounts for this change, assuming that the two sites are independent (i.e. distributing Hg⁴⁺ randomly among the 4 metal sites of a protein dimer), and that population of two adjacent sites (the functional site and the C-terminal site) leads to formation of HgS₃ because there are no more cysteines locally available to form HgS₄. The experimental data were not presented, Stoyanova and Brown indicated that the selectivity of reaction with other, unspecified metal ions was not affected. To further explore this issue, a series of in vitro and in vivo transcriptional assays should be conducted.
In summary, we have demonstrated that up to two Hg$^{2+}$ ions bind with high affinity to WT CueR, one at the functional (C112 and C120) metal binding site, and the other at the C-terminal CCHH motif. Moreover, under conditions where the protein is not saturated by Hg$^{2+}$, a higher coordination number (presumably Hg$_2$S$_2$) is observed for WT CueR but not for ΔC7-CueR, indicating that side chains from the CCHH motif may be recruited as auxiliary ligands at the functional metal site (or vice versa). This implies a mechanism where the specificity of CueR for monovalent coinage metal ions and against divalent metal ions is achieved by coordination to divalent metal ions by the CCHH motif, preventing the docking of the C-terminal helix into the hydrophobic pocket,[5] and consequently inhibiting activation of transcription. Indeed, the CCHH motif provides a selection of ligands that may participate in coordination of both soft and intermediate metal ions. As the findings presented here on Hg$^{2+}$ do represent a special case, the generalization to other divergent metal ions should be considered carefully.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: coordination modes · CueR metalloregulatory protein · mercury · metal ion selectivity · perturbed angular correlation (PAC) spectroscopy

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