Specificity of the Metalloregulator CueR for Monovalent Metal Ions: Functional Role of a Coordinated Thiol?

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Abstract: Metal ion responsive transcriptional regulators within the MerR family effectively discriminate between mono- and divalent metal ions. In this work we address the origin of the specificity of the CueR protein for monovalent metal ions. Several spectroscopic techniques were employed to study Ag^{I}, Zn^{II}, and Hg^{II} binding to model systems encompassing the metal ion binding loop of CueR from *E. coli* and *V. cholerae*. In the presence of Ag^{I} a conserved cysteine displays a pK_a for deprotonation of the thiol close to physiological pH. This is unique to the monovalent metal ion. Quantum chemically optimized structures of the CueR metal site with Cys112 protonated demonstrate that the Ser77 backbone carbonyl oxygen is "pulled" towards the metal site. A common allosteric mechanism of the metalloregulatory members of the MerR family is proposed. For CueR the mechanism relies on a protonation switch of Cys112.

Metal ion homeostasis and cellular defense mechanisms against toxic metal ions are central topics in biochemistry.[1] One facet of this theme is the function of metalloregulatory proteins of the MerR family.[1,2] Examples include MerR and ZntR which act as specific sensors of Hg^{II} and Zn^{II}, respectively, and CueR that senses monovalent metal ions such as Cu^{I} and Ag^{I}.[3] The MerR proteins function as DNA bound homodimers with a dimerization domain, a metal ion binding loop and a DNA binding domain. A striking feature of the Cu^{I} binding site of CueR, is that the metal ion coordinating S_{Cys112} is positioned 3.6 Å from the backbone carbonyl oxygen of Ser77,[3] i.e. at a typical hydrogen bonding distance for a cysteine SH donor to a backbone amide C=O.[4] This fact has lead Brown and coworkers to suggest that Cys112 might be protonated.[5] Metal ion binding allosterically affects the protein-DNA interaction, changing DNA structure and regulating transcription.[1,6] In a very recent and interesting study, the structure of both the repressor (i.e. metal free) and activator form of the CueR protein, the Ag^{I}-CueR-DNA complex, was determined, providing unprecedented insight into the changes of DNA structure accompanied by metal ion binding.[6]

In this work we employ spectroscopic techniques to explore characteristic differences between binding of mono- and divalent metal ions to peptides encompassing the metal ion binding loops of the CueR proteins from *V. cholerae* and *E. coli,* as well as quantum chemical methods to model various possible metal site structures. The amino acid sequences of the two peptides are shown in Scheme 1. In a series of structures of the CueR protein from *E. coli*, O'Halloran and coworkers have demonstrated that the two cysteine residues (Cys112 and Cys120) coordinate to monovalent metal ions in an almost linear coordination geometry.[3,7]

MBL-VC: Ac-S**C**PGDQGSD**C**PI-NH_2
MBL-EC: Ac-A**C**PGDDSAD**C**PI-NH_2

Scheme 1: Amino acid sequences of the peptides encompassing the metal ion binding loop of CueR from *E. coli* (MBL-EC) and *V. cholerae* (MBL-VC), here both N- and C-terminally protected. The two cysteine residues coordinating monovalent metal ions in the CueR protein[3] are indicated in bold face.

The binding of monovalent metal ion is investigated using Ag^{I} as a probe. The binding of divalent metal ions is probed with 1) Zn^{II} as the most relevant biometal against which discrimination must be exercised by CueR, and 2) Hg^{II} as a divalent cation with considerable similarity to Cu^{I} in terms of ligands and coordination number preferences. Most notably Hg^{II} commonly binds with high affinity to bisthiolato ligands, such as those present in CueR.

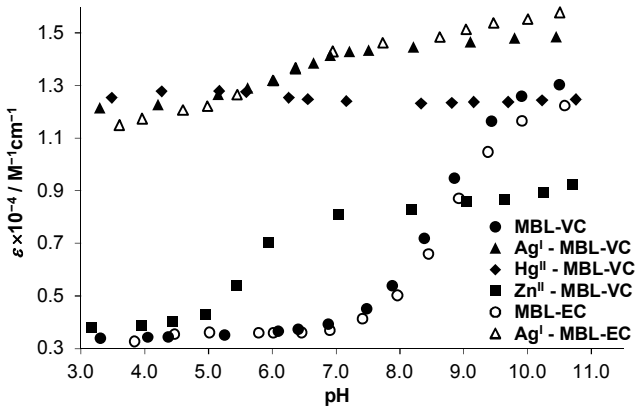

Figure 1: UV absorbances at 230 nm as a function of pH for the various metal ion-MBL-VC and free MBL-VC (filled symbols), and for the Ag^{I}-MBL-EC and free MBL-EC (open symbols) systems, divided by the total ligand concentrations. $c_{MBL\text{-}VC}$ or $c_{MBL\text{-}EC}$ = 1–2×10^{-4} M. Full UV spectra are in Figures S1-6.

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A pH series of UV absorption spectra were recorded in order to monitor the metal ion binding via observation of ligand to metal charge transfer (LMCT) bands, see Figure 1.

The two peptides show the same qualitative changes of the absorption spectra with pH. The free pepides display a large increse in absorbance from pH ~7.5 to ~9.5, reflecting the deprotonation of the cysteine thiols. None of the metal-peptide complexes show this change in the same pH range, illustrating that the thiols are deprotonated at lower pH in the presence of metal ions. For the Hg^{II}-MBL-VC complexes, there are only minor changes in the absorption over the entire pH range, indicating that both Cys residues coordinate to Hg^{II} already at the lowest pH investigated. The metal site structure was probed by ^{199m}Hg PAC spectroscopy, see Figure S7 and Table S1, where a characteristic signal of bisthiolatomercury(II) is observed at pH 9.5. At pH 6.0 the signal is highly similar, albeit with a marginally lower frequency, possibly indicating the presence of an additional very weak ligand. In the Zn^{II}-MBL-VC system the absorbance is very similar to the free peptide at low pH, indicating that the metal ion does not bind at this pH. An increase in absorbance is observed in the pH range from 5 to 6, indicating that the pK_a of the two Cys residues fall in this range in the presence of Zn^{II}. For the Zn^{II}-MBL-VC complexes an additional increase in absorbance is observed at high pH (>10), presumably reflecting deprotonation of a coordinating water molecule.[8] In the presence of Ag^{I} the absorbance at 230 nm of the two peptides is comparable to that of the Hg^{II}-MBL-VC complexes at low pH, and considerably higher than for the free peptide, implying coordination of both Cys sulphurs. However, a surprising change in absorbance is observed at pH ~6.5 both for the Ag^{I}-MBL-VC and Ag^{I}-MBL-EC systems, apparently reflecting deprotonation of an ionizable group. The modest change in absorbance accompanying the titration might imply that it could be the deprotonation of a thiol already coordinating, or alternatively coordination of a thiolate replacing another ligand which in complex with Ag^{I} gives similar spectroscopic properties. This pK_a value is unique to the monovalent metal ion, i.e. not observed for the two divalent metal ions, and it is intriguing that it occurs relatively close to physiological pH, thus potentially relevant to the function of CueR.

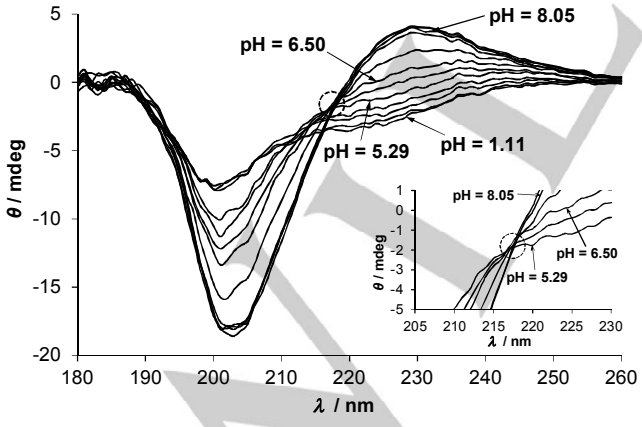

Figure 2: CD spectra for the Ag^{I}-MBL-VC system as a function of pH. The isodichroic point (marked by dashed circle) in the pH range from 5 to 8 at ~218 nm is highlighted in the insert. c_{MBL-VC} = 1×10^{-4} M.

To further explore this pK_a we conducted CD and potentiometric experiments for the Ag^{I}-MBL-VC system, see Figures 2 and 3.The CD spectra display an isodichroic point at ~218 nm, demonstrating that Ag^{I} is bound to the peptide, as this is the only chiral molecule present, and that the titration in the range of pH ~5-8 is mainly occurring between two species, i.e. the protonated and deprotonated forms. The potentiometric data, shown in Figure 3, further support the presence of a unique pK_a of ~6.5 for the Ag^{I}-MBL-VC system. In addition, the data clearly demonstrate that the first deprotonation process for Ag^{I}-MBL-VC takes place at pH much lower than for the free peptide (p$K_{a,Cys}$ = 8.25 and 9.06, see Table S2) and the Zn^{II}-MBL-VC complex, implying that the pK_a for the first thiol is low for the Ag^{I}-MBL-VC complex.

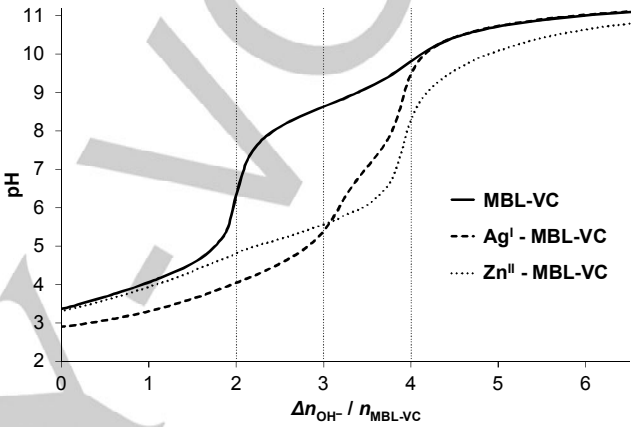

Figure 3: Titration curves for MBL-VC in the absence (solid line) and presence of metal ions (Ag^{I}: dashed line; Zn^{II}: dotted line). Volumes are normalized as consumed base equivalents per ligand. The vertical lines identify the points of the curves where 2, 3 and all the 4 protons of the ligand are titrated. c_{MBL-VC} = 1×10^{-3} M.

The Ag^{I}-MBL-VC complex and the free peptide titrations at high pH are very similar, indicating that no more than the expected four groups undergo titration (2Asp, 2Cys). Contrary to this, the Zn^{II}-MBL-VC system titrates in a highly similar manner to the free peptide at low pH, indicating that Zn^{II} binds only above pH ~4 (see also Figure S8), after which the deprotonation of the two thiols occurs at pH ~5-6, and shows an additional group titrating at high pH assigned as the proton release of a metal-bound water ligand (pK_a = 9.53, Table S2), in agreement with the UV data, vide supra. (A more detailed description of the speciation in the Zn^{II}-MBL-VC system is found in the Supporting Information.)

Having established that a unique pK_a of ~6.5 is present for the Ag^{I} peptide systems, we conducted ^{1}H-NMR experiments to identify the titrating group, see Figure 4. The assignment of the resonances, as deduced from 2D ^{1}H-^{1}H COSY, TOCSY and ROESY experiments, is indicated in the Supporting Information. In Figure 4 we focus on the $C_\beta H_2$ protons of the two Asp and two Cys residues of the MBL-VC peptide in the absence and presence of the metal ions. The free peptide shows the expected deprotonation of the carboxyl groups and the thiols with pK_as of ~4.0 and ~8.5 (see also Table S2). In the presence

of Ag^{I} and Hg^{II} the pK_as of the two carboxylic acids are only marginally perturbed, indicating that they do not participate in metal ion binding, whereas coordination of at least one of the Asp-carboxylates can be proposed is likely in the Zn^{II}-containing system.

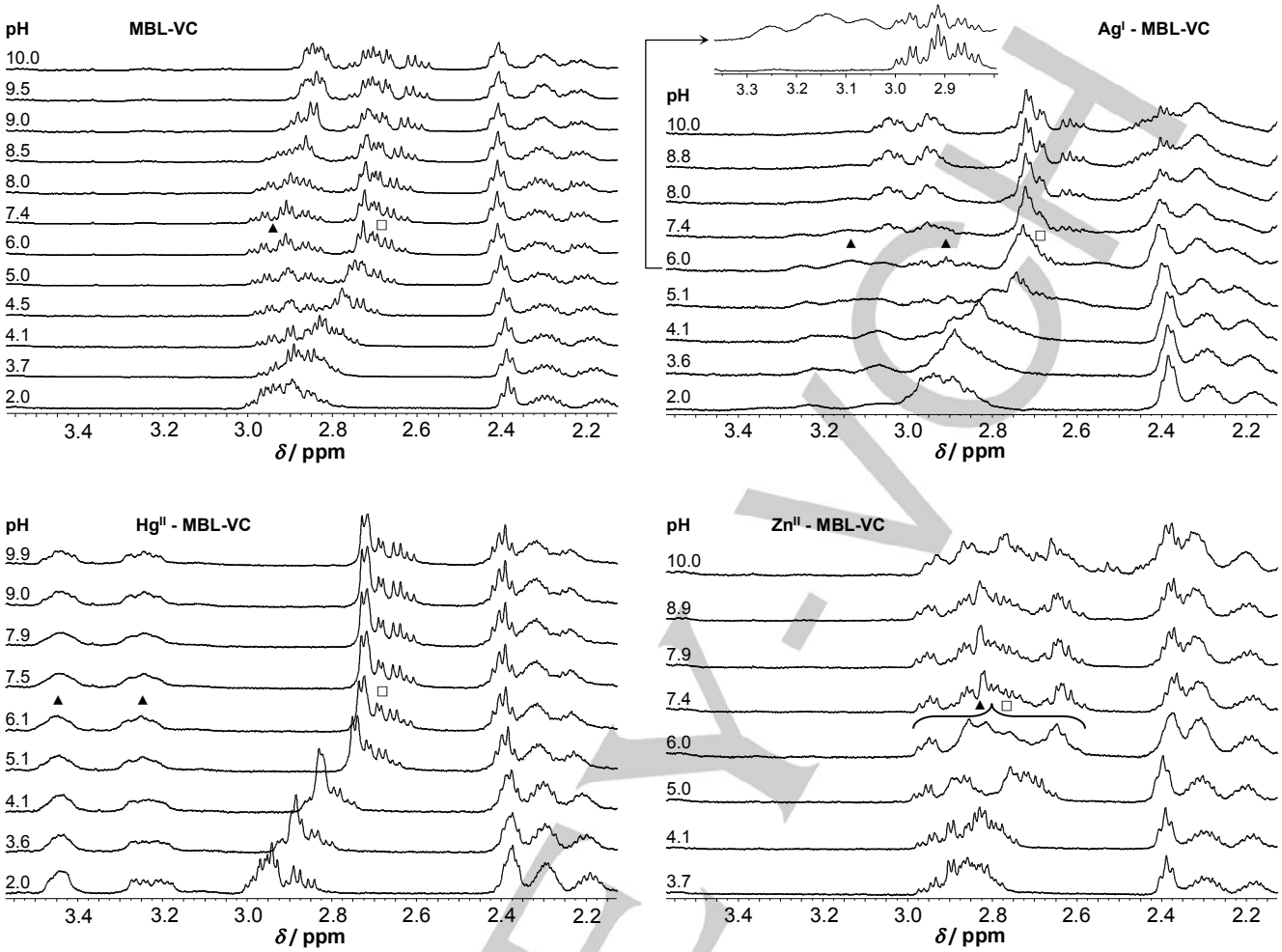

Figure 4: pH-dependent series of ^{1}H-NMR spectra recorded for free MBL-VC and metal ion - MBL-VC systems, in the range of the Cys and Asp $C_\beta H_2$ resonances (δ ~ 2.5 – 3.5 ppm). ▲ and □ denote the resonances of Cys $C_\beta H_2$ and Asp $C_\beta H_2$, respectively. Comparison of spectra in the absence and presence of Ag^{I} ions at pH = 6.0 in the insert (upper right series, recorded at higher concentration) demonstrates that free ligand-like signals are clearly present even if one equivalent of Ag^{I} is added. (Signals appearing in the spectra between δ ~ 2.15 – 2.45 ppm originate from Gln-6 $C_\beta H_2$, Pro-3 and Pro-11 $C_\beta H_2$ and Gln-6 $C_\gamma H_2$.) $c_{MBL\text{-}VC}$ = 1.3×10^{-3} M, H_2O/D_2O = 90 : 10% v/v.

In the presence of Hg^{II} the $C_\beta H_2$ proton resonances of the two Cys residues are shifted significantly downfield at all pH values, reflecting coordination of both thiolates from pH 2 and upwards. Similarly, in the presence of Zn^{II} above pH 5-6, the $C_\beta H_2$ protons of the two Cys are affected, demonstrating that they coordinate to the metal ion. For the Ag^{I}-MBL-VC system a somewhat different change with pH is observed, with broad downfield resonances already at pH 2, revealing that at least one thiolate is coordinating. More interesting is the group of resonances near 2.9 ppm at pH 5 and 6, which very closely resembles those observed for the free peptide. This strongly suggests that one of the thiols is protonated at this pH. At pH 6.0 the integral of the broad bands corresponding to Ag^{I} bound thiolate (3.01-3.29 ppm) amount to slightly more than two protons, while the resonances resembling thiol in the free peptide (2.80-3.01 ppm) amount to slightly less than two protons (using the Asp $C_\beta H_2$ protons as a reference at 2.65-2.79 ppm, corresponding to four protons), consistent with a pK_a of about 6.5. This demonstrates that the pK_a of ~6.5 for the Ag^{I}-MBL-VC peptide originates from deprotonation of the second Cys thiol. The same conclusions may be drawn for the MBL-EC peptide, but the spectra display more line broadening, see Figures S9 and S10. Whether the protonated thiol coordinates cannot be concluded, as the NMR data suggest that this is not the case, while the UV absorption data might indicate the contrary. A search in the Cambridge Structural Database[9] demonstrates that Cu^{I} and Ag^{I} coordination by protonated thiols is rare, but does exist.[10–12] Further, the aqua ion of Ag^{I} is a rather weak acid, with a pK_a of ~12 (extrapolated to zero ionic strength) and ~10 at an ionic strength > 0,[13] i.e. decreasing the pK_a of water by ~4-6 pH units. Thus, it seems plausible that Ag^{I}, in a formally neutral Ag^{I}-SSH unit, will be a very weak acid, lowering the pK_a of the second

thiol by only a few pH units. Thus, spectroscopic, structural and thermodynamic data indicate that the presence of a protonated Cys at the metal site of CueR cannot be excluded.

To explore the effect of protonating Cys112 and if a thiol may coordinate Cu^{I} and Ag^{I} at the CueR metal site, we carried out quantum chemical geometry optimizations of various model systems, see Figures 5 and S11-S14. The metal site was optimized with and without protonated Cys112 1) within a fixed protein structure model, allowing only the Cys side chains, Cu^{I} or Ag^{I}, Ser77 backbone atoms and hydrogen atoms to relax, and 2) for small model systems allowed to relax completely, i.e. with no constraints from the protein. The large model system with both cysteines deprotonated is highly similar to the experimentally determined structure. Thus, the latter structure likely represents the deprotonated state for both coordinating cysteines, despite the peculiar proximity of the Ser77 backbone oxygen. Importantly, the effect of protonating Cys112 is to "pull" the Ser77 backbone oxygen ~0.2 to 0.4 Å closer to the metal site in a combined interaction involving both the hydrogen bond to Cys112 and a shorter Cu^{I}-O and Ag^{I}-O distance. Surprisingly, this is the only major structural change accompanying the protonation, i.e. the Cu^{I}-S and Ag^{I}-S bond lengths only change marginally (< 0.03 Å). This is also the case for the small model systems, see Figures S11-S14. That is, the metal site reorganization energy upon protonation is minimized, while giving rise to considerable movement of Ser77, allowing for an efficient allosteric mechanism.

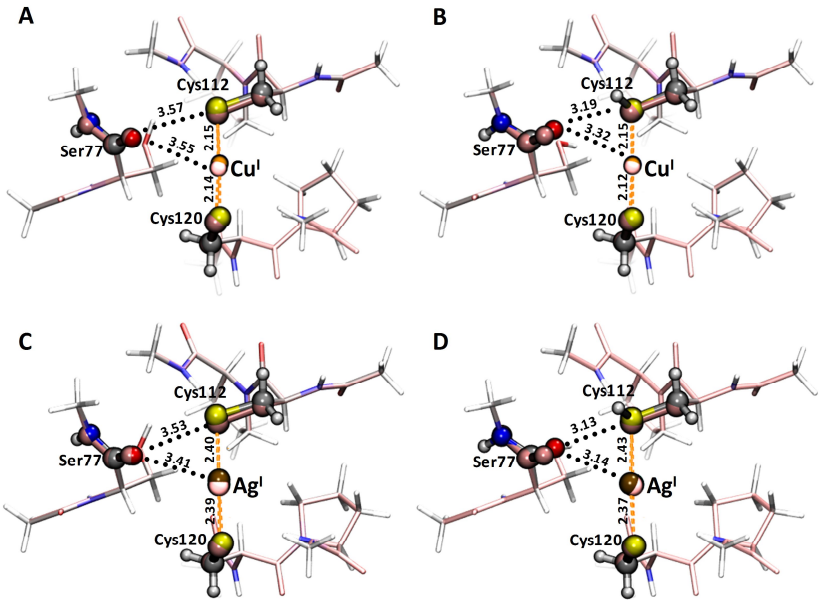

Figure 5: Overlay of the experimentally determined structures (pale red, PDB id 1Q05 in A, B and 1Q06 in C, D) and geometry optimized structures of the CueR metal site. Atoms allowed to relax in the geometry optimization are shown as ball-and-stick (H: white, C: grey, N: blue, O: red, S: yellow, Cu: orange, Ag: brown) A: Cu^{I}-model with deprotonated Cys112, and B: protonated Cys112. C: Ag^{I}-model with deprotonated Cys112, and D: protonated Cys112. The geometry optimizations were carried out in ORCA[14] at the BP86-D3/def2-TZVP level of theory.[15–19]. Selected interatomic distances for the optimized structures are indicated. Notice that the Ser77 backbone oxygen is "pulled" towards both Cu^{I} (Ag^{I}) and the Cys112 sulphur upon protonation, even in this constrained optimization. Figures were made with VMD.[20]

Finally, we investigated if the metal site with Cys112 protonated was strained. The overall structures of the small model systems optimized without any constraints, see Figures S11-S14, are similar to those of the large model systems, and the thiol remains coordinated, although the hydrogen bond geometry is more optimal. The strain energy is 55 kJ/mol for the large Cu^{I} model system and 57 kJ/mol for the large Ag^{I} model system. This is comparable to estimates of strain energies at metal sites in other proteins,[21,22] although the strain energy is defined slightly differently in this work, see the Supporting Information. This indicates that the CueR metal site may exist with a coordinating protonated Cys112. Similarly, in a thorough theoretical study of binding of various metal ions to the CueR metal site,[23] it was demonstrated that the metal site discriminates against formal positive charge, but that discrimination between $Cu^{I}SS$ and $Cu^{I}SSH$ is less obvious. That is, one of the thiols, might be protonated in the metal bound form. Additionally, S_{Cys112} takes part in only one other hydrogen bond, accepting a hydrogen bond from the Gly114 amide nitrogen. Another important aspect is how the local structure around the metal site in the protein may affect and tune the pK_a of Cys112. The metal site is shielded from the solvent,[3] and should be stabilized in the uncharged $Cu^{I}SSH$ form. Similarly, the Cys120 sulphur accepts hydrogen bonds from two amide nitrogens, and is positioned directly over the N-terminus of a small helix, and thus may be stabilized as a thiolate by hydrogen bonding and the helix dipole. Although the argument is the same as that put forward by O'Halloran and coworkers, we argue here that the effect of the helix dipole is not to stabilize the negative charge of the $Cu^{I}SS$ species, but to selectively lower the pK_a of Cys120, and thus ensure that Cys112 is the least acidic of the two cysteines. Correspondingly, thioether coordination to Cu^{I} is well

established in cellular compartments where the conditions are less reducing than in the cytosol,[24] implying that the affinity of such sites – although presumably lower than bisthiolato-Cu^{I} coordination – is high enough to have physiological significance. Finally, EXAFS data recorded at pH 8.0 demonstrate that Cu^{I} is bound in a bisthiolato complex.[7] Under such conditions both cysteines are expected to be deprotonated, in agreement with the experimental results in this work. Very recently it was demonstrated that mutating Ser77 to Ala77 or Cys77 did not significantly affect the response of CueR to Cu^{I}, but that introduction of Cys at position 77 gave rise to sensitivity to divalent metal ions as well.[25] This agrees well with a functional unit for transmission of the effect of monovalent metal ion binding occurring via the backbone carbonyl of the amino acid at position 77. Thus, the model proposed in this work coherently accounts for the sensitivity profile observed by Ibanez et al..[25] For the MerR family members responding to divalent metal ions, the conserved Ser is substituted by a Cys residue,[3,25] which has been demonstrated to coordinate to Zn^{II} in ZntR.[3] Based on a previously observed change from bisthiolato to tristhiolato coordination mode in the Hg^{II} complexes of de novo designed TRIL9C peptides[26] it is conceivable that a pH dependent structural switch is also operational in the Hg^{II}-dependent MerR protein, where the corresponding Cys79/Cys82 completes the trigonal planar coordination geometry.[27–29] Thus, it seems plausible that a common allosteric mechanism may be functional in the metalloregulatory MerR family members, where metal ion specificity and transmission of altered structure and dynamics upon metal ion binding is propagated by "pulling" a specific residue from the other monomer of the homodimer: Either by direct metal ion coordination of a conserved Cys for divalent metal ions, or via a hydrogen bond to a conserved Ser for monovalent metal ions. O'Halloran and coworkers very recently demonstrated that amino acids 75 to 77 of one monomer undergo considerable movement upon Ag^{I} binding to the other monomer of the CueR-DNA complex.[6] Similarly, the structure of a MtaN, a MerR protein family member, displays the largest difference in backbone Φ/Ψ angles upon binding to DNA for residues 71-75, constituting a hinge region. A structural alignment of CueR (pdb id 1q05) and MtaN in complex with DNA (pdb id 1r8d),[30] see Figure S15, demonstrates that the same hinge region is present for CueR,[30] and that it encompasses the conserved Ser which we propose to be "pulled" upon metal ion binding, thus further supporting the hypothesis put forth in this work.

In summary, in a spectroscopic study of Ag^{I}, Zn^{II}, and Hg^{II} binding to model systems of the CueR metal ion binding site, we observed a unique pK_a of a thiol close to physiological pH for the Ag^{I} complexes. Using quantum chemical methods we explored the structural consequences of protonating Cys112. We propose a functional model that may be general to MerR family members, where the allosteric mechanism involves a conserved residue (Ser77 in the case of CueR) in the other monomer of the homodimer.

Experimental Section

See Supporting Information.

Acknowledgements

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Keywords: metal sensor proteins • metal site structure • peptides • molecular modeling • thiol coordination

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