Reports

The MerR Metalloregulatory Protein Binds Mercuric Ion as a Tricoordinate, Metal-Bridged Dimer

JOHN D. HELMANN, BARRY T. BALLARD, CHRISTOPHER T. WALSH*

Bacterial MerR proteins are dimeric DNA-binding proteins that mediate the Hg(II)-dependent induction of mercury resistance operons. Site-directed mutagenesis of the *Bacillus* sp. RC607 MerR protein reveals that three of four Cys residues per monomer are required for Hg(II) binding at the single high-affinity binding site. Inactive mutant homodimers can exchange subunits to form heterodimers active for Hg(II) binding. Studies of a heterodimer retaining only three of eight cysteine residues per dimer reveal that Cys^{79} in one subunit and Cys^{114} and Cys^{123} in the second subunit are necessary and sufficient for high-affinity Hg(II) binding in an asymmetric, subunit bridging coordination complex.

THE EXPRESSION OF GENETIC INFORMATION is frequently regulated by transcription factors that modulate the synthesis of mRNA (*1*). Bacterial mercury resistance is mediated by an inducible set of genes arranged in a single operon under control of the metal-sensing MerR protein (*2–6*). MerR, like other metalloregulatory proteins (*2, 3*), is thought to respond to changing intracellular metal ion concentrations by a rapid and reversible interaction with its regulatory ligand. Mercury resistance depends on expression of proteins for the import of toxic mercuric ion [Hg(II)], the reduction of Hg(II) to volatile Hg(0) by NADPH (the reduced form of nicotinamide-adenine dinucleotide phosphate), and, in the "broad spectrum" determinants, the cleavage of organomercurial compounds to the corresponding hydrocarbon and Hg(II) (*4–6*). This ensemble of proteins acts to cleanse the local microenvironment, primarily through the MerA-catalyzed reduction of the highly toxic Hg(II) ion to the less toxic, and readily volatized Hg(0) metal (*4–6*).

Mercury resistance is regulated both positively and negatively by the MerR protein. In the absence of Hg(II), MerR protein represses transcription by binding tightly to the *mer* operator region. When Hg(II) is present, the dimeric MerR protein binds a single Hg(II) ion to become a potent transcriptional activator, while remaining bound at the *mer* operator site (*7–11*). All three biochemically characterized MerR proteins (Tn501, Tn21, and *Bacillus* sp. RC607) are similar in DNA recognition specificity (*11*) and contain a single, high-affinity Hg(II) binding site per protein dimer (*8–10, 12*). Three Cys residues are evolutionarily conserved in all sequenced MerR proteins and, given the strength of Hg–S bonds, are obvious candidates as Hg(II) ligands (*2, 11*). Indeed, genetic studies of both the Tn501 and Tn21 MerR proteins show that these three conserved residues are all required for high-affinity Hg(II) binding and in vivo transcriptional activation (*9, 13, 14*). From studies with model compounds, it was anticipated that the bound Hg(II) ion could interact with two, or perhaps even three or four Cys residues (*2, 3, 15*).

To determine those amino acid residues responsible for Hg(II) binding, we have used site-directed mutagenesis to alter each of the four Cys residues in the *Bacillus* MerR protein to Ala both individually and in combination (Fig. 1). Only the wild-type and C12A (Cys to Ala substitution at position 12) proteins show any detectable Hg(II) binding in the presence of 1 m*M* 2-mercaptoethanol as a competitor (Table 1). Both of these proteins bind one Hg(II) ion per dimer with high affinity. In contrast, no binding of ^{203}Hg(II) above background could be detected to any of the other mutationally altered proteins by high-performance liquid chromatography (HPLC) analysis. Consistent with their failure to bind Hg(II), the C79A, C114A, and C123A mutant proteins all fail to activate transcription in vitro, and none show a Hg(II)-dependent alteration in their affinity for the *mer* operator sequence. Similar effects were observed when either Cys^{79} or Cys^{123} was replaced with His. However, none of these mutationally altered MerR proteins are significantly affected in their other biochemical activities. All purified proteins are dimeric in solution, bind tightly to the *mer* operator sequence, and function to repress transcription from the *mer* operon promoter in in

Table 1. Biochemical properties of wild-type and mutant MerR proteins. Wild-type and all mutationally altered MerR proteins were overexpressed in *Escherichia coli* K38 (*23*) and purified with heparin-Sepharose essentially as described (*11, 12*) and were >90% homogeneous by SDS–polyacrylamide gel electrophoresis (PAGE). Oligomeric state was assessed by chromatography on an SEC-125 HPLC size-exclusion column as described for the wild-type protein (*8, 9*). Protein fractions were assayed for ^{203}Hg(II) binding by either SEC-125 HPLC gel filtration chromatography (Bio-Rad) or nitrocellulose filtration to separate bound from free Hg(II) as described for the wild-type MerR protein (*8, 9*). MerR protein concentrations were typically 1 to 2 μ*M* dimer for these assays, except where otherwise indicated. Recognition of the *Bacillus mer* operator sequence was determined by gel-mobility shift assays in the presence or absence of 1 μ*M* Hg(II) as described previously (*11*). This dissociation constant (K_d) for MerR binding to the *mer* operator was estimated from the amount of protein required to alter the mobility of about 50% of the labeled, operator-containing DNA fragment under conditions of protein to DNA excess as previously described (*11*). Transcription assays were performed with purified MerR protein, purified *B. subtilis* σ^{43} RNA polymerase, and purified DNA template in reactions similar to those described previously (*11*). The reaction products were quantitated by either autoradiography of denaturing polyacrylamide gels or trichloroacetic acid precipitation and filtration. As a control, the ability of purified MerR proteins to affect the transcription of supercoiled pAR1707 (containing the strong bacteriophage T7 A1 promoter site) by *B. subtilis* RNA polymerase was assayed in parallel. No inhibition of transcription from this site was observed by any of the purified MerR proteins, nor was there evidence of ribonuclease contamination in the purified protein preparations by gel analysis of the resulting transcripts.

Protein	Dimers	K_d Hg(II) (*M*)	K_d DNA (*M*)		Transcription*	
			−Hg(II)	+Hg(II)	−Hg(II)	+Hg(II)
Wild type	+	$<10^{-7}$	10^{-10}	10^{-8}	R	A
C12A	+	$<10^{-7}$	10^{-10}	3×10^{-9}	R	A
C79A	+	ND†	10^{-8}	10^{-8}	R	R
C114A	+	ND	3×10^{-9}	3×10^{-9}	R	R
C123A	+	ND	3×10^{-10}	3×10^{-10}	R	R

*R indicates repressor and A indicates activator of transcription. †ND indicates binding not detected.

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

*To whom correspondence should be addressed.

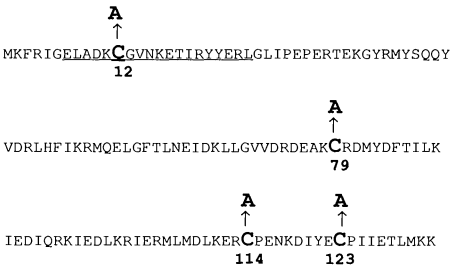

Fig. 1. Amino acid sequence of wild-type and mutationally altered *Bacillus* MerR proteins. The amino acid sequence of the MerR protein from *Bacillus* sp. RC607 is represented in the one-letter amino acid code (*22*). The Cys residues that have been altered to Ala are shown in boldface type. The underlined region designates the helix-turn-helix homology proposed to mediate site-specific recognition of the *mer* operator sequence (*11*). The *Bacillus merR* gene, present in a bacteriophage T7 promoter-based overexpression plasmid (pJHT7-2) (*12*), was used to prepare template for site-directed mutagenesis with the Amersham Mutagenesis Kit (Version 2.0) and the indicated oligonucleotide primers: (i) 5′GGCTGACAAGGCTGGTGTTAAC3′ (C12A), (ii) 5′GAAGCGAAGGCTCGTGATAT3′ (C79A), (iii) 5′TAAAGAAAGAGCTCCCGAAA3′ (C114A), and (iv) 5′ATTTACGAAGCTCCCATTATT3′ (C123A). The underlined residues are those that are different from the wild-type sequence (*23*). All mutant *merR* genes were completely sequenced to confirm the presence of the desired mutation and to verify that no additional mutations had occurred. Double and triple mutants were generated by additional rounds of mutagenesis or by recombination of restriction fragments and were confirmed by DNA sequence analysis.

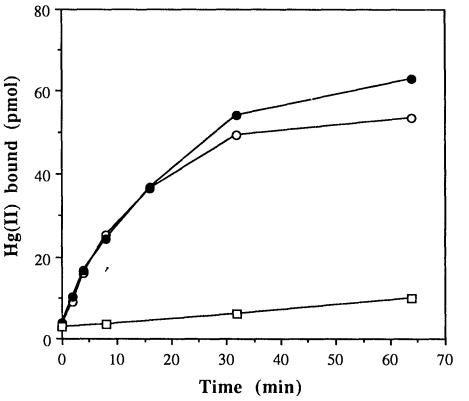

Fig. 3. Heterodimer formation is temperature-dependent and concentration-independent. Heterodimers were formed between MerR proteins as described for Fig. 2 with the indicated modifications. (○) 1 μ*M* C79A and C114A, incubated at 37°C; (●) 5 μ*M* C79A and C114A, incubated at 37°C; (□) 1 μ*M* C79A and C114A, incubated at 4°C. Samples containing 100 pmol of total MerR protein (50 μl of 2 μ*M* total MerR protein or 10 μl of 10 μ*M* MerR protein) were filtered onto nitrocellulose at the indicated times and bound Hg(II) determined by liquid scintillation counting. Qualitatively similar results are obtained with the combination of C79A and C123A or with the double- and triple-mutant combination shown in Fig. 2.

vitro transcription reactions (Table 1).

The ability of these mutant MerR homodimers to exchange subunits and reform an active Hg(II) binding site (heterodimer complementation) was investigated. When purified mutant proteins were incubated together in the presence of ^{203}Hg(II), a clear reconstitution of Hg(II) binding is observed with some, but not all, combinations of mutant proteins (Fig. 2). As expected for a statistical mixture of the two homodimers and heterodimers (1:1:2), ~50% of the Hg(II) binding activity of an equal concentration of wild-type protein was recovered. This binding site reconstitution reaction is temperature-dependent and occurs with a half-time ($t_{1/2}$) of ~10 min at 37°C (Fig. 3). This rate is due to slow subunit exchange rather than slow Hg(II) binding, as heterodimers first formed in the absence of Hg(II) will bind ^{203}Hg(II) as rapidly as the wild-type protein ($t_{1/2}$ < 15 s under these conditions). This result also shows that heterodimer formation proceeds in the absence of Hg(II). The metal binding site formed by heterodimer complementation has at least as high an affinity for Hg(II) as the intact, wild-type MerR protein. In all cases, ≥300 m*M* 2-mercaptoethanol is required to displace 50% of the bound Hg(II) ion from a 1 μ*M* solution of MerR protein or heterodimer in equilibrium binding experiments. This remarkably high Hg(II) affinity is presumably necessary for MerR to sense very low levels of intracellular Hg(II) in the presence of millimolar concentrations of glutathione.

The heterodimer complementation observed in these experiments appears to proceed by the dissociation of MerR dimers to monomers, followed by subsequent reassociation to dimers. The rate of heterodimer formation is first order with respect to protein concentration, as expected for a reaction in which dimer dissociation is the rate-limiting step (Fig. 3). In addition, this heterodimer complementation reaction appears to be species specific. The C79A *Bacillus* MerR protein does not reform an active Hg(II) binding site when mixed with a C117Y mutant MerR protein from the Gram negative–derived transposon Tn21 (*9, 13*), although the reaction with the *Bacillus* protein altered at the homologous position (C114A) proceeds readily. This suggests that the dimerization interface of these related MerR proteins (37% identical amino acids) has not been sufficiently conserved to allow facile subunit exchange.

Analysis of such heterodimer complementation experiments began with the single Cys to Ala mutants (Fig. 2, lanes 1 to 7) and allows a model for the high-affinity Hg(II) binding site in MerR proteins to be proposed. The C79A mutation can complement mutations at either Cys^{114} or Cys^{123} for Hg(II) binding, but mutations at Cys^{114} cannot complement mutations at Cys^{123} (Fig. 2). These data suggest that thiolate ligands from Cys^{114} and Cys^{123} must be present on the same subunit, whereas Cys^{79} from the opposing subunit can complete the coordination sphere. The simplest model consistent with these results is tricoordinate ligation to three thiolates as shown in Fig. 4. The salient features of this model are that: (i) Cys thiolates from both subunits are involved in a metal-mediated, subunit bridging interaction; (ii) all three of the evolutionarily conserved Cys residues are coordinated to Hg(II); and (iii) Cys^{114} and Cys^{123}

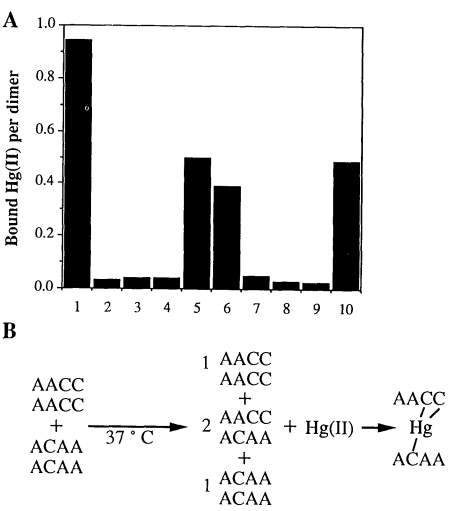

Fig. 2. In vitro reconstitution of Hg(II) binding activity from inactive mutant homodimers. (**A**) Hg(II) binding by homodimeric and heterodimeric MerR proteins. Lane 1, C12A; lane 2, C79A; lane 3, C114A; lane 4, C123A; lane 5, C79A + C114A; lane 6, C79A + C123A; lane 7, C114A + C123A; lane 8, C12A, C79A double mutant; lane 9, C12A, C114A, C123A triple mutant; and lane 10, C12A, C114A, C123A + C12A, C79A. (**B**) Schematic of the reaction represented in lane 10. In this abbreviated nomenclature, AACC represents MerR protein with Ala at positions 12 and 79 and Cys at positions 114 and 123 (Fig. 1). To allow heterodimer formation, purified samples of MerR protein were diluted in buffer A (10 m*M* tris-HCl, *p*H 7.5, 5% glycerol, 400 m*M* NaCl, and 10 m*M* 2-mercaptoethanol) to a concentration of ~2 μ*M* protein dimer, and equal amounts of two mutant MerR proteins were combined and incubated at 37°C for ≥60 min in the presence of a molar excess of ^{203}Hg(II). The amount of bound ^{203}Hg(II) was quantitated by nitrocellulose filtration and liquid scintillation counting as described (*12*). All data were collected in triplicate, averaged, and normalized to the amount of total MerR protein present in the reaction. Maximum observed variation (range) between replicate samples corresponded to 0.04 Hg(II) per dimer.

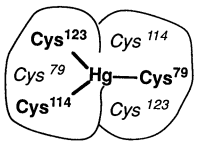

Fig. 4. The tricoordinate Hg(II) binding site of the MerR metalloregulatory protein. The Cys residues in italics represent the symmetrically related site that is presumed to overlap the site represented here.

from one subunit and Cys^{79} from the other subunit are coordinated to Hg(II). This model is also consistent with results from 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) protection experiments and disulfide-mediated cross-linking of MerR (*16*). The binding of Hg(II) to MerR leads to a coordination asymmetry in the protein. As MerR proteins only bind one Hg(II) ion per dimer, we suspect that the two symmetrically related potential Hg(II) binding sites may physically overlap, thereby precluding their simultaneous occupation.

To rigorously test this tricoordinate model of Hg(II) ligation, we investigated the ability of various double and triple Cys to Ala mutant proteins to form heterodimers active for Hg(II) binding. Purified MerR protein that retains only Cys^{79} (the triple mutant C12A, C114A, C123A) can complement the MerR protein that retains only Cys^{114} and Cys^{123} (C12A, C79A) to reconstitute a high-affinity Hg(II) binding site (Fig. 2, lane 10). The resulting mixture contains both types of homodimers together with heterodimers that retain only three of the eight Cys residues of the wild-type protein (Fig. 2B). The active Hg(II) binding species is a dimer, as judged by HPLC–gel filtration chromatography, and is at least as high in Hg(II)-binding affinity as the Hg(II) complex formed with wild-type MerR protein as judged by thiol competition assays. Thus, the three evolutionarily conserved Cys residues are both necessary and sufficient for formation of the high-affinity Hg(II) binding site in the MerR protein dimer.

Physiological evidence in support of this tricoordinate model is provided by a genetic analysis of the Tn21 MerR protein (*13*). In vivo, MerR proteins with Cys to Tyr alterations at position 82 (C82Y) can complement mutations at positions 117 and 126 (C117Y and C126Y) and restore a low but detectable level of Hg(II)-responsive transcriptional activation (*13*). In contrast, the C117Y and C126Y mutations cannot complement each other. The tricoordinate ligation model presented here can account for these genetic results. As this genetic complementation pattern mirrors that observed with the *Bacillus* point mutants for Hg(II) binding, the heterodimers reported here are likely to be functional under physiological conditions. In preliminary studies, the heterodimer mixtures we have produced do not strongly activate transcription in in vitro reactions, although this may simply reflect the presence of competing homodimeric species that act as transcriptional repressors, even in the presence of Hg(II) (Table 1), and the relatively tighter DNA binding affinity of the metal-free MerR protein (*10, 12*). It is likely that homogeneous populations of heterodimers will be required to answer the question of transcriptional activation definitively.

Tricoordinate ligation may also pertain to MerR:Hg(II) complexes of the Tn501 and Tn21 proteins, with the additional complication that a fourth, nonconserved Cys (Cys^{115}) is present in this region as well. Interestingly, replacement of Cys^{115} with Ala leads to a protein that is a better transcriptional activator than wild-type MerR (*14*). This super-activator phenotype is consistent with the notion that alternative coordination geometries can occur in the wild-type MerR protein, some of which may be less effective for transcriptional activation (*14*). For example, an alternative coordination geometry in which Cys^{115} replaces Cys^{117} could account for the residual in vitro Hg(II) binding and the weak Hg(II) response shown in vivo by the C117A mutant Tn501 MerR protein (homologous with the *Bacillus* sp. RC607 C114A MerR protein) in the presence of high concentrations of Hg(II) (*14*). In addition, recent characterization of Tn501 MerR:Hg(II) complexes by extended x-ray absorption fine structure spectroscopy (EXAFS) has led to a model in which bound Hg(II) is three coordinate to Cys thiolates with an average S-Hg bond length of 2.43 Å (*17*). This structure is consistent with the bond lengths observed in crystallographically determined $Hg(SR)_3$ structures (*18, 19*).

In addition to their essential catalytic and structural role in many enzymes, metal ions are increasingly recognized as an essential component of many transcription factors. Bound zinc atoms appear to have a structural role in the many transcriptional regulatory proteins of the zinc-finger family (*20*). Other metal ions interact with regulatory factors that mediate metal ion–specific regulation of gene expression. Such metalloregulatory proteins include the Fe(II) binding Fur protein from the enteric bacteria and the copper-binding protein, ACE1/CUP2, from *Saccharomyces cerevisiae* (*21*). The results of the present study provide a model for the structural features that allow the MerR family of metalloregulatory proteins to bind their regulatory metal ligand with extremely high affinity and specificity while retaining the kinetic lability essential in a rapidly responding genetic system.

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16. The ability of bound Hg(II) to protect Cys residues from reaction with the thiol-specific reagent DTNB was investigated for the wild-type MerR protein (*12*) and the ACCC mutant protein. In both cases, the presence of bound Hg(II) reduced the number of DTNB accessible thiols by three to four per protein dimer, consistent with tricoordinate ligation and partial occlusion of an additional Cys residue. In addition, as has been reported elsewhere for the Tn501 protein (*8*), MerR readily forms disulfide-linked dimers by autoxidation. This reaction is observed to occur with the wild-type *Bacillus* MerR protein and a number of the mutant proteins lacking particular Cys residues. This cross-linking reaction is greatly diminished, however, in mutant homodimers that lack either Cys^{79} or both Cys^{114} and Cys^{123}. This suggests that the predominant disulfide-linked species may be a cross-link between Cys^{79} on one subunit and either Cys^{114} or Cys^{123} on the other subunit. This agrees with the physical proximity predicted for these residues in the tricoordinate model we now propose.
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22. The one-letter amino acid code is as follows: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.
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