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A design for life: prokaryotic metal-binding MerR family regulators

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Abstract

The MerR family of metal-binding, metal-responsive proteins is unique in that they activate transcription from unusual promoters and coordinate metals through cysteine (and in the case of ZntR, histidine) residues. They have conserved primary structures yet can effectively discriminate metals *in vivo*.

Introduction

Bacteria are intimately associated with their natural environments and have to sense their immediate external environment and regulate their own intracellular environment, in order to maintain normal functions. Intracellular bacterial homeostasis systems have to be capable of adjusting rapidly to either shortage or excess of specific nutrients, metals and other chemicals, as well as to alterations in more general physical changes in pH, redox, ionic strength and anaerobiosis, for example, so that a rapid cellular response occurs. Key to these processes are response regulator proteins. When the signalling and response systems that maintain internal cellular homeostasis cannot effectively cope with these chemical and physical fluctuations, the bacteria become stressed, possibly lethally so.

Metals can pose a particular problem to bacteria, because essential metals may be limiting in the environment, requiring active uptake mechanisms to import minimum concentrations of essential metals (Butler 1998; Outten *et al.* 2000). Bacteria can encounter purely toxic metals, such as Hg, Cd and As, which have no beneficial role in cellular metabolism, and must be avoided, removed or neutralized. These two problems overlap, because many metals, such as Cu, Fe and Zn, are essential for normal cellular functioning, yet toxic if their concentration within the cell becomes too high. Clearly, the metal ion uptake and homeostasis mechanisms, and metal resistance systems that bacteria possess have to be highly discriminatory, so that the cellular response is specific to a specific metal. The key first step in how bacteria respond to varying levels of both toxic and essential metals in the internal environment of the cell is due to the metal sensor and regulator proteins that they encode. There are several known types of prokaryotic metal ion sensing regulators. These include the MerR (MerR, ZntR, CueR, PbrR, CadR, CoaR) and Fur (Fur, Zur) families of regulators, the ArsR/SmtB family repressors, two-component sensor-regulator systems (*e.g.*, PcoRS, CusRS, SilRS), and several other structural regulator types including NikR from *Escherichia coli*, the DtxR/MntR family of regulators and the *E. hirae* copper responsive CopY repressor. Many of these families of regulators are discussed in more detail by Penella and Giedric in this issue of *Biometals*, and in the recent (2003) thematic issue of *FEMS Microbiology Reviews*, vol. 27 (issue 2–3).

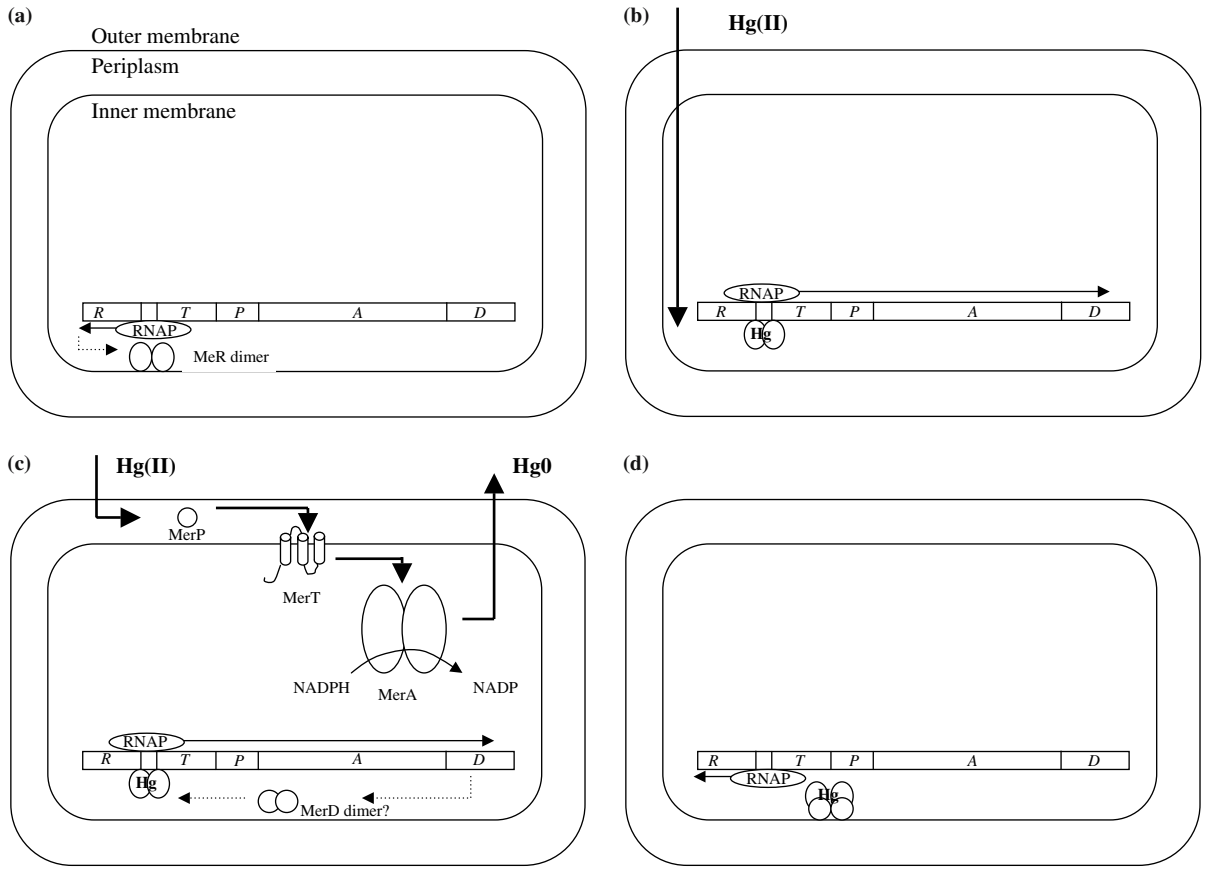

Figure 1. Schematic representation of an *E. coli* cell containing the Tn*501* mercury resistance operon. (a) RNA polymerase (RNAP) transcribes *merR* from P_{merR}. MerR binds to the mer promoter/operator region (*merOP*) as a homodimer, recruits RNA polymerase, and represses transcription of *merTPAD* from P_{merT}. (b) Hg(II) enters the bacterial cell by diffusion through the outer membrane, cytoplasm and inner membrane, and binds to three cysteine residues in the apo-MerR homodimer. The Hg-bound MerR homodimer causes an underwinding of *merOP* DNA, allowing RNAP to proceed with transcription of the resistance genes. (c) The resistance proteins are translated and localized to the periplasm (MerP), the inner membrane (MerT) and the cytoplasm (MerA, and MerD). A pair of cysteine residues in MerP binds mercuric ions in the periplasm. The Hg(II) ions are transferred to cysteine residues in MerT, allowing them to pass through the inner membrane, and are reduced to Hg(0) by mercuric reductase (MerA) in the cytoplasm, in an NADPH dependent reaction. Mercury vapour (Hg(0)) diffuses from the cell into the immediate local environment. (d) When all of the mercury has been lost from the bacterial cell, MerD (which may exist as an oligomer) forms a ternary complex with MerR-*merOP*, and destabilizes the interaction between MerR and *merOP*. This allows the production of new apo-MerR, which will bind to *merOP*. As yet nothing is known about how quickly the *mer* resistance proteins are turned over.

MerR – a mercury sensing gene activator

A key class of prokaryotic metal ion responsive activators is the MerR family, of which the mercuric ion sensing MerR is the archetype. The closely-related 144 amino-acid MerR proteins from the mercuric ion resistance (*mer*) operons from transposons Tn*501* and Tn*21* have been the most heavily studied of these proteins (reviewed in Summers 1992, 1986; Hobman & Brown 1997; Outten *et al.* 2000a; Barkay *et al.* 2003; Brown *et al.* 2003, and references therein), and the mechanism of Hg(II) resistance is now well known (reviewed most recently by Barkay *et al.* (2003)). In the simplest mercury resistance system from Tn*501*, Hg(II) ions bind to MerP in the periplasm, and are transferred to the inner membrane MerT protein. Hg(II) passes from MerT to MerA in the cytoplasm, where it is reduced from Hg(II) to volatile Hg(0), and diffuses from the cell as mercury vapour (Figure 1). Coordination of Hg(II) in the mercury resistance proteins is via cysteine pairs in each protein. MerR regulates its own expression and that of the resistance genes.

In the *mer* operon from Tn*501*, MerR binds to operator DNA within a divergent promoter and regulates both its own expression and, via a polycistronic mRNA, expression of the structural genes for mercury resistance *merT, P* and *A*, and of the co-regulator *merD* (Figure 1). Activation of transcription from the P_{merT} promoter by MerR in response to Hg(II) is hypersensitive (Ralston & O'Halloran 1990; Rouch *et al.* 1995), with virtually total induction of promoter activity occurring across a very narrow Hg(II) concentration range. Promoter response also occurs to very low concentrations of mercury $\sim 10^{-8}$ M (Ralston & O'Halloran 1990; Condee & Summers 1992), with full induction of expression of the *mer* structural genes occurring at sublethal levels of Hg(II). MerR has a strong selectivity for Hg(II) (Ralston & O'Halloran 1990) and Hg(II) has a very high affinity for MerR (Shewchuk *et al.* 1989b).

The model for regulation of the *mer* promoter

The current model for MerR activation of transcription proposes that expression of MerR (in the absence of MerR and Hg (II) in the cell) proceeds from the P_{merR} promoter (Figure 1a). In the absence of Hg(II), the MerR homodimer binds to the operator region within the divergent promoter (the *merOP* region – see Figures 1b and 2) with binding centered on the dyad symmetrical DNA sequence between the −35 and −10 sequences of P_{merT}, slightly repressing transcription of the structural gene promoter, and repressing transcription of *merR* from the P_{merR} promoter. This is probably due to MerR interfering with RNA polymerase (RNAP) binding, or open complex formation. P_{merT} is unusual, it has a 19 bp spacing between the −35 and −10 sites (Figure 2), rather than the 16–18 bp spacing found in most prokaryotic promoters (Harley & Reynolds 1987). This makes P_{merT} suboptimal for RNAP recognition of, and binding to, the −35 and −10 sequences, preventing formation of the open complex and transcriptional activation (see Browning & Busby 2004). Deletion mutants of P_{merT}, in which the spacer has been shortened, are constitutively transcriptionally active (Parkhill & Brown 1990). In the absence of Hg(II), the tight binding of the apo-MerR homodimer to P_{merT} causes a further bending of the promoter DNA to itself, making it even less ideal for RNAP binding (Ansari *et al.* 1995). Once the MerR homodimer has bound to *merOP*, recruitment of RNAP to the *mer* promoter occurs (Heltzel *et al.* 1990), and MerR has been shown to cross-link to several subunits of RNAP (Kulkarni & Summers 1999). In the absence of Hg(II) the ternary complex of MerR, RNAP, and *merOP* represses transcription of the *mer* structural genes. In the presence of mercuric ions, one Hg(II) per MerR homodimer (O'Halloran *et al.* 1989; Shewchuk *et al.* 1989a) coordinates in a trigonal manner to three essential cysteine residues of the MerR homodimer, two cysteines from one monomer, and one from the other (Helmann *et al.* 1990; Utschig *et al.* 1995). Hg(II) binding to the MerR homodimer results in both a relaxation of the DNA bends induced by apo-MerR, and both DNA distortion (Frantz & O'Halloran 1990) and an allosteric underwinding of the promoter sequence by approximately 33° (Ansari *et al.* 1992). The underwinding of the promoter DNA aligns the −10 and −35 sequences, such that RNA polymerase can recognize and bind to these sites, initiating transcription from P_{merT} (Figures 1b and 2) (Heltzel *et al.* 1990; Ansari *et al.* 1992, 1995).

Recent work on the interaction between MerD and MerR from Tn*4378* at *merOP* (Champier

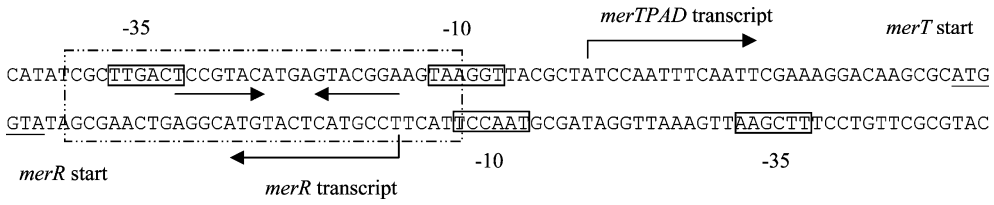

Figure 2. DNA sequence of the Tn*501 mer* operator promoter region. The MerR binding site on P_{merT} is marked by a box. MerR protects this region from DNA'ase I digestion in footprinting assays. The −35 and −10 regions for both P_{merR} and $P_{merTPAD}$ are marked with boxes, and the dyad symmetrical DNA sequence that MerR recognizes and binds to is marked with arrows under the DNA sequence. Modified from Brown *et al.* (2003).

et al. 2004) seems to have resolved the outstanding question on regulation of the P_{merT} promoter by MerR: namely, how is transcription of P_{merT} switched off, once the intracellular levels of Hg(II) have decreased due to volatilization of Hg(0) by MerA? It has been known for some time that MerD bears some similarity to MerR, particularly in the N-terminal DNA binding domain (Brown *et al*. 1986), and that MerD is located after the resistance genes *merTP* and *A* (Figure 1). Initial work showed that Tn*21 merD* was translated (Lee *et al*. 1989) and, although the evidence was slightly contradictory, MerD appeared to be involved in co-regulation of the *mer* promoter (Nucifora *et al*. 1989; Mukhopadhyay *et al*. 1991). Recent experimental evidence (albeit in the absence of RNAP) supports the hypothesis that MerD dissociates the metallated MerR/*merOP* complex, thus allowing the synthesis of apo-MerR, which can in its turn bind to *merOP*, repressing expression of the *mer* structural genes if Hg(II) concentrations are at zero (Figure 1d) (Champier *et al*. 2004). This regulatory mechanism makes biological sense because Hg(II) has no known beneficial role in the bacterial cell and should be eliminated totally, and once Hg(II) is eliminated from the cell, regulation of the *mer* operon should very quickly switch off *mer* gene expression, in order to minimize the production of proteins that are no longer required.

The current model describes a resistance mechanism whose expression is repressed until Hg(II) ions enter the cytoplasm of the cell, but is primed to initiate transcription of the resistance genes at very low levels of Hg(II), and to fully induce resistance gene expression across a narrow increase in external Hg(II) concentration. The model now also describes how resistance gene expression can be turned off, once Hg(II) has been eliminated from the cell.

Metal binding homologues of MerR

Three metal ion binding MerR homologues from *E. coli*, SoxR, ZntR and CueR are now well characterized, and structural information for CueR and ZntR is now available (Changela *et al*. 2003). There are now many other known metal binding MerR-family regulators from other organisms, which are less well characterized (reviewed in Brown *et al*. 2003). SoxR is an extremely important regulator of oxidative stress response, and in response to oxidation or nitrosylation of its [2Fe–2S] centre (reviewed in Pomposiello & Demple 2001) activates expression of *soxS* (the product of which regulates 40 or so other genes (Pomposiello *et al*. 2001)). CueR responds to Cu(I), Ag(I) and Au(I), and ZntR responds to Zn(II), Pb(II) and Cd(II). The mechanisms of copper and zinc homeostasis in prokaryotes are beginning to be well understood (Outten & O'Halloran 2001; Blencowe & Morby 2003; Finney & O'Halloran 2003; Rensing & Grass 2003), and both ZntR and CueR play important roles in the regulation of intracellular levels of these metals. ZntR regulates the expression of *zntA*, encoding a Zn(II)/Cd(II)/Pb(II) efflux ATPase. ZntR binds as a homodimer to the unusual promoter, P_{zntA}, which contains a 20 bp spacing between the −35 and −10 sequences (Brocklehurst *et al*. 1999; Outten *et al*. 1999). CueR regulates the expression of *copA*, a Cu(I)/Ag(I) efflux ATPase (Outten *et al*. 2000b; Peterson & Moller 2000; Stoyanov *et al*. 2001), as well as that of the *cueO* gene encoding a multicopper oxidase (Outten *et al*. 2000b). Both of these promoters have a 19 bp spacing between the −35 and −10 sequences, and CueR binds to them. Unlike MerR, which upon binding to *merOP* represses expression of P_{merT}, neither ZntR or CueR appear to repress transcription from their cognate promoters in the absence of Zn or Cu, respectively. No evidence has so far been found for a co-regulator role of MerD homologues in the regulatory mechanism of other metal binding MerR homologues.

MerR family cysteine coordination of metals

The MerR design template for the wider family of metal responsive family of regulators (CueR, ZntR, SoxR) also applies to the role that cysteines play in co-ordination of metals, and the consequent activation of transcription from their cognate promoters. In some elegant early work the cysteine residues in MerR were mutagenized to serine or alanine, showing that there were three cysteines: Cys 82, 117 and 126 that were essential in mercury coordination (Shewchuk *et al*. 1989b; Helmann *et al*. 1990). The mercuric ions bound to the MerR homodimer in a tricoordinate

manner, with one cysteine from one monomer (C82) and two cysteines from the other monomer (C117, C126) involved (Helmann *et al.* 1990). Recent work on an engineered single chain polypeptide mimic of the MerR metal binding domain, normally created by the interaction of 2 MerR homodimers, has confirmed earlier data that it will bind thiophilic metals as well as Hg(II) (Song *et al.* 2004).

Mutagenesis of cysteine and histidine residues in CueR and ZntR has also shown that cysteine coordination is important for binding of copper and zinc. In ZntR, cysteine residues C79, C114, C115 and C124 are essential for Zn(II) induction from P_{zntA} *in vivo* (Khan *et al.* 2002), whilst mutagenesis of histidine residues H29, H53, and H119 has shown that they are important in Zn(II)-mediated ZntR activation of P_{zntA}. C115 is not required for Pb(II) and Cd(II) induction from P_{zntA} *in vivo*, nor are H29, H53 or H119. For CueR, C112 and C120 are essential for *in vivo* activation of P_{copA} by Cu(I), Ag(I) and Au(I) (Stoyanov & Brown 2003).

Deletion mutants of MerR from Tn*21* (Zeng *et al.* 1998), and subsequent structural studies of metallated CueR and ZntR (Changela *et al.* 2003) have clearly demonstrated that there is a short region close to the dimerization interface helix in which key cysteine residues are located, and where metal binding occurs. One Cu(I) coordinates to C112 and C120 in the metal-binding loop of one of the CueR homodimers. For ZntR, two Zn(II) atoms are coordinated by the metal binding loop. The first Zn(II) is coordinated by C114 and C124 from one monomer, and by C79 of the other monomer. The second Zn(II) is coordinated by C115 and H119 of the metal binding loop and C79 of the other monomer (Changela *et al.* 2003), echoing earlier findings on Hg(II) coordination in MerR between one cysteine (C82) in one monomer and C117 and C126 in the other monomer (Helmann *et al.* 1990).

In the case of SoxR, which normally exists as a homodimer, and contains one [2Fe–2S] cluster per monomer, mutagenesis of cysteine residues has shown C119, C122, C124 and C130 to be essential for *in vivo* activation of transcription of the *soxS* promoter (Bradley *et al.* 1997). Changela *et al.* (2003) have predicted that cysteine residues will coordinate to Fe(II) in a similar manner to the binuclear Zn(II) site in ZntR.

Amino-acid sequence alignments highlight extensive similarities in the sequences of the metal-responsive MerR family regulators, and are consistent with the shared overall fold apparent in the crystal structures of CueR and ZntR (Changela *et al.* 2003). Furthermore, Cysteine residues implicated in the binding of Hg(II) by MerR and Pb(II) by PbrR align with their counterparts in the CueR and ZntR sequences indicating that the metal-binding loops of these two proteins are located in similar positions to those demonstrated in the crystal structures of CueR and ZntR. Homology modelling of the MerR structure suggests a trigonal coordination of the bound metal ion by C82, C117 and C126, as shown experimentally. In contrast to this, the sequence of the organic-responsive regulator BmrR shows little similarity in the ligand binding domain with differing loops being responsible for binding the ligand. Despite this, however, it is clear that BmrR shares the same fold and overall structure as the metal-responsive regulators (Heldwein & Brennan 2001). Similarly, MtaN, a C-terminally truncated regulator that retains only the N-terminal DNA-binding domain and the interdomain helix, and is a constitutive activator, shares the common MerR-family fold for its DNA-binding domain and interdomain helix. Thus it is likely that all members of this family (both metal sensing and non-metal sensing) share a common mechanism for transmission of the effects of ligand binding into a transcriptional response when these proteins are bound to their cognate promoter DNA.

Unfortunately, an understanding of the mechanism by which this signal-response communication is achieved is hindered by the fact that all of the current X-ray crystal structures are of activator forms of the regulators. However, structures have been determined for MtaN both in the presence and absence of DNA (Newberry & Brennan 2004). Comparison of these two structures shows that there is no change in the internal conformation of the DNA-binding domain of MtaN upon binding DNA, but an overlay of the interdomain helices of the two forms shows an 11° rotation and 6 Å translation of the DNA-binding domains relative to each other. This movement is achieved by flexing a hinge region spanning residues 71–75 in MtaN (corresponding to 75–79 in CueR and 77–81 in ZntR) that lies at the N-terminus of the interdomain helix. It is perhaps significant that the central

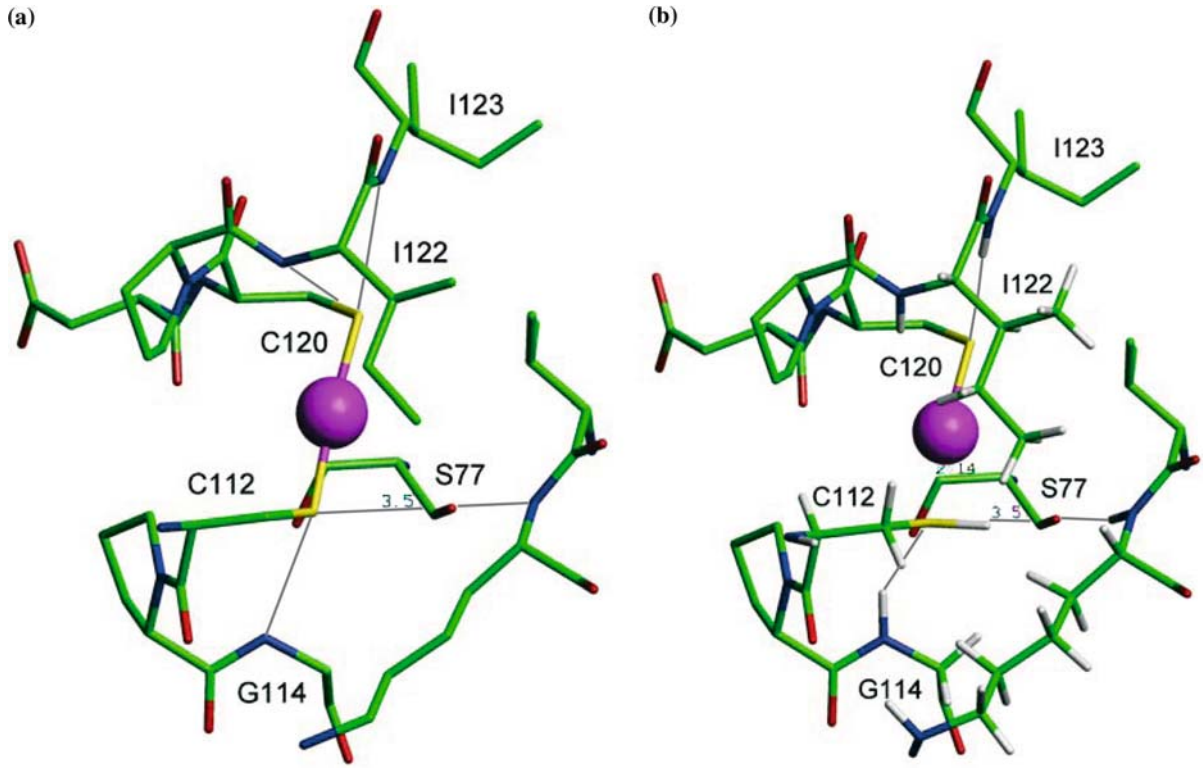

Figure 3. (a) X-ray crystal structure of CueR (entry 1q05) showing hydrogen-bonding contacts in the metal-binding site and (b) showing the proposed hydrogen locations for C112, C120 and interacting groups.

residue of this hinge region, C79, is involved in metal binding in both ZntR and MerR (C82) and it is tempting to suggest that binding of the metal ion results in a change in the position of this residue which, as it lies at the N-terminus of the rigid interdomain helix, serves to propagate this positional change into the rest of the DNA-binding domain. Against this however, the hinge region of CueR does not participate directly in metal binding. Instead, we can observe a close contact between the carbonyl O-atom of S77 (positionally analogous to the metal-binding C79 of ZntR) and the metal-binding S-atom of C112 (3.57 Å). Though O'Halloran and co-workers described C112 as a *thiolate* (Changela *et al.* 2003) it is more reasonable to suppose that this residue is in the *thiol* form, with the proton involved in a hydrogen-bond to the carbonyl O-atom of S77. This suggestion is supported by the hydrogen-bonding environment of the two metal-binding cysteines in the crystal structure of CueR – C112 accepts only a single hydrogen-bond (from the backbone NH of G114) whereas C120 accepts hydrogen-bonds from backbone amides of both I122 and I123 and is a more convincing thiolate (Figure 3).

In this example, binding a Cu(I) ion to the thiol of C112 will modify its proton affinity and subsequently impact on both the strength and length of any hydrogen-bond to S77, and may therefore fulfill a similar role to direct interaction between the equivalent cysteine (C79) and the Zn(II) ion seen for ZntR.

One of the unresolved questions about the response of one of the regulator-promoter systems to its cognate metal is where the specificity of response resides. Is it in metal coordination and binding? Is it in the conformational change induced by the bound metal? Or is it in the deformation of DNA by the metal-protein complex? In reality it may be a combination of all three. The coordination of the metal by cysteine or histidine residues may affect the binding affinity of the protein for the metal; CueR, for example, may be able to compete successfully with other intracellular ligands for Cu(I), but not for Zn(II). Even if both Cu(I) and Zn(II) bind to ZntR with similar

affinities, say, only Zn(II) may coordinate the correct residues to alter the conformation of the protein. Even if metal binding changes the conformation of the protein, there may be a difference in the extent of change. The fact that P_{zntA} has a spacer region of 20 bp between the −10 and −35 sequences, whereas that of P_{copA} is 19 bp, suggests that the deformation of P_{zntA} must be greater, as both are recognized by the σ^{70} subunit of RNAP.

Conclusions

Recent experimental data and structural models of the MerR family of regulators are revealing remarkable insights into how bacteria can differentiate between different metals, and activate expression of genes accordingly. This is essential for cell viability, as efflux of a metal in response to another stimulus (*e.g.*, copper efflux in response to elevated zinc concentrations) could deplete the cell of an essential nutrient. Despite this requirement for specificity, the fact that some toxic metals such as Ag(I), Au(I); or Cd(II), Pb(II) can activate gene expression by CueR and ZntR, respectively indicates the difficulties of discriminating between different metals, and may explain in part their toxicity to bacteria.

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