Transcriptional Switching by the Metalloregulatory MerR Protein: Initial Characterization of DNA and Mercury(II) Binding Activities†

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Received September 22, 1988

ABSTRACT: The MerR protein from the Tn*501* mercury resistance operon is a metalloregulatory transcriptional switch, converting from repressor to activator on binding of Hg(II). We have determined via binding studies with ^{203}Hg(II) that a single Hg(II) atom binds to the MerR dimer (32 kDa) with a half-saturation concentration of 10^{-7} M in the presence of up to 10^{-3} M exogenous thiols. This 10^4 selective binding is specific for the binding of Hg(II) and corresponds to concentrations of metal that induce mercury(II) resistance in vivo. K_d values for MerR binding, in the absence and presence of Hg(II), to a 305 bp DNA fragment containing the 18 bp dyad symmetry element, DS1, located at −35 to −10 upstream of the *mer* structural genes, were determined by a gel shift assay. A K_d of 10^{-10} M for free MerR and 10^{-11} M for Hg(II)–MerR complexes was revealed. Measurements of k_{off} values, by this assay, show equally long-lived complexes of MerR–DNA (51-min half-life) and Hg(II)–MerR–DNA (49-min half-life), suggesting that Hg(II) accelerates MerR binding to DNA rather than influencing the dissociation rate of the protein–DNA complex. In contrast, ^{203}Hg(II) studies reveal that mercuric ions rapidly dissociate and associate with MerR–DNA complexes. Extensive footprinting studies by DNase I, methylation protection, and hydroxyl radicals indicate MerR stays bound to DS1 even on addition of Hg(II) and shares no interaction in vitro with a second dyad symmetry element, DS2, centered at −79/−80. Studies with DTNB and pHMB titration of protein thiols and alkylation studies with iodo[^{14}C]acetamide, in the presence and absence of stoichiometrically bound Hg(II), allow initial assessment of roles for Cys-82, -115, -117, and -126 as potential ligands for Hg(II). A tryptic fragment of 1–120 amino acids (or 1–121 aa) still dimerizes and binds specifically to *mer* DNA but has lost ^{203}Hg(II) binding capacity.

Bacteria can acquire resistance (Hg^R) to the toxic effects of heavy-metal mercury(II) salts via the genes of the *mer* operon, frequently located on plasmids and transposable elements. The best characterized *mer* operons to date are from Tn*501* and from Tn*21* [for a review, see Summers (1986) and Foster (1987)]. Mercury resistance is effected at the molecular level by the coordinate action of the operon-borne *merT*, *merP*, and *merA* genes (Figure 1); *merT* and *merP* encode membrane and periplasmic proteins, respectively, involved in Hg(II) uptake; *merA* encodes a unique enzyme, mercuric ion reductase, that detoxifies internalized Hg(II) salts by reduction to nontoxic, volatile Hg(0). Mercury-resistant bacteria respond to inducing doses (10^{-8}–10^{-6} M) of Hg(II) by amplifying the coordinate transcription rate of the *merTPA* (abbreviated hereafter as *mer*) genes roughly 100-fold (Lund & Brown, 1987). This positive transcriptional control in the *mer* operon is effected by a metalloregulatory DNA binding protein, MerR, the product of the divergently transcribed *merR* gene (Figure 1). MerR activates transcription from the *mer* promoter in the presence of Hg(II) and represses transcription from the *mer* promoter in the absence of Hg(II) (Lund et al., 1986). In addition, MerR negatively regulates its own synthesis, both in the presence and in the absence of Hg(II), from the overlapping but divergent *merR* promoter. The transcriptional control region of the Tn*501* *mer* operon possesses two regions of strong dyad symmetry that are potential binding sites for MerR: an 18 base pair inverted repeat, DS1, embedded between the "−35" and "−10" recognition elements of the *mer* promoter; and a 16 bp dyad, DS2, centered on positions −79/−80 with respect to the startpoint of *mer* mRNA.

Our recent attempts to elucidate the molecular basis of heavy-metal-mediated genetic switching in the *mer* operon have resulted in the subcloning, overexpression, and partial purification of the Tn*501* MerR protein as a 32-kDa dimer. Initial DNase I footprinting studies demonstrated protection by MerR of a 26 bp sequence encompassing DS1 (O'Halloran & Walsh, 1987). Inspection of the primary amino acid sequence of the 144-residue Tn*501* MerR led us and others to postulate that the protein has two domains, an amino-terminal DNA binding domain and a carboxy-terminal Hg(II) binding domain. Specifically, two regions in the N-terminal half may be predicted to form the helix–turn–helix DNA binding motif common to many bacterial repressor/activator molecules (Pabo & Sauer, 1984); a high density of potential metal binding residues in the C-terminal half, particularly the four cysteine residues at positions 82, 115, 117, and 126, strongly suggests that this region is involved in Hg(II) ligation.

In this paper, we examine more fully the interactions of MerR protein with operator DNA, including the thermodynamics and kinetics of specific DNA association. Detailed footprinting studies have been undertaken in order to (i) assess the roles of DS1 and DS2 in transcriptional regulation and (ii) determine how the binding of Hg(II) to MerR affects its DNA binding. Further, we report our findings on the specificity and stoichiometry of Hg(II) binding by MerR. Preliminary observations on the role of individual cysteine residues of the MerR–Hg(II) complex, as derived from the results of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[1] titrations and iodo[^{14}C]acetamide alkylations, are presented. The findings

† This work was supported in part by a grant from the National Institutes of Health (GM20011).

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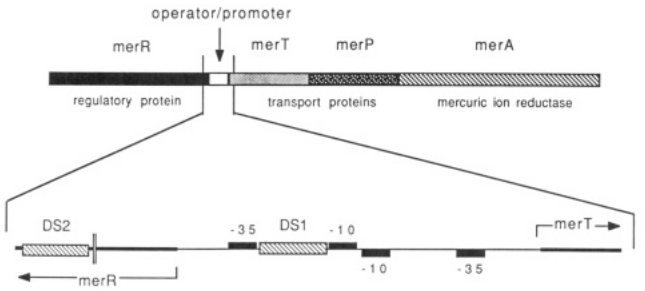

FIGURE 1: Structure of the *mer* operon from Tn*501*.

reported here are discussed in the context of prokaryotic transcriptional regulation and metal–protein interactions. In the following paper (Shewchuk et al., 1989), we describe the purification and initial structural and functional characterization of mutant MerR proteins answering a genetic selection for an activation minus (a^-) and/or a repression minus (r^-) phenotype; these studies complement and extend those reported in the present paper with respect to the identification of separate, distinct functional domains in MerR, and the assignment of individual cysteine residues to roles in MerR structure and Hg(II) ligation.

Materials and Methods

Materials

Restriction endonucleases and DNA modification enzymes were from New England Biolabs. Deoxyadenosine [α-^{32}P]-triphosphate ([α-^{32}P]dATP) and $^{203}HgCl_2$ were purchased from New England Nuclear. Iodo[1-^{14}C]acetamide was from Amersham. Proteases were from Sigma Chemical Co. TPCK and TLCK were from Boehringer Mannheim. Molecular weight standards were from Pharmacia. All chemicals were of the highest grade available and were used without further purification.

Methods

Protein Purification. MerR was purified from *Escherichia coli* JM105 harboring the plasmid pTO90-16 (O'Halloran & Walsh, 1987), using a modified method [see Shewchuk et al. (1989)]. The purity of the protein preparations was assessed by SDS–PAGE according to the method of Laemmli (1970).

Atomic absorption analysis of the protein was performed by Dr. B. Holmquist, Harvard Medical School, according to standard practice.

Gel Binding Assay: Determination of Equilibrium and Kinetic Constants of Operator DNA Binding. A 305 bp *Hin*dIII/*Eco*RI fragment from the plasmid pJOE114 (Brown et al., 1986) was used as a source of *mer* operator DNA. DNA of high specific activity was generated by replacement synthesis with [α-^{32}P]dATP using T4 DNA polymerase as described by Maniatis et al. (1982). DNA concentrations were determined fluorometrically according to the method of Le Pecq and Paoletti (1966). Labeled fragment (approximately 500 cpm/reaction) was gently mixed with MerR in the presence and absence of 1 μM Hg(II) in gel binding assay buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 μg/mL BSA, 5 μg/mL salmon sperm DNA, 5% glycerol, 0.05% Nonidet P40, and 10 μM 2-mercaptoethanol] and allowed to equilibrate at 4 °C for 2 h. At that time, aliquots were loaded onto an 8% polyacrylamide gel and electrophoresed at 250 V in Tris–acetate buffer (pH 8.0) for 1 h. The gel was subsequently blotted onto filter paper, dried under vacuum, and exposed to Kodak XAR-5 film using a DuPont Cronex Lightning Plus intensifying screen. Band intensities were quantified by scanning laser densitometry using an LKB densitometer.

The rate of dissociation of the MerR–operator complex was measured by mixing protein with DNA at concentrations at which most of the operator was bound and allowing the mixture to equilibrate for 2 h. A 100-fold molar excess of cold plasmid operator DNA was then added, and aliquots were removed at fixed time points and immediately loaded onto a running gel.

DNA Footprinting. A 685 bp *Ava*I/*Eco*RI fragment from pJOE114 (Brown et al., 1986) was the source of operator DNA for all footprinting reactions. DNA fragments were end-labeled with ^{32}P on the 3′ or 5′ end using Klenow fragment or T4 DNA kinase, respectively, as described by Maniatis et al. (1982).

DNase I footprinting reactions were carried out essentially as described by Galas and Schmitz (1978). Reactions were performed in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 2 mM $CaCl_2$, 10 μM 2-mercaptoethanol, 250 μg/mL BSA, and 25 μg/mL salmon sperm DNA. MerR, in the presence or absence of 1 μM Hg(II), was incubated with operator DNA for 30 min at 24 °C. DNase I was added to a final concentration of 22 ng/mL and incubation continued for an additional 10 min. Reactions were quenched with 5 M ammonium acetate; the DNA was precipitated, resuspended in deionized formamide loading dye, and loaded onto a 6% polyacrylamide sequencing gel.

Dimethyl sulfate (DMS) methylation protection experiments were performed as described by Siebenlist and Gilbert (1980). MerR, in the presence or absence of 1 μM Hg(II), was incubated with operator DNA in 50 mM sodium cacodylate (pH 7.5), 10 mM $MgCl_2$, 200 mM KCl, 10 μM 2-mercaptoethanol, 1 mM $CaCl_2$, 250 μg/mL BSA, and 5 μg/mL salmon sperm DNA for 30 min at 24 °C. Reactions were cooled to 4 °C, followed by addition of DMS for 10 min. Further methylation was stopped by addition of 1.5 M NaOAc, 1 M 2-mercaptoethanol, and 100 μg/mL yeast tRNA. DNA was precipitated and electrophoresed as above.

Hydroxyl radical footprinting reactions were carried out as described by Tullius and Dombroski (1986). MerR, in the presence or absence of 1 μM Hg(II), was incubated with operator DNA in the same buffer as for the DNase I reactions. The radical reactions were initiated by the addition of 0.2 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.4 mM EDTA, 0.6% H_2O_2, and 20 mM ascorbic acid. Reactions were allowed to proceed for 2 min and then quenched with 0.1 M thiourea and 3 M NaOAc. Samples were extracted with TE-saturated phenol, precipitated, and electrophoresed as above.

Gel Filtration and Nitrocellulose Filter Binding Assays with ^{203}Hg(II): Measurement of Hg(II) Stoichiometry and Binding Affinity. MerR in gel filtration buffer (GFB)[50 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 5% glycerol] was incubated with ^{203}Hg(II) (1000 Ci/mol) in the presence of 1 mM 2-mercaptoethanol for 30 min on ice; 100-μL aliquots were loaded onto a Bio-Sil TSK-125 HPLC gel filtration column (7.5 mm × 300 mm) equilibrated in GFB. The column was run at 0.5 mL/min, and 1-mL fractions were collected. The fractions of bound and free ^{203}Hg(II) were quantified by liquid scintillation counting on a Beckman LS-100 scintillation

[1] Abbreviations: BSA, bovine serum albumin; DMS, dimethyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; pHMB, *p*-(hydroxymercuri)benzoate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

counter. The protein concentration in each fraction was determined by the Bradford assay using the peptide bond absorbance (A_{205}) of purified MerR as a standard.

For nitrocellulose filter binding assay, ^{203}Hg(II) was added to protein samples in 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM 2-mercaptoethanol, 50 μg/mL BSA, and 5% DMSO. Aliquots (1 mL) were filtered on a Millipore filtration apparatus at 15-s intervals after the addition of Hg(II) and washed with 2 mL of the same buffer. The percent of ^{203}Hg(II) bound to the protein was quantified by liquid scintillation counting of the filter disks (BA85, Schleicher & Schuell). The rate of exchange between protein-bound Hg(II) and free Hg(II) was determined by adding a 100-fold molar excess of Hg(II) to a preformed ^{203}Hg(II)–MerR complex. Aliquots were removed at 15-s intervals, filtered, washed, and counted as described above.

Thiol Titrations. Thiol titrations with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) on denatured and native samples, in the presence or absence of a stoichiometric amount of Hg(II), were carried out by the method of Riddles et al. (1983) using a 100-fold molar excess of DTNB. Extinction coefficients of 13 700 and 14 150 M^{-1} cm^{-1} at 412 nm were used for TNB^- under denaturing and native protein conditions, respectively.

Thiol titrations of native MerR with *p*-(hydroxymercuri)-benzoate (pHMB) were performed in 50 mM Na_2HPO_4 (pH 7.2) and 10 μM cysteine buffer as described by Boyer (1954). Aliquots of pHMB were added until no further change in absorbance at 250 nm was observed.

All spectroscopic measurements described above were performed on a Hewlett-Packard 8452A diode array spectrophotometer.

Iodoacetamide Alkylation and Isolation of Labeled Peptides. MerR, in the presence or absence of a stoichiometric amount of Hg(II) [ca. 0.5 mg of protein in 1 mL of 10 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5% glycerol, and 10 μM 2-mercaptoethanol], was alkylated with a 100-fold molar excess of iodo[^{14}C]acetamide (2.65 mCi/mmol) for 10 min in the dark at 25 °C. The reaction was quenched by the addition of 10 μL of 2-mercaptoethanol, and the protein was precipitated with 10% trichloroacetic acid. Protein pellets were washed twice with cold acetone and resuspended in 8 M urea. The remaining free thiols were exhaustively alkylated by addition of cold iodoacetamide to a final concentration of 4 mM. After incubation in the dark for 1 h, the reactions were again quenched with 2-mercaptoethanol and the samples extensively dialyzed against 2 M urea and 100 mM Tris-HCl (pH 8.0) at 25 °C.

Iodoacetamide alkylations were also carried out under denaturing conditions. Samples of MerR were denatured with 8 M urea, incubated with 10 μM DTT for 1 h at 37 °C, and then alkylated in the same manner as above.

Following dialysis, samples were digested with TPCK-treated trypsin (1% w/w) for 12 h at 25 °C. Peptides were separated on a Vydac Analytical Protein and Peptides C18 HPLC column and eluted with a 120-min linear gradient run at 1 mL/min from 5 to 60% acetonitrile in aqueous 0.1% trifluoroacetic acid; 1.5-mL fractions were collected and counted on a Beckman LS-100 liquid scintillation counter. Total amino acid analyses of the radiolabeled peptides were performed by W. Lane and D. Andrews of the Harvard University Microchemistry facility.

Partial Proteolysis with Trypsin. MerR [ca. 5 mg in 10 mM TrisHCl (pH 7.5), 250 mM NaCl, 5% glycerol, and 1 mM 2-mercaptoethanol] was digested with TPCK-treated trypsin (1% w/w) for 30 min at 4 °C. The reaction was stopped by addition of 10 μL of TLCK (50 mg/mL in ethanol) to give a 120 or 121 amino acid polypeptide in approximately 50% yield. The peptide was purified from undigested MerR and other minor proteolytic products by sequential chromatography on G75 (superfine) and G50 (superfine) gel filtration columns (1 × 30 cm), both equilibrated with 10 mM Tris-HCl (pH 7.5), 250 mM NaCl, 2 mM 2-mercaptoethanol, and 5% glycerol. N-terminal sequencing and total amino acid analyses were performed by the Harvard University Microchemistry facility. Native molecular weight determinations were performed on a Bio-Sil TSK-125 HPLC gel filtration column calibrated with ribonuclease A (13 700) chymotrypsinogen (25 000), ovalbumin (43 000), and bovine serum albumin (67 000); 100-μL samples were applied to the column, which had been equilibrated with 10 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM 2-mercaptoethanol, and 5% glycerol and eluted at 0.5 mL/min. The elution profile was monitored at 280 nm. All other assays performed on the tryptic peptide were as described above.

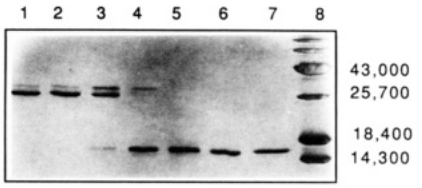

FIGURE 2: SDS–polyacrylamide gel electrophoresis of MerR in the presence of increasing concentrations of 2-mercaptoethanol. MerR (10^{-6} M) was incubated with 2-mercaptoethanol for 10 min at 24 °C followed by electrophoresis on a 16% SDS–polyacrylamide gel. Proteins were stained with Coomassie blue. (Lanes 1–7) MerR incubated in the following concentrations of 2-mercaptoethanol: (1) 10^{-6} M, (2) 10^{-5} M, (3) 10^{-4} M, (4) 10^{-3} M, (5) 10^{-2} M, (6) 10^{-1} M, (7) 1 M. Lane 8: molecular weight standards.

RESULTS

Overproduction, Purification, and Properties of MerR. We have developed an adaptation of our published method (O'-Halloran & Walsh, 1987) for rapid purification of Tn*501* MerR to homogeneity on a large scale (up to 25 mg of pure protein per preparation) [see Shewchuk et al. (1989)]. Chromatography on heparin–Sepharose, often useful in purifying DNA binding proteins, is the key step in the purification of wild-type Tn*501* and Tn*21* MerRs and *Bacillus* RC607 MerR (J. Helmann, unpublished data), as well as for the specific Tn*21* MerR mutants described in Shewchuk et al. (1989). This procedure removes tightly associated DNA, which was variably associated with MerR in the original preparations, the removal of which is important for the kinetic and thermodynamic studies on *mer* operator/promoter-containing restriction fragments, as well as for assessing the fraction of active MerR molecules in each preparation. Typically, the MerR preparations obtained by this method are >95% homogeneous as judged by SDS–PAGE analysis.

We have found that upon storage of pure MerR in thiol-free buffer, even in the frozen state, the 16-kDa monomer by SDS–PAGE is rapidly converted to a 32-kDa disulfide-linked dimer. Substantial quantities of 2-mercaptoethanol are required to quantitatively prevent or reverse this behavior, as shown in Figure 2. Prior determination of the oxidation state of MerR samples is important for evaluation of the metalloregulatory properties, since the disulfide-linked dimer still binds Hg(II) but is less active in specific DNA binding (vide infra). On the other hand, for studies on the stoichiometry of ^{203}Hg(II) binding to MerR and to MerR–DNA complexes, too much buffer thiol prevents Hg(II) association with MerR

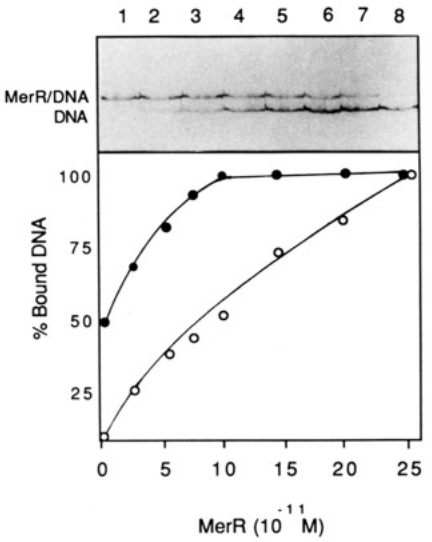

FIGURE 3: Concentration dependence of MerR–operator binding in the presence and absence of Hg(II). (Top) Autoradiogram of a gel shift assay, in the absence of Hg(II), performed as described under Materials and Methods. The concentration of MerR in each lane is as follows: (1) 2.5×10^{-10} M, (2) 2×10^{-10} M, (3) 1.5×10^{-10} M, (4) 1×10^{-10} M, (5) 7.5×10^{-11} M, (6) 5×10^{-11} M, (7) 2.5×10^{-11} M, (8) 1×10^{-11} M. (Bottom) Percent of bound operator DNA vs the concentration of MerR and MerR–Hg(II). Half-maximal binding was observed at 10^{-10} and 10^{-11} M, in the absence (○) and presence (●) of Hg(II), respectively.

by mass action competition. Thus, the buffer thiol:MerR ratio must be carefully controlled; in experiments that demand removal of exogenous thiols, this step is carried out immediately prior to use, ensuring that MerR is in its quantitative 8 thiol/dimer state (vide infra). In our hands, Tn*501* MerR can be stored, in the presence of 10 mM 2-mercaptoethanol, at 4 °C or −20 °C for 2 weeks without appreciable disulfide formation.

It has previously been noted that the arrangement of cysteine and histidine residues in the C-terminal domain of MerR bears some similarity to the zinc binding domain of so-called zinc finger proteins (O'Halloran & Walsh, 1987); thus, we were interested to determine whether MerR is able to coordinate Zn(II) at physiologically accessible concentrations. By atomic absorption analysis, MerR protein, prepared as above or supplemented with 10 equiv of Zn(II) followed by gel filtration to remove unassociated metal, contains ≤0.1 equiv of Zn(II)/subunit (J. Helmann, unpublished data).

Binding of Tn501 MerR to mer Operator/Promoter DNA. (A) K_d Measurements. Binding of homogeneous MerR protein to the 18 bp DS1 element (Figure 1) was examined with a ^{32}P-labeled 305 bp restriction fragment. A polyacrylamide gel shift electrophoresis assay was used to obtain the equilibrium dissociation constant (K_d) for MerR in the presence or absence of Hg(II), and also to determine the dissociation and association rates of the MerR–operator complex (vide infra). In order to obtain reproducible results, it was necessary to carry out all binding reactions and subsequent electrophoresis steps at 4 °C. Thus, the equilibrium and kinetic constants obtained here in vitro may differ somewhat from what would be observed at the more physiologic temperatures of 25 or 37 °C. A typical autoradiogram of a K_d determination, in the presence or absence of Hg(II), is shown in the top panel of Figure 3: the upper band corresponds to a MerR–operator DNA complex and the lower band to free DNA. The relative amounts of bound and free DNA were quantitated by scanning laser densitometry, and the percent of bound DNA vs the concentration of MerR is shown in the bottom panel of Figure 3. The concentration of operator DNA in all experiments ($\sim 10^{-12}$ M) was well below that of the protein. Under these conditions, the apparent K_d, calculated as the concentration of active protein required for half-maximal DNA binding, is determined to be 1×10^{-10} M in the absence of Hg(II) and 1×10^{-11} M in the presence of Hg(II). The concentration of active protein was determined by titration against a known amount of operator DNA at concentrations at least 10-fold above the equilibrium constant. MerR preparations typically contain 70–90% active molecules by this criterion. These equilibrium binding experiments suggest that binding of Hg(II) results in a ca. 10-fold increase in the affinity of the protein for its operator site on *mer* DNA.

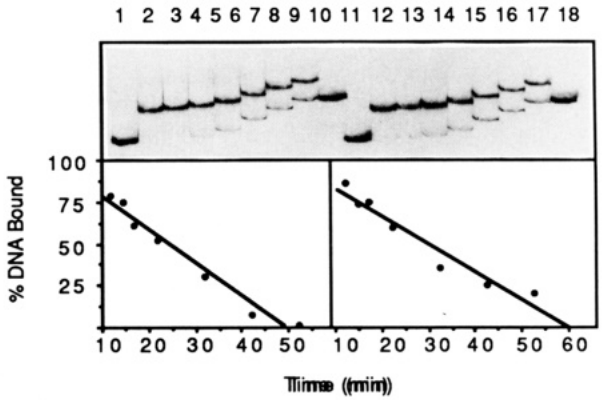

FIGURE 4: MerR–operator DNA dissociation kinetics, in the absence (lanes 1–9) or presence (lanes 10–18) of Hg(II), as determined by the gel shift assay. Autoradiogram of a dissociation time course performed as described under Materials and Methods. Reaction times for each lane are as follows: (2 and 12) 7 min, (3 and 13) 12 min, (4 and 14) 17 min, (5 and 15) 22 min, (6 and 16) 32 min, (7 and 17) 42 min, (8 and 18) 52 min, (1, 9, 10, and 18) contain no protein. Dissociation rates of 2.4×10^{-4} and 2.3×10^{-4} s^{-1} and half-lives of 49 and 51 min, in the presence and absence of Hg(II), respectively, were determined from this experiment.

(B) k_{off} Rates of MerR–^{32}P-mer DNA Complexes in the Presence and Absence of Hg(II). The observed high affinity of MerR for *mer* operator DNA suggests long lifetimes for the protein–DNA complex. The dissociation rate constants (k_{off}'s), in the presence or absence of Hg(II), were determined by the addition of a large (100-fold) excess of unlabeled plasmid DNA, containing the *mer* operator, to a preformed MerR–^{32}P-*mer* DNA complex. Once dissociation of the initial radiolabeled complex occurs, the vast majority of the released protein will reassociate with unlabeled rather than labeled DNA. Dissociation was stopped by loading the samples on a running polyacrylamide gel, and the percent of bound and free DNA was quantified by the gel shift assay (Figure 4). Dissociation of the MerR–operator complex follows first-order kinetics with a half-life independent of the DNA concentration. No significant difference, within experimental error, was observed if the concentration of the MerR–operator complex or the competitor DNA was varied over a 10-fold range (data not shown). The k_{off} measured in the absence of Hg(II) was 2.3×10^{-4} s^{-1}, corresponding to a half-life of the MerR–*mer* DNA of 51 min. In the presence of Hg(II), the k_{off} and half-life were 2.4×10^{-4} s^{-1} and 49 min, respectively. Thus, Hg(II) ligation to MerR does not influence the dissociation of the protein from its operator; this requires that the observed ca. 10-fold difference in K_d be reflected in the k_{on} step, indicating a faster association of Hg(II)–MerR than apoMerR with the operator site on *mer* DNA.

Mode of Interaction of MerR with mer Operator DNA: DNA Footprinting Analysis. (A) DNase I Footprinting. Our preliminary study on the binding of MerR to its operator

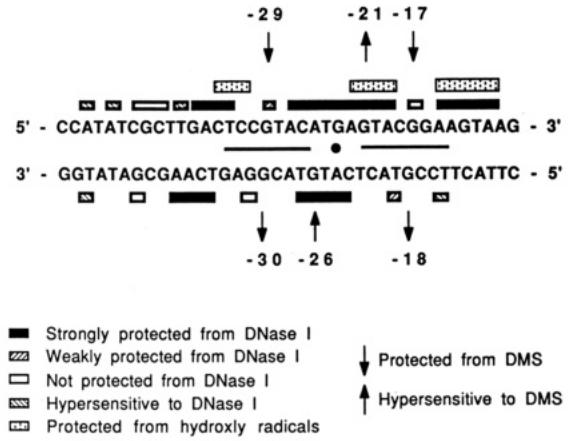

FIGURE 5: Summary of DNase I, DMS protection, and hydroxyl radical footprinting data for MerR binding to *mer* operator DNA (DS1).

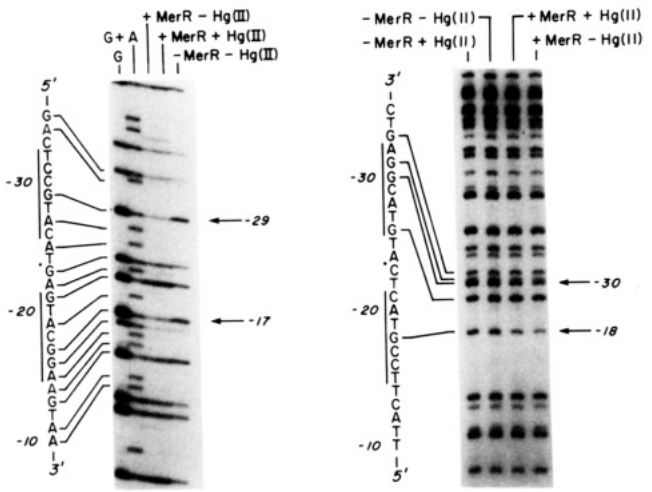

FIGURE 6: Dimethyl sulfate (DMS) protection experiments in the presence and absence of Hg(II). An end-labeled operator fragment (10^{-10} M) was incubated with MerR (10^{-8} M) in the presence and absence of Hg(II) (10^{-6} M). Reactions were performed as described under Materials and Methods. (Left) DMS protection of the top strand. (Right) DMS protection of the bottom strand.

DNA, utilizing DNase I footprinting, led to the conclusion that MerR binds to a 26 bp segment between the *mer* promoter's "−35" and "−10" sequence elements which encompass an 18 bp region of strong dyad symmetry, DS1 (O'Halloran & Walsh, 1987). Essentially identical DNase I footprints were obtained in the presence and absence of Hg(II). We have now repeated these DNase I reactions with MerR purified to homogeneity, on both the 3′ and 5′ strands of the operator DNA, and Figure 5 summarizes the results from several representative sets of experiments (data not shown). Several regions of differential protection, nonprotection, and hypersensitivity to DNase I are apparent on both strands of the operator DNA, and the presence or absence of Hg(II) has no discernible effect.

(*B*) *Dimethyl Sulfate Protection.* Methylation protection experiments, also summarized in Figure 5, were performed to detect any sequence-specific contacts made by MerR to guanines (N7) in the major groove or adenines (N3) in the minor groove of the DS1 region of *mer* DNA. Four guanine residues (−17, −18, −29, −30), two on the top strand (−29, −17; see Figure 6, left) and two on the lower strand (−30, −18; see Figure 6, right), appear to be partially protected from attack by DMS. Full protection of these guanines was never observed even under conditions which give rise to complete protection from DNase I or complete gel retardation in the gel shift assay.

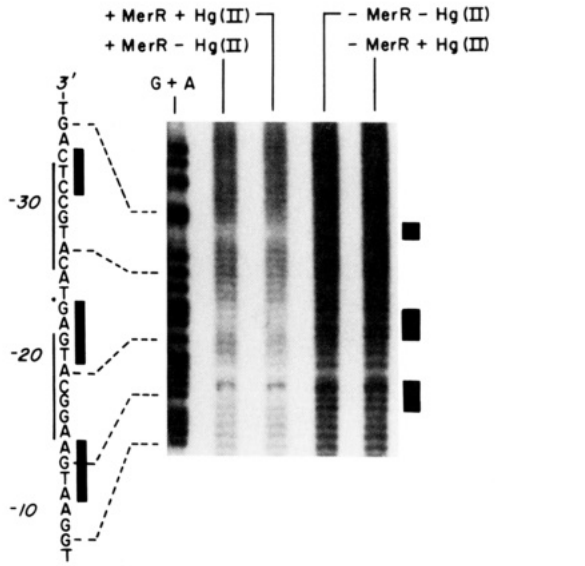

FIGURE 7: Hydroxyl radical footprinting of MerR in the presence and absence of Hg(II). Reactions were performed as described under Materials and Methods.

This result suggests that perhaps MerR does not bind directly to the N7 atom of the protected guanine but rather to a neighboring base, thereby partially occluding attack by DMS. Binding of MerR also results in hypersensitivity of an adenine at −22 and two guanines at −21 and −26 (Figure 6). As noted, DMS protection experiments performed in the presence of Hg(II) afforded essentially the same footprint, corroborating the DNase I footprinting (vide supra) results which indicate that MerR occupies DS1 both in the presence and in the absence of Hg(II). On occasion, one upstream G at −150 becomes less accessible to dimethyl sulfate when Hg(II) is added to MerR–DNA complexes (data not shown). While the significance of this phenomenon is as yet unknown, it does suggest at least minor reorganization of this stretch of DNA on Hg(II) ligation to MerR.

(*C*) *Hydroxyl Radical Footprinting.* As shown in Figure 7, we have also carried out hydroxyl radical footprinting experiments (only on the upper strand in Figure 5) in an attempt to detect even more subtle differences induced by the binding of Hg(II) by MerR. MerR appears to protect three regions within its operator from radical attack, the protected bases being a subset of those protected by DNase I. As with the results of the previous two footprinting methods, no difference is apparent upon addition of Hg(II).

Nature of the Interactions of MerR with Its Metalloregulatory Ligand Hg(II). Having outlined the tight and specific interactions of MerR with its operator DNA, we next focused attention on delineating the affinity and specific interactions of MerR with Hg(II). A priori, we reasoned that one or more of the four cysteine residues would be prime candidates for Hg(II) ligation given the enormous avidity of Hg(II) for bidentate thiol ligation [estimated K_d of 10^{-35}–10^{-44} M (Casas & Jones, 1980)]. To this end, we assessed Hg(II) binding stoichiometry and then the oxidation state and kinetic protection of cysteine thiols upon Hg(II) binding to MerR.

Stoichiometry and Specificity of Hg(II) Binding to MerR. Gel filtration experiments with ^{203}Hg(II) were used to establish the stoichiometry of the MerR–Hg(II) complex and the relative affinity of MerR for Hg(II) in the presence of competing thiols and other heavy metals. MerR (10^{-6} M) was incubated with increasing amounts of ^{203}Hg(II) and applied to a gel filtration column, resulting in the separation of protein-bound Hg(II) from free Hg(II). The stoichiometric ratio of MerR

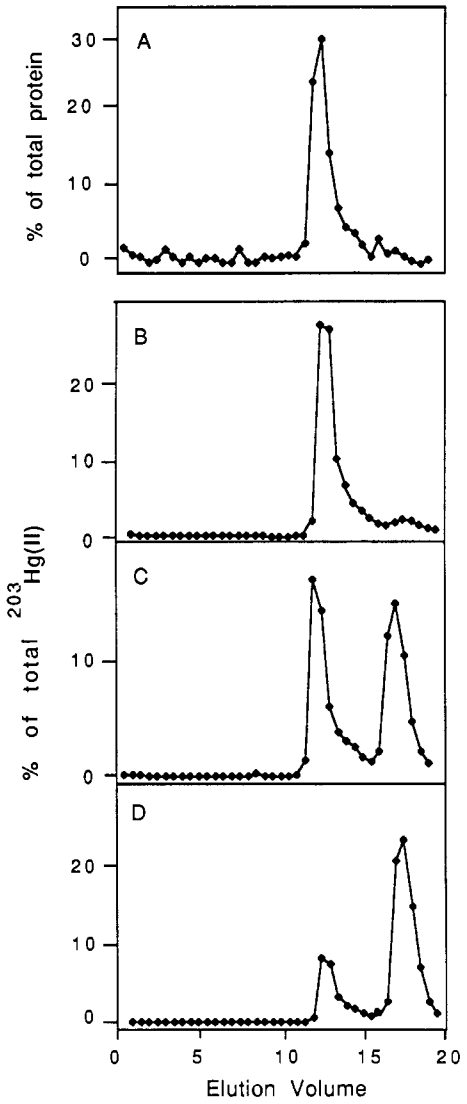

FIGURE 8: HPLC gel filtration profiles of MerR incubated with ^{203}Hg(II). (A) Chromatographic profile as determined by the Bradford assay. (B, C, and D) Profile, determined by liquid scintillation counting, of MerR in the presence of 1, 2, and 4 equiv of ^{203}Hg(II)/dimer, respectively.

to Hg(II) was calculated by determining the protein and radioactive Hg(II) concentrations in the MerR–Hg(II) complex, as well as determining the ratio of bound to free Hg(II). Typical gel filtration profiles are shown in Figure 8A–D. At 1 equiv of Hg(II)/protein dimer, the ^{203}Hg(II) is quantitatively bound to the protein, under these conditions. At 2 equiv of ^{203}Hg(II)/protein dimer, 50% is bound, and at 4 equiv, 25% is bound, clearly establishing that one atom of Hg(II) is bound per MerR dimer. The same result was obtained under forcing conditions, in which a 10-fold molar excess of ^{203}Hg(II) per MerR dimer was used (data not shown). It should be noted that these studies were conducted in the presence of a 1000-fold molar excess of buffer thiol relative to MerR, emphasizing the specificity of the interaction and the effectiveness of MerR as a specific trap for its heavy-metal ligand. The avidity and specificity with which the MerR dimer binds a single Hg(II) atom are not only remarkable in itself but also permits interpretation of the cysteine thiol protection experiments presented in the next section.

An estimate of the equilibrium dissociation constant for the MerR–Hg(II) complex may be obtained from thiol competition experiments, which measure the relative affinity of MerR versus simple thiol compounds for Hg(II). In these experiments (data not shown), varying concentrations of MerR were incubated with ^{203}Hg(II) in the presence of 1 mM 2-mercaptoethanol. Gel filtration (as in Figure 8), followed by scintillation counting, directly gives the extent of partitioning of the Hg(II) between MerR and the buffer thiols. Under these conditions, MerR is half-saturated with Hg(II) at approximately 1×10^{-7} M protein, indicating that MerR binds Hg(II) 10^4 times more tightly than a simple thiol ligand. Placed in energetic terms, the observed 10^4 discrimination indicates that Hg(II) bound to MerR is stabilized by ca. 5.5 kcal/mol relative to simple Hg(II)–bis(thiolate) complexes.

Preliminary attempts to determine the kinetics of Hg(II) binding to MerR utilized a nitrocellulose filter binding assay. ^{203}Hg(II) (1 equiv) was added to MerR samples (10^{-6} M) containing a 1000-fold molar excess of competing thiol and the percent of bound Hg(II) determined by filtration at 15-s time intervals after addition. Association was essentially complete by the first time point. The rate of association was at least as rapid if a stoichiometric amount of operator DNA was present as well.

The filter binding assay was also used to demonstrate the fact that the exchange of Hg(II) into and out of MerR, in the presence of exogenous thiols, is both rapid and facile. Addition of a 100-fold molar excess of Hg(II) to a preformed ^{203}Hg(II)–MerR complex, followed by filtration at fixed time points (earliest time point at 15 s), resulted in complete exchange before the first time point could be measured. The complementary experiment, addition of excess ^{203}Hg(II) to a Hg(II)–MerR complex, yielded the same result. Thus, while the half-life of MerR–DNA complexes approach 1 h in the presence and absence of Hg(II), complete kinetic exchange of the bound Hg(II) with buffer thiols occurs on a time scale of seconds; this observation is consistent with the known kinetic lability of Hg(II)–thiolate complexes.

Additional competition experiments (data not shown) were conducted to investigate the ability of other heavy metals to bind specifically to MerR in the presence of Hg(II). Our results indicate that MerR specifically binds 1 equiv of Hg(II) even in the presence of 1000 equiv of Zn(II), Cd(II), Ag(I), or Tl(II). It has been reported that MerR in the presence of Cd(II) effects a low level of transcriptional activation in vitro from the *mer* promoter (T. O'Halloran, personal communication).

Indications That Stoichiometric Binding of Hg(II) to MerR Involves Cysteine Residues. Given the exceptional thermodynamic stability of the MerR–Hg(II) complex, we reasoned that the protein must possess a highly refined metal binding domain with specific ligand donating residues. Our preliminary attempts to define the metal binding residues of MerR, described below, centered on thiol titrations using DTNB and pHMB in addition to iodoacetamide alkylation.

(A) DTNB/pHMB Titration of MerR. DTNB titrations on MerR gave different values of titratable SH groups depending on the recent history of the protein sample. Freshly prepared MerR, gel filtered to remove 2 mM buffer thiol, yielded 6 thiols/dimer (of 8 possible). Titration of a protein sample denatured in 6 M guanidine also yielded 6 thiols/dimer. MerR samples that had been stored in low (or no) thiol buffer only possessed 4 titratable thiols/dimer, indicative of oxidation and disulfide bond formation. To assess whether 2 of 8 cysteines/dimer, not titratable with DTNB, were in fact disulfide linked, the titration was repeated with *p*-(hydroxymercuri)-benzoate (pHMB) and yielded all 8 cysteines/dimer. Assuming no pHMB-induced disulfide scission, it appears that all four cysteines per subunit are present in the free thiol form,

though two remain kinetically inaccessible to DTNB even in 6 M guanidine. Further analysis of DTNB titration data, monitoring MerR on denaturing acrylamide gels, suggests that DTNB actually catalyzes the conversion of the noncovalently associated MerR dimer to a disulfide-linked dimer (as in Figure 2; data not shown). This thiol/disulfide exchange reaction presumably proceeds via initial formation of a mixed Cys–S–S–TNB disulfide which undergoes displacement of the TNB anion by attack of a Cys SH from the adjacent subunit. Facile DTNB-mediated oxidation of a cysteine pair has previously been observed with the enzyme thiolase, in this laboratory (Davis, 1986). The fact that this reaction occurs in the case of MerR implies that one cysteine residue of each monomer is disposed close to the dimer interface.

Formation of the stoichiometric Hg(II)–MerR dimer complex followed by DTNB titration reveals that 2 thiols/dimer are kinetically protected from titration, consistent with ligation of Hg(II) to two cysteine residues. Anticipated conservation of C_2 symmetry would require that the single Hg(II) atom be ligated in a bridging fashion across the dimer–dimer interface in MerR. While this binding motif remains to be unequivocally demonstrated, it has been noted that occasionally ^{203}Hg(II) binding to MerR can still be detected on SDS gels and in that event the associated protein migrates as a dimer rather than a monomer. It remains to be proven if this indeed represents a Hg(II)-linked dimer rather than an oxidized dimer. Interestingly, Frankel et al. (1988) have recently demonstrated the existence of a metal-linked dimer formed in the binding of Zn(II) and Cd(II) to the HIV Tat protein.

(*B*) *Iodo[^{14}C]acetamide Alkylations of MerR.* In an attempt to identify the ligand donor residues in MerR and to count thiols under nonoxidative conditions, alkylation studies with iodo[^{14}C]acetamide were conducted. The alkylated apoprotein was subjected to tryptic digestion, peptide purification on HPLC, and quantification of the radiolabeled peptides. These experiments were then repeated on the stoichiometric Hg(II)–MerR complex. With the prior knowledge that two of the cysteines per dimer are extemely prone to oxidation and that both thiol/disulfide and Hg(II)/thiol interchanges are rapid, alkylations were carried out for only 10 min with a 100-fold molar excess of iodo[^{14}C]acetamide (just enough to give detectable radiolabel incorporation). Tryptic digestion of iodo[^{14}C]acetamide-alkylated MerR should generate three labeled tryptic peptides: Leu-76 to Lys-92 (containing Cys-82), Met-106 to Arg-120 (containing both Cys-115 and Cys-117), and Gly-122 to Pro-144 (containing Cys-126). As shown in Figure 9, it is possible to detect three labeled peptides (**1**–**3**) under suitable conditions; however, the presence of the radiolabeled peptide **1** was highly variable among MerR preparations, being virtually absent whenever the MerR sample was extensively oxidized to the disulfide-linked form (vide supra). Total amino acid analysis of peptide **1** established it to be the Leu-76–Lys-92 fragment, which contains Cys-82. These data are then consistent with Cys-82 in each subunit being the readily autoxidized cysteine which forms a symmetrically cross-linked Cys-82–S–S–Cys-82 dimer. It also suggests Cys-82 is not involved in Hg(II) ligation and, consequently, in *mer* operon transcriptional activation.

The remaining two radiolabeled tryptic peptides obtained from iodo[^{14}C]acetamide-alkylated native (Figure 9, bottom, B) and denatured MerR (Figure 9, bottom, A) typically yielded 2:1 ratios of ^{14}C radioactivity, respectively, as displayed in Figure 9, suggesting that peptide **2** contains two cysteines versus one cysteine in peptide **3**. Total amino acid analysis confirmed that peptide **2** contained both Cys-115 and Cys-117

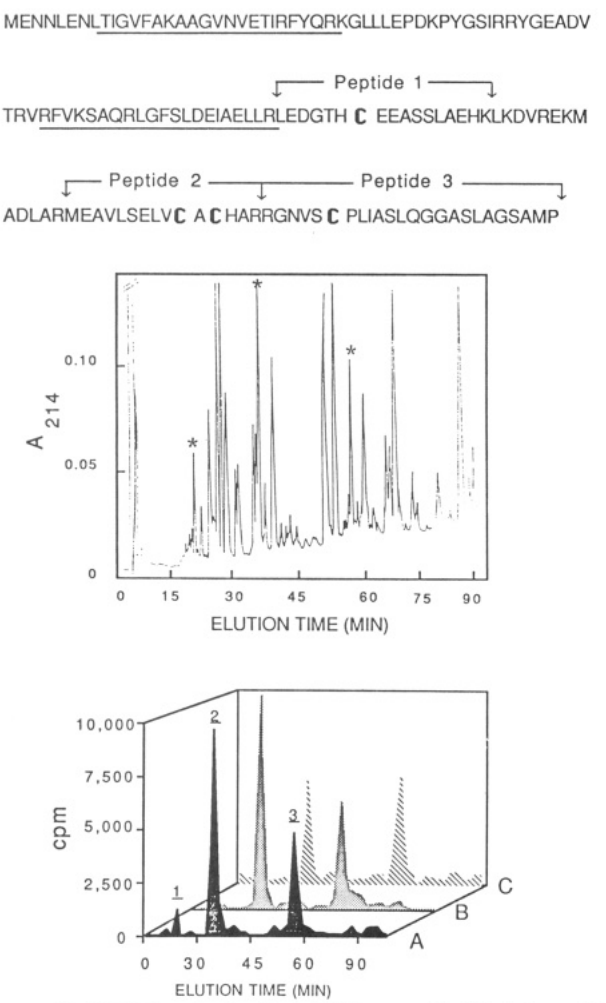

FIGURE 9: HPLC analysis of iodo[^{14}C]acetamide-labeled peptides of MerR. MerR, in the presence of 100 μM 2-mercaptoethanol, was alkylated and digested with trypsin as described under Materials and Methods. Peptides were resolved on a C_{18} reverse-phase column. (Upper panel) Amino acid sequence of MerR. The four cysteines in MerR are shown in boldface, and the two regions proposed to form helix–turn–helix supersecondary structures are underlined. The three radiolabeled peptides are as indicated. (Middle panel) The chromatographic profile as monitored at 214 nm. Radiolabeled peptides are denoted by an asterisk. (Lower panel) Chromatographic profile as determined by scintillation counting: (A) denatured protein, (B) native protein, (C) native protein in the presence of Hg(II).

while peptide **3** contained the fourth cysteine in MerR, namely, Cys-126. Upon addition of a stoichiometric amount of Hg(II) to MerR [10 μM protein and Hg(II) in the presence of 100 μM 2-mercaptoethanol] prior to iodo[^{14}C]acetamide alkylation, peptide **2** (containing Cys-115, -117) reproducibly experienced a 2-fold decrease in radiolabel incorporation, while the Cys-126-containing peptide **3** (and the poorly labeled Cys-82-containing peptide **1**) showed no difference in sensitivity to alkylation (Figure 9, bottom, C). When alkylations were performed with lower concentrations of exogenous thiol, both native and denaturing conditions gave similar labeling patterns on MerR peptides; however, no label was detected when Hg(II) was prebound in the presence of 10 μM 2-mercaptoethanol. It may be that these conditions (i) greatly increase the rate of Cys-82–Cys-82 disulfide formation, as qualitatively observed at several times during the course of this work, and (ii) with Hg(II) ligated to two of the remaining cysteines access to the other four Cys per dimer was kinetically blocked in this Hg(II)-induced, disulfide-linked dimer conformer. While the structural basis for this anomalous kinetic protection remains unclear, these results again emphasize the

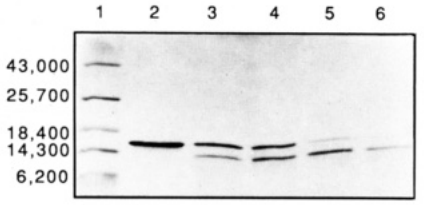

FIGURE 10: Time course of partial digestion of MerR with trypsin. The lane assignments are as follows: (1) undigested MerR; (2) 15-min digestion; (3) 30-min digestion; (3) 60-min digestion; (4) molecular weight standards.

balance between exogenous thiol, Hg(II), and MerR concentrations needed to reveal accurate structural and functional aspects of this metalloregulatory protein.

Limited Proteolysis To Assess Existence and Function of DNA Binding and Hg(II) Binding Domains in MerR. In an effort to map the regions of MerR functionally equipped to bind DNA, Hg(II), and the other dimer subunit, susceptibility to controlled proteolysis was examined. Several residue-specific and -nonspecific proteases were initially examined, including trypsin, α-chymotrypsin, and subtilisin, in an attempt to obtain stable functional peptides. It was noted that the addition of a stoichiometric amount of Hg(II) did not substantially alter the proteolysis patterns (data not shown), suggesting that the binding of Hg(II) by MerR does not result in any gross structural changes.

Partial proteolysis with trypsin yielded one particularly stable fragment (Figure 10), corresponding to a molecular weight of ca. 13 000, which could be purified away from intact MerR and examined for DNA and Hg(II) binding activity and dimerization. N-Terminal sequencing and total amino acid analysis (data not shown), along with the molecular weight determination of the fragment and the placement of Arg and Lys residues in the MerR sequence, established the proteolytic fragment as the N-terminal 120 or 121 amino acids and thus lacking Cys-126. This fragment still dimerizes (to a 26-kDa species) and binds in an analogous fashion to its operator, as assessed by DNase I footprinting (data not shown). Gel shift assays, in the presence or absence of Hg(II), indicate that Hg(II) no longer influences the equilibrium constant of this MerR fragment for the operator and that the truncated protein possesses an approximately 10-fold lower affinity than native MerR for its operator. Gel filtration assays with ^{203}Hg(II) have revealed that this protein, lacking Cys-126, is no longer able to bind Hg(II) with a measurable stability over that of exogenous thiols. This fragment, however, like the intact protein, is still sensitive to oxidation to a disulfide-linked dimer. These observations suggest that Cys-126 plays a required role in either direct Hg(II) ligation or stabilization of a functional metal binding domain. These results, as well as other preliminary observations on the role of individual cysteine residues in MerR, are examined in further detail in the following paper by analysis of a collection of mutant MerR molecules (Shewchuk et al., 1989).

Discussion

The results in this paper provide characterization of the MerR metalloregulatory protein's specific interactions with its promoter/operator region, as well as its specific interaction with Hg(II), the in vivo inducer species. Genetic analysis of the *mer* operon has shown that MerR is both a repressor and a metal-activated transcriptional inducer of the *mer* structural genes (Ni'Bhriain et al., 1983; Foster et al., 1979). We have shown that MerR binds, with high affinity, to dyad symmetry 1, DS1, both in the presence and in the absence of Hg(II), and that the MerR dimer ligates a single mercuric ion. From chemical modification studies, cysteine residues appear to be involved in metal ligation.

Specific interactions between MerR and its DNA operator were characterized by DNA footprinting. DNase I, dimethyl sulfate, and hydroxyl radical protection data, in the presence and absence of saturating Hg(II), allow identification of the specific points contacted by MerR in DS1(−10 to −35, overlapping the *mer* promoter region). These data are summarized in Figure 5. Significantly, no difference was detected in any of the DNA contacts upon addition of Hg(II). Four guanines in DS1 are obstructed from DMS methylation while two become hypersensitive upon MerR binding. These contacts, related by a local C_2 symmetry axis, suggest that the MerR dimer makes symmetric contacts in the major groove region of two contiguous turns of B-like DNA, both in the presence and in the absence of Hg(II). This binding geometry is characteristic of other prokaryotic gene regulators possessing a helix–turn–helix DNA binding motif [for a review, see Pabo and Sauer (1984)].

One wonders to what extent these in vitro footprinting results mimic the in vivo situation. In vivo footprinting, via dimethyl sulfate, has recently been reported for the highly homologous Tn*21* MerR (Heltzel et al., 1988). In addition to complete protection of the same four guanines partially occluded in the in vitro experiments, protection of the guanine at −33 and hypersensitivity of two adenines, A-32 and A-36, were detected, possibly due to the presence of RNA polymerase in the in vivo complexes. Interestingly, the proposed RNA polymerase contacts were MerR dependent but Hg(II) independent. We do note that footprinting of the DS2 element at −80/−70 was not detected in any of our studies, precluding a simple "looping mechanism", as proposed for other positive/negative regulatory proteins (Ptashne, 1986; Schleif, 1987). Confirmation of this assumption, in the presence of RNA polymerase and by in vivo studies, is still required.

The kinetics and thermodynamics of MerR binding to *mer* operator DNA were studied by using a gel shift assay. K_d values of 10^{-10} and 10^{-11} M were determined for MerR in its Hg(II)-independent repressor and Hg(II)-dependent inducer modes, respectively. These values compare with K_d's in the range of 10^{-9} M for P22 Arc (Vershon et al., 1987) to 10^{-13} M for *lac* repressor (Winter & von Hippel, 1981), binding to their cognate operator regions. The half-life of ca. 50 min, for the MerR–DNA complex, as determined by the in vitro assay, is unaffected by the presence of Hg(II). Thus, Hg(II) ligation must result in a 10-fold increase in the pseudo-first-order rate of protein–DNA association.

The second focus of this paper is on the specific binding and recognition of the toxic heavy-metal inducer of the *mer* operon, Hg(II), by the MerR protein. Of particular note is the demonstration of highly specific, albeit rapidly reversible, binding of Hg(II) at a stoichiometry of one Hg(II) per MerR dimer. The protein is an effective Hg(II) trap, even in the presence of a 1000-fold excess of buffer thiols, perhaps reflecting an entropic advantage of bis(thiol)Hg(II) chelation by MerR. Half-saturation at 10^{-7} M Hg(II) correlates well with reported in vivo concentrations of 10^{-8}–10^{-6} M required for induction of the Hg^R phenotype (Lund et al., 1986).

Efforts were made to assess the role of the four cysteines, at 82, 115, 117, and 126, in the 144 aa MerR monomer, in stoichiometric Hg(II) binding. Titration of cysteine thiols with DTNB and iodoacetamide suggests that Cys-82 is not involved in Hg(II) ligation but may be the cysteine involved in intersubunit disulfide (Cys-82–S–S–Cys-82) formation in the facile

oxidation processes described. DNA sequence comparisons of all *merR* genes reported to date (T. Misra, A. Summers, and I. Mahler, personal communications) reveal the conservation of three of the four cysteines but specific replacement of Cys-115, suggesting that Cys-115 may be irrelevant for MerR negative and positive gene regulation. Of the remaining two cysteine residues, Cys-117 is implicated in metal binding, on the basis of Hg(II) protection of the Cys-117/Cys-115-containing tryptic peptide from iodo[^{14}C]acetamide alkylation, while Cys-126 appears consequential from studies on the 1–120 (121) tryptic fragment which still binds DNA but not Hg(II). Neither set of experiments can distinguish direct roles for Cys-117 or Cys-126 in either maintenance of a metal binding domain structure or direct ligation to Hg(II). Therefore, either could participate in intersubunit bis ligation to Hg(II) as homoligands [e.g., Cys-117–S–Hg(II)–S–Cys-117 or Cys-126–S–Hg(II)–S–Cys-126] or as heteroligands [e.g., Cys-117–S–Hg(II)–S–Cys-126]. The intrasubunit Cys-126–S–Hg(II)–S–Cys-117 is not entirely ruled out. Other methods such as X-ray analysis and/or NMR as well as site-specific mutagenesis may be useful to address these alternatives.

We note the fact that MerR remains in a tightly associated complex with its operator DNA in both its repressor [–Hg(II)] and its activator [+Hg(II)] modes and that DNA contacts at DS1 are essentially unaffected upon Hg(II) addition. These observations strongly argue that the repressor behavior of MerR does not simply represent physical obstruction of RNA polymerase from its primary binding site, consistent with the previously mentioned in vivo footprinting results (Heltzel et al., 1988).

Given the high degree of homology between the −35 and −10 elements of the *mer* promoter and the *E. coli* consensus sequence (Hawley & McClure, 1983) (consensus "−35", 5′-TTGACA-3′, *mer*, 5′-TTGACT-3′; consensus "−10", 5′-TATAAT-3′, *mer*, 5′-TAAGGT-3′), one would expect the *mer* promoter to be very strong and thus not require positive control. However, the spacing between the −10 and −35 sequences is 19 bp, rather than the consensus 17+/−1 bp spacing. Thus, from the data at hand, a model for Hg(II)-dependent transcriptional activation and repression can be proposed. MerR acting as a repressor may control promoter structure and thereby inhibit transcriptional initiation by RNA polymerase. Ligation of Hg(II), via strong Hg(II)–S–Cys bonds, may result in favorable interactions between a Hg(II)-induced MerR conformer and RNA polymerase, leading to possible reorientation of a MerR–DNA–RNA polymerase complex and efficient gene transcription. These ideas are currently under investigation in a reconstituted in vitro system.

In the following paper (Shewchuk et al., 1989), we report the biochemical characterization of a series of mutant MerR proteins which were selected for defects in either repression (r^-) or activation (a^-). Together with the studies on wild-type MerR reported in this paper, the domain organization and identification of Hg(II) ligand donor residues of this transcriptional metalloregulatory protein switch are further defined.

ACKNOWLEDGMENTS

We gratefully acknowledge Melissa Moore for helpful advice. We also thank Dr. A. Summers for helpful discussion and Dr. S. Goldin for use of his laser densitometer.

Registry No. Hg, 7439-97-6.

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