Accelerated Publications

Mutagenesis of the Cysteines in the Metalloregulatory Protein MerR Indicates That a Metal-Bridged Dimer Activates Transcription†

Lisa M. Shewchuk,‡ Gregory L. Verdine,§ Huw Nash,§ and Christopher T. Walsh*,‡

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

Received April 14, 1989; Revised Manuscript Received May 25, 1989

ABSTRACT: Bacterial resistance to mercury(II) compounds is controlled by the metalloregulatory MerR protein, a transcriptional repressor and a mercuric ion dependent activator of the *mer* operon. Site-directed mutagenesis of all four cysteine residues in the Tn*501* MerR protein has led to the specific replacement of C82, C115, and C117 with alanine and of C126 with serine. Mutation of C82 and C126 abolishes transcriptional activation in vivo while mutation of C115 and C117 leads to a slight increase and dramatic decrease in transcriptional activation, respectively. All four mutants are competent, to varying degrees, to repress *mer* transcription. Characterization of the four purified mutant proteins in vitro demonstrates that only the C126S MerR mutant is most notably deficient in stoichiometric Hg(II) binding. All four mutant proteins possess similar DNA binding properties, and the C82 mutant is most affected in the ability to form stable dimers. Given an observed stoichiometry of one Hg(II) per MerR dimer, it is likely that the transcriptionally activating MerR species is a metal-bridged dimer. It is most likely that one C126 per subunit provides high-avidity bidentate ligation to Hg(II), but it remains possible that C82 may be a secondary Hg(II) ligand (e.g., in a tetracoordinate thiol ligation array).

Bacterial resistance to the toxic effects of mercury(II) salts is mediated via the genes of the *mer* operon [for review, see Helmann et al. (1989a), O'Halloran (1989), Walsh et al. (1988), and Silver and Misra (1988)]. Mercury resistance is effected at the molecular level by the coordinate action of the operon-borne *merT*, *merP*, and *merA* gene products. *merT* and *merP* encode membrane and periplasmic proteins, respectively, involved in Hg(II) uptake, while *merA* encodes a unique enzyme, mercuric ion reductase, that reduces internalized Hg(II) to less toxic, volatile Hg(0). Transcriptional control of the Tn*501* and Tn*21* *mer* operons is effected by a metalloregulatory DNA-binding protein, MerR, the product of the divergently transcribed *merR* gene. MerR both activates transcription from the *merTPAD* promoter (P_{mer}) in the presence of Hg(II) and represses transcription from P_{mer} 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 (P_{merR}) (Ni'Bhriain et al., 1983).

The MerR protein appears to have two domains, an N-terminal domain possessing a helix-turn-helix DNA-binding motif (residues 9-29) and a C-terminal metal-specific mercury-binding domain (Shewchuk et al., 1989a,b; O'Halloran & Walsh, 1987). Taking into account the exceptional thermodynamic stability of Hg(II)-thiolate complexes, it can be predicted that one or more of the four cysteine residues located in the MerR C-terminal domain (C82,[1] C115, C117, and C126 in the Tn*501* and the homologous Tn*21* proteins) are the mercury ligands which, upon metal binding, induce the switch from repression to activation. Previously, a series of biochemical and genetic experiments were utilized to begin to assess the individual role of each cysteine in metalloregulation (Shewchuk et al., 1989a,b; Ross et al., 1989). With Tn*501* MerR protein, Hg(II) ligation protected one of the four cysteines against [^{14}C]iodoacetamide alkylation; tryptic mapping identified the protected peptide as one containing both C115 and C117 (Shewchuk et al., 1989a). Since C115 is the only cysteine residue in MerR that is not evolutionarily conserved (Misra et al., 1984; Barrineau et al., 1984; Laddaga et al., 1987; Helmann et al., 1989b; Nucifora et al., 1989), it was suggestive that C117 was the protected cysteine. Random mutagenesis studies on the Tn*21* *merR* gene generated a class of activation minus, repression plus (a^-r^+) mutants, including three of the four cysteine residues, each altered to tyrosine (C82Y, C117Y, and C126Y) (Ross et al., 1989). In vitro studies with the purified mutant proteins suggested that C82Y was defective in dimerization and that C126Y was the most defective in high-affinity Hg(II) binding (Shewchuk et al., 1989b). Thus from these previous studies, C117 and C126 were identified as the two most likely Hg(II) ligand residues. Unfortunately, the nonconservative nature of the C → Y mutations used in this preliminary study left open the possibility that the observed biochemical effects were due to artifactual changes in MerR structure.

To clarify further the structural and functional roles of the cysteines in MerR, we have turned to site-directed mutagenesis in the Tn*501* system, mutating each of the cysteine codons at positions 82, 115, and 117 to an alanine codon and mutating the C126 codon to a serine codon, to yield mutant proteins

† This work was supported in part by NIH Grant GM20011.

‡ Harvard Medical School.

§ Harvard University.

[1] Abbreviations: C82, cysteine 82; C82A, mutant MerR with cysteine 82 changed to alanine; dATP, deoxyadenosine triphosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

0006-2960/89/0428-6140$01.50/0

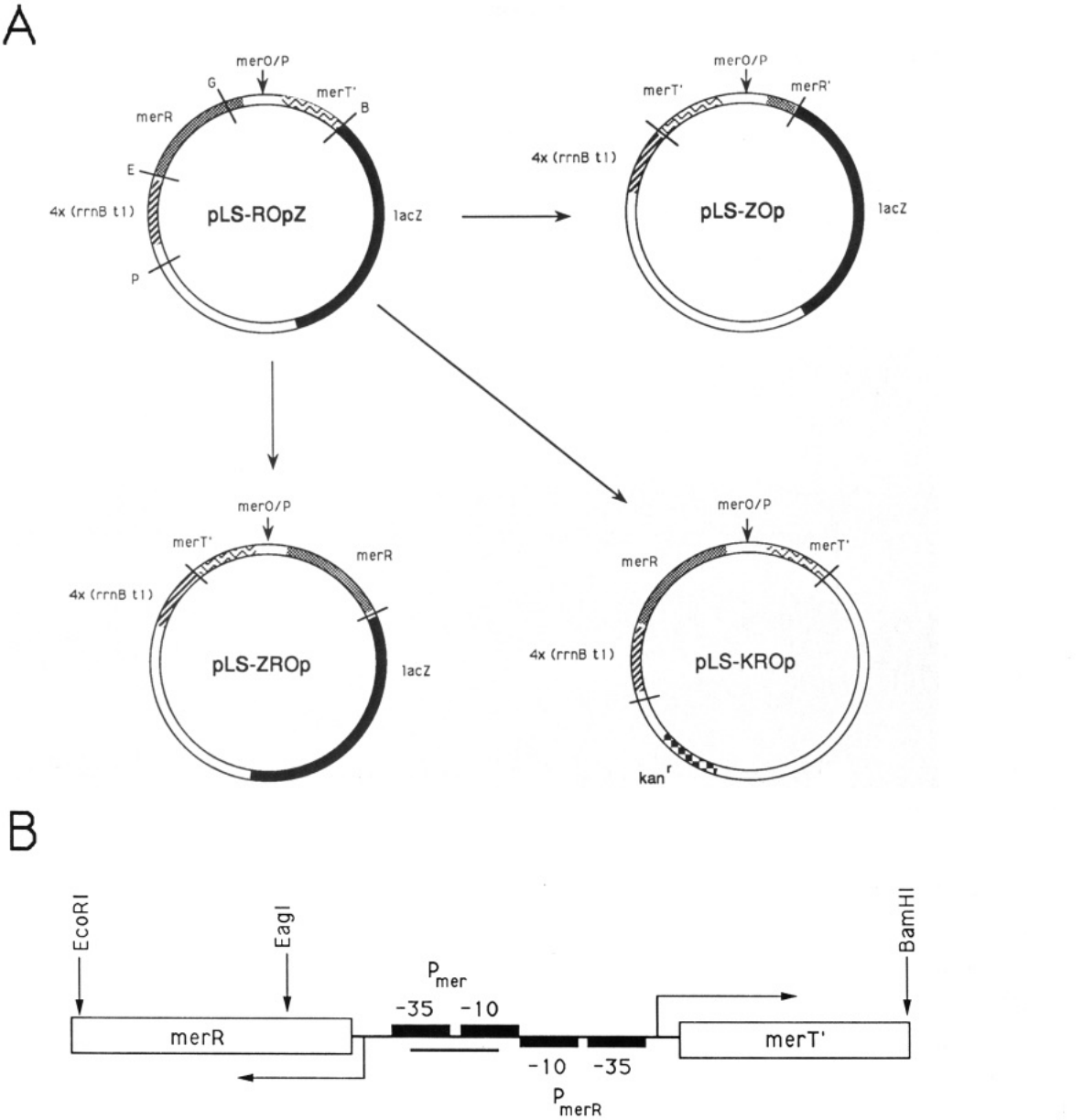

FIGURE 1: (A) Plasmid constructions. Restriction sites are indicated on pLS-ROpZ by letters: B, *Bam*HI; E, *Eco*RI, G, *Eag*I; P, *Pst*I. (B) Schematic of the *mer* operator/promoter region of the Tn*501 mer* operon. Transcriptional regulation of P_{mer} and $P_{mer}R$ is mediated by the binding of MerR to an operator located between the −35 and −10 elements of P_{mer}, as indicated by a bold line.

having more conservative side-chain replacements. We report here the characterization of these four Tn*501* MerR mutants for in vivo transcriptional activation and repression and for in vitro DNA binding, Hg(II) binding, and subunit dimerization.

Materials and Methods

Materials. Oligonucleotide–directed in vitro mutagenesis system Version 2 was purchased from Amersham Corp. $^{203}HgCl_2$, [α-^{35}S]dATPαS and [α-^{32}P]dATP were from New England Nuclear. Sanger sequencing reagents were from U.S. Biochemical (Sequenase kit). Restriction endonucleases and DNA modifying enzymes were from New England Biolabs. All other reagents were of the highest grade available and used without further purification.

Site-Directed Mutagenesis. Replacement of cysteines was accomplished by oligonucleotide-directed mutagenesis of an *EcoRI* fragment (encoding the entire *merR* gene) from pTO90-16 (O'Halloran & Walsh, 1987) subcloned into M13 mp18. Mutagenesis reactions were carried out as described by Amersham Corp. with oligonucleotides synthesized on a Pharmacia gene assembler. Mismatched base pairs are shown in boldface:

5′-GGCTTCCTCG**GC**ATGGGTGCC-3′ Cys 82 to Ala

5′-ATGGCAGGCG**GC**CACCAACTC-3′ Cys 115 to Ala

5′-TCGCGCATGG**GC**GGCCGTGTG-3′ Cys 117 to Ala

5′-GATCAGCGGG**G**AGGAAACGTT-3′ Cys 126 to Ser

Mutations were confirmed by Sanger sequencing (Sanger et al., 1977).

Plasmid Constructions. For overproduction of mutant proteins, *merR* genes were subcloned behind the tac promoter by replacement of a 500-bp *Eco*RI fragment in pTO90-16 (O'Halloran & Walsh, 1987) with the corresponding mutant fragment.

In vivo characterization of the mutant MerRs' ability to repress and activate transcription was accomplished by the constructure of *lacZ* fusions to both the *merR* and *merTPAD* promoters. A pWR2 (Ross et al., 1989) derivative, pLS-ROpZ, was used to monitor transcriptional regulation by

wild-type and mutant MerRs at P_{mer} (Figure 1A). pWR2 contains the Tn*21* *merR* gene, the *mer* operator/promoter region (P_{merR} and P_{mer}), and a *merT:lacZ* fusion. pLS-ROpZ was constructed by replacement of codons 15–144 in Tn*21* *merR* gene with the corresponding region of the Tn*501* gene as a 484-bp *Eag*I/*Eco*RI fragment. Each of the mutant *merR* genes was similarily subcloned into pWR2.

pLS-KROp was constructed to assess the dominance or recessiveness of each of the mutants. This plasmid, containing the *merR* gene under the control of its own promoter, P_{merR}, was constructed by ligation of a 2100-bp *Bam*HI/*Pst*I fragment from pLS-ROpZ and a 2900-bp *Bam*HI/*Pst*I fragment, containing a kanamycin-resistance marker and P15A origin, from pGP1-2 (Tabor & Richardson, 1985).

pLS-ZOp and pLS-ZROp were used to monitor transcription from P_{merR}. pLS-ZOP contains the *mer* operator/promoter region and *lacZ* fused to the first 15 amino acids of the *merR* gene. This plasmid was constructed by ligation of a blunt 275-bp *Eag*I/*Bam*HI fragment and a blunt 7100-bp *Bam*HI/*Eco*RI fragment, both from pLS-ROpZ. pLS-ZROp contains the *merR* and *lacZ* genes both under the control of P_{merR} and was constructed by ligation of a blunt 675-bp *Eco*RI/*Bam*HI fragment and a blunt 7100-bp *Eco*RI/*Bam*HI fragment, both from pLSROpZ. All plasmids were expressed in *Escherichia coli* JM109 [recA1, endA1, gyrA96, thi, hsdR17(r_{k^-}, m_{k^+}), supE44, relA1, (λ^-), Δ(lac-rpoAB), {F′, traD36, proAB, $lacI^qZ_{\Delta}M15$}].

Protein Purification. Mutant proteins were expressed and purified from *E. coli* JM109 as previously described for the wild-type protein (Shewchuk et al., 1989b).

In Vitro Characterization. Dimerization, DNA-binding, and Hg(II)-binding assays were performed as previously described (Shewchuk et al., 1989a).

β-Galactosidase Expression Assays. Assays were performed essentially as described by Miller (1977) with a $CHCl_3$/SDS lysis procedure. Overnight cultures were diluted 1:100 into T-broth and grown at 37 °C for 1 h. Aliquots were then induced with 0, 1, and 10 μM $HgCl_2$ for 20–60 min at 37 °C.

In Vitro Heterodimer Formation. Equimolar quantities of pairs of MerR proteins were dialyzed against 1.5 M KSCN in buffer A [10 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5% glycerol, and 2 mM β-mercaptoethanol] for 8 h at 4 °C followed by dialysis against buffer A overnight at 4 °C. Disulfide cross-linking and $^{203}Hg(II)$-binding assays were performed as previously described (Shewchuk et al., 1989a).

Results

In Vitro Characterization of Mutant Proteins. Site-directed mutagenesis of the Tn*501* MerR protein had led to the specific replacement of each of the four cysteines to give the mutant proteins C82A, C115A, C117A, and C126S. These four mutant proteins were overexpressed and purified to homogeneity (in milligram quantities), which has allowed for in vitro characterization of their Hg(II)-binding, DNA-binding, and dimerization abilities, described below.

$^{203}Hg(II)$ Binding Affinity. Wild-type MerR binds a single mercuric ion per homodimer with high specificity and avidity (Shewchuk et al., 1989a). To determine the effect of specific cysteine mutations on Hg(II) affinity, gel-filtration assays with $^{203}Hg(II)$ were performed. Each mutant protein was incubated with a 10-fold molar excess of $^{203}Hg(II)$ (in a dilution series from 10^{-5} to 10^{-8} M protein in the presence of 1 mM β-mercaptoethanol), followed by gel filtration to separate protein-bound $^{203}Hg(II)$ from free $^{203}Hg(II)$. The four MerR mutants were variably affected in Hg(II) binding affinity relative to the wild-type protein (Figure 2A). At high protein

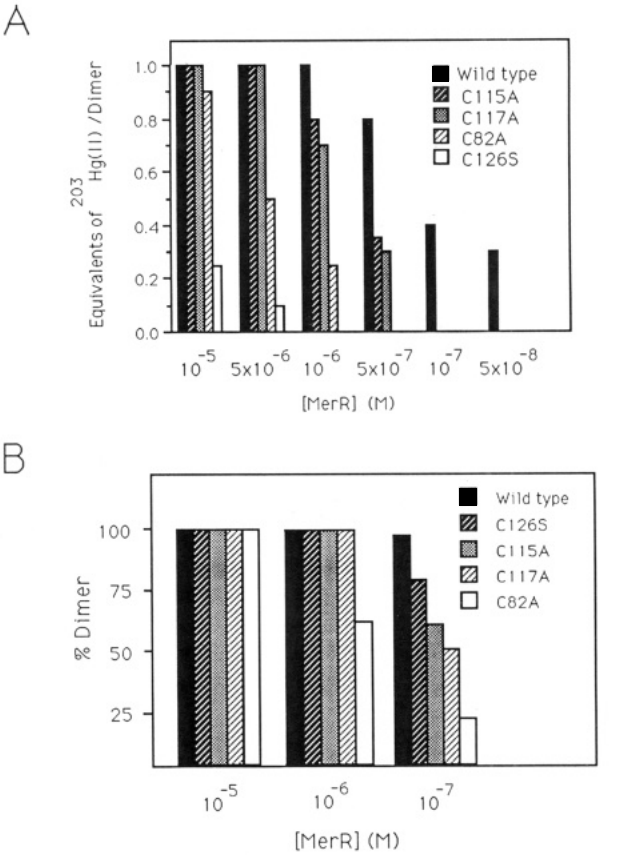

FIGURE 2: (A) Relative Hg(II) affinity of wild-type and mutant MerR proteins. Equivalents of Hg/dimer were determined as described under Materials and Methods. All assays were performed in the presence of 1 mM β-mercaptoethanol. (B) Concentration dependence of dimerization of wild-type and mutant MerRs. Extent of dimer formation was determined as described under Materials and Methods.

and Hg(II) concentrations (10^{-5} and 10^{-4} M, respectively), C82A, C115A, and C117A MerRs can achieve a stoichiometry of one Hg(II)/dimer; however, upon dilution of both the protein and Hg(II), metal binding is diminished. C126S MerR is the most attenuated in metal ligation, binding a maximum of 0.25 equiv of Hg(II)/dimer, even at the highest MerR concentration tested (10^{-5} M), and uniquely showing no Hg(II) ligation at 10^{-6} M MerR. These results mirror the results of Hg(II)-binding studies with the three C → Y mutants described for the homologous Tn*21* MerR (Shewchuk et al., 1989b).

DNA Binding Affinity. The MerR protein, like many procaryotic transcriptional regulators, binds as a dimer to an operator element possessing dyad symmetry. MerR binds its operator sequence with a K_d of 10^{-10} M, and in the presence of its coinducer, Hg(II), up to a 10-fold increase or 3-fold decrease in operator affinity is observed depending on the assay conditions (Shewchuk et al., 1989a; O'Halloran et al., 1989). The relative binding affinity of the four mutant MerR proteins to *mer* operator DNA was examined by a gel-mobility shift assay with ^{32}P end-labeled restriction fragments. All four mutant proteins exhibit near wild-type affinity for *mer* DNA in the absence of Hg(II), but no measurable difference in the dissociation constant is observed in the presence of Hg(II) (Table I). Failure to observe any difference in DNA affinity in the presence of Hg(II) may reflect the decreased affinity for Hg(II) of all four mutants. None of the cysteine mutations affect the sequence specificity of the protein (data not shown).

Effects of Mutations on Subunit Dimerization. Both activation and repression are mediated by MerR in its dimeric

Table I: Summary of in Vitro Properties of Purified Tn*501* MerR Cysteine Mutant Proteins

mutant	phenotype[a]		Hg(II) binding[b]	DNA binding [$\sim K_d$ (M)]	dimerization
C82A	a^-	r^{+++}	+	$\sim 5 \times 10^{-10}$	+
C115A	a^{++++}	r^+	++	$\sim 10^{-10}$	++
C117A	$a^{+/-}$	r^{+++}	++	$\sim 10^{-10}$	++
C126S	a^-	r^{+++}	+/−	$\sim 10^{-10}$	++
wild type	a^{+++}	r^{+++}	+++	$\sim 10^{-10}$	+++

[a] Phenotype determined from β-galactosidase expression assays. [b] Assays performed as described under Materials and Methods. (+++) implies wild-type activity, (++) and (+) imply less than wild-type activity, and (+/−) implies almost no activity.

form. Therefore, mutations that disturb the equilibrium between active dimers and monomers may influence the observed phenotype. With this in mind, the four mutant MerR proteins were tested for functional defects in dimerization by native gel filtration. Over a concentration range of 10^{-5}–10^{-7} M, wild-type MerR exists exclusively as a dimer, while the four mutant proteins all show a concentration-dependent dissociation to the monomeric form as the protein concentration is lowered to 10^{-7} M (Figure 2B). The most compromised mutant protein is C82A. We have previously shown that this cysteine (C82) is located at the dimer interface such that the symmetry-related C82 residues from each monomer can oxidatively couple in a disulfide bridge (Shewchuk et al., 1989a). Thus, one would expect that mutations in such a critical interface residue would impair dimerization, and this is precisely what is observed with both the C82A mutant protein studied here and the more radically impaired C82Y protein studied earlier (Shewchuk et al., 1989b).

Phenotypic Analysis. Fusion of the *lacZ* gene to both the *merR* and *merTPAD* promoters, as described under Materials and Methods and in Ross et al. (1989), has allowed for in vivo characterization of the mutant MerR proteins' ability to activate and repress transcription at either promoter. In these constructs wild-type MerR represses and activates the expression of β-galactosidase from P_{mer} in the absence and presence of Hg(II), respectively, while expression from P_{merR} is repressed (negative autoregulation) regardless of whether Hg(II) is present or absent. All four MerR mutants were examined individually and in combination with the wild-type and other mutant proteins. Representative data are displayed in Table II.

Even in the presence of 10μM Hg(II), C126S and C82A are completely deficient in P_{mer} activation, although they retain near wild-type levels of repression in the absence of Hg(II). The remaining two mutants, C115A and C117A, retain transcriptional activation behavior but are differentially affected in their regulatory abilities. Mutation of C117 leads to a significant decrease in activation but no difference in repression, relative to the wild-type protein. The residual activation behavior of C117A (~5% of wild-type activity) was most readily quantitated during 60-min inductions with 10 μM Hg(II). In contrast, mutation of C115, the only MerR cysteine that is not evolutionarily conserved, leads to a decreased level of repression but surprisingly results in greater activation than the wild-type protein.

The a^+r^{+++} phenotype of C117A is analogous to those observed with L113F, A116T, and H118A of the Tn*21* protein (Ross et al., 1989; S. Silver, personal communication). This clustering of mutations with similar phenotypes may suggest that this region of the protein is involved in metal-mediated activation but not directly responsible for Hg(II) binding. The ability of the C117A mutant to activate P_{mer} transcription at high Hg(II) concentrations implies that the a^- phenotype of the previously described C117Y Tn*21* mutant (Shewchuk et al., 1989b; Ross et al., 1989) may have arisen from a tyrosine-induced perturbation of structure.

Table II: Repression and Activation of β-Galactosidase Expression from P_{mer} by Wild-Type and Mutant MerR Proteins

	β-galactosidase (Miller units)[a]		
	0 μM Hg(II)	1 μM Hg(II)	10 μM Hg(II)
wild type (WT)	77	10 500	7 950
merR deletion	838	777	662
C82A	55	50	85
C115A	365	15 850	8 150
C117A	65	55	420
C126S	105	90	87
WT/C82A	117	1 150	1 000
WT/C115A	420	11 150	10 240
WT/C117A	55	1 550	5 650
WT/C126S	150	1 810	2 300

[a] Assays performed in triplicate as described under Materials and Methods. Cultures were induced with Hg(II) for 20 min.

The increased transcriptional activation capacity of C115A versus that of wild-type MerR over a range of Hg(II) concentrations was monitored and observed to be most dramatic at lower Hg(II) concentrations (10^{-7}–10^{-8} M) where up to a 4-fold increase in Hg(II)-mediated *lacZ* response is observed (data not shown).

Transcriptional regulation from P_{merR} was also monitored for each of the mutants (data not shown). All four mutant proteins possess near wild-type levels of repression from P_{merR}, suggesting that the mutant phenotypes are not the result of increased or decreased intercellular levels of MerR.

In Vivo Complementation and in Vitro Heterodimer Formation. In vivo complementation analysis and in vitro heterodimer experiments were performed to examine whether or not the MerR–Hg(II) complex is a metal-bridged dimer. If the metal-ligand residues are located in a single subunit, then heterodimers containing a wild-type subunit should be active for metal ligation. Conversely, if the metal is involved in a bridging ligation motif, then the presence of a single wild-type subunit will not restore specific Hg(II) binding.

In vivo complementation experiments were conducted to assess transcriptional regulation in cells harboring both wild-type and mutant MerR proteins. Pairs of MerR proteins were expressed from coresident plasmids, each with an alternative antibiotic resistance marker. Transcriptional regulatory activity was reported from a *merT–lacZ* fusion. The most readily interpreted set of mixtures were those arising from coexpression of wild-type and individual mutants (Table II). Only coexpression of WT and C115A gave wild-type levels of transcriptional activation. In contrast, coexpression of WT/C82A, WT/C117A, and WT/C126S gave significantly lower levels of β-galactosidase, most likely reflecting inactive heterodimer formation since a 2-fold dilution of wild-type homodimer by mutant homodimer, having nearly equal P_{mer} affinity (Table I), would have left 50% of wild-type *lacZ* activity. These data suggest in vivo heterodimer formation in which Hg(II) ligation, required for transcriptional activation, cannot proceed.

In vitro experiments with MerR heterodimers were performed to verify that subunit mixing could occur. Heterodimer formation between pairs of MerR proteins was induced by the addition of a chaotropic salt (1.5 M KSCN) followed by extensive dialysis. Evidence for the mixing of wild-type and mutant subunits was obtained from DTNB cross-linking experiments and $^{203}Hg(II)$-binding assays. It was previously noted that DTNB catalyzed the quantitative formation of

Table III: Hg(II) Stoichiometry of MerR Heterodimers

MerR proteins	equiv of Hg(II)/MerR dimer (±0.05)[a]
WT	1.00
WT/C82A	0.86
WT/C115A	0.98
WT/C117A	0.97
WT/C126S	0.34
C82A/C115A	0.90
C82A/C117A	0.87
C82A/C126S	0.26
C115A/C117A	0.97
C115A/C126S	0.33
C117A/C126S	0.31

[a] Assays were performed as described under Materials and Methods. Equimolar mixtures of MerR proteins (10^{-5} M total) were incubated with 5 equiv of Hg(II) and 1000 equiv of β-mercaptoethanol.

disulfide-linked dimers and that the disulfide was specifically formed between C82 in each subunit (Shewchuk et al., 1989a). Nonreducing SDS–PAGE analysis, following treatment with DTNB, was used to quantitate the proportion of dimeric species still competent for disulfide cross-linking (data not shown). In the presence of C82A, all three mutants (C115A, C117A, and C126S) as well as the wild-type protein show a 2–3-fold decrease in disulfide-linked dimer formation, while all other combinations of mutant and wild-type proteins exhibited complete oxidation to the covalently liked form, suggesting subunit mixing had occurred.

One-to-one preparations of heterodimer mixtures, prepared as described above, were examined for their ability to bind ^{203}Hg(II) in nitrocellulose filtration-binding assays (Shewchuk et al., 1989a). Only preparations in which one of the proteins was C126S exhibited a 60–70% decrease in Hg(II) binding (Table III). The observed decrease was greater than the average binding that would be observed if no subunit mixing had occurred, again implying that C126 in each subunit is required for metal ligation.

Discussion

The construction and purification of the four cysteine mutants of Tn*501* MerR have allowed assignment of individual roles to each cysteine in dimerization, Hg(II) binding, and transcriptional activation. The C82A mutant can bind Hg(II), can bind *mer* DNA with near wild-type affinity, and can function as a repressor, but it is not a transcriptional activator. This is probably due to a decrease in the K_{eq} for dimerization, but the possibility that C82 functions as an ancillary ligand to Hg(II) (see below) is not yet ruled out. Why competence for transcriptional activation is affected more than repression (Table I) is not yet fully clear.

The C115A mutant is actually a transcriptional superactivator. As mentioned previously, C115 is not conserved in MerR proteins from diverse sources and therefore cannot per se be crucial in P_{mer} activation. However, when C115 is present, it may compete for Hg(II) ligation with C126 thiolate and yield a conformer incompetent for transcriptional activation. Removal of the thiolate side chain of C115 may alleviate such a competition for Hg(II) and so increase the fraction of MerR–Hg(II) species active for transcription. This finding excludes C115 as an essential Hg(II) ligand and provides a particularly useful mutant for further studies on the mechanism of activation in the MerR/*mer* DNA/RNA polymerase complex. It should be noted that C115 mutants were not available in our previous study.

C117A, while much less activation competent than wild-type MerR, is still a sufficient transcriptional activator that one can now rule out C117 as a key thiol for binding Hg(II). This work removes the ambiguity from the previous Tn*21* random mutagenesis data in which C117Y and C126Y were determined to be activation-minus species (Ross et al., 1989). The C117A mutation described here is undoubtedly less disruptive than the former C117Y change. The previously observed protection, by Hg(II) ligation, of either C115 or C117 from iodoacetamide alkylation may either be an indirect conformational effect or reflect a secondary ligation event of Hg(II) by the 115, 117 thiol pair [the C126S mutant can bind 0.25 Hg(II)/dimer at very high Hg(II) levels].

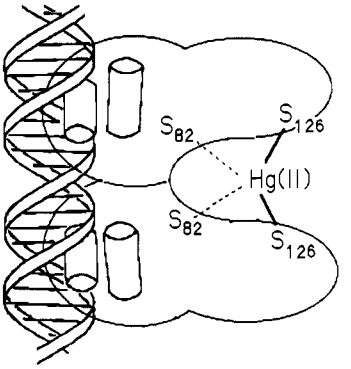

FIGURE 3: Schematic of transcriptionally active MerR as a metal-bridged dimer. The observed stoichiometry of one Hg(II)/dimer is most likely a bis Cys 126 thiol–Hg(II) ligation. The possibility of higher coordinate (tri- or tetradentate) ligation to an additional cysteine, in particular C82 as shown, is not yet ruled out.

These data taken in combination clearly implicate C126 as the primary Hg(II) ligand in MerR. Indeed, the C126S mutant is exceedingly defective in Hg(II) ligation and is not a transcriptional activator. These data corroborate the C126Y data gained from random mutagenesis of Tn*21* MerR (Shewchuk et al., 1989b; Ross et al., 1989). Given the observed stoichiometry of one Hg(II)/dimer and that subunit mixing of C126S with wild-type MerR does not restore metalloactivation, we propose that (i) one C126 from each subunit ligates Hg(II) in a linear bis-coordinate geometry and (ii) the transcriptionally active metalloregulatory complex is a metal-bridged dimer. While linear bis-coordinate $(Cys)_2$–Hg(II) ligation is most likely, the failure of C82A to function as a transcriptional activator leaves open the possibility that the thiol of C82 may function as an ancillary ligand to Hg(II) in a tri- or tetradentate ligation complex as schematized in Figure 3. Tri- and tetradentate thiol–Hg(II) complexes are known (O'Halloran, 1989), and it may be that MerR–Hg(II) EXAFS studies with wild-type and these cysteine mutants of MerR will give information on the coordination number of MerR. To date MerR has been refractory to crystallization and insufficiently soluble for NMR study. We note that a metal-bridged dimer of the HIV tat protein has recently been proposed as a transcriptionally active species (Frankel et al., 1988). Occupancy of the $(C126)_2$ intersubunit bis thiol site by Hg(II) is transmitted either back to the DNA-binding site or to an adjacent RNA polymerase molecule in the transcriptionally active ternary complex (O'Halloran et al., 1989) to switch from gene repressor to activator mode.

Acknowledgments

We thank Wilma Ross for plasmid pWR2 and Ann Summers and John Helmann for helpful discussion.

References

Barrineau, P., Gilbert, P., Jackson, W. J., Jones, C. S., Summers, A. O., & Wisdom, S. (1984) *J. Mol. Appl. Genet. 2*, 601.

Frankel, A. D., Bredt, D. S., & Pabo, C. O. (1988) *Science 240*, 70.
Helmann, J. D., Shewchuk, L. M., & Walsh, C. T. (1989a) *Adv. Inorg. Biochem.* (in press).
Helmann, J. D., Wang, Y., Mahler, I., & Walsh, C. T. (1989b) *J. Bacteriol. 171*, 222.
Laddaga, R. A., Chu, L., Misra, T. K., & Silver, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5106.
Lund, P. A., Ford, S. J., & Brown, N. L. (1986) *J. Gen. Microbiol. 132*, 465.
Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Misra, T. K., Brown, N. L., Fritzinger, D. C., Pridmore, W. M., Barnes, W. M., Haberstroh, L., & Silver, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5975.
Ni'Bhriain, N. N., Silver, S., & Foster, T. J. (1983) *J. Bacteriol. 155*, 690.
Nucifora, G., Chu, L., Silver, S., & Misra, T. K. (1989) *J. Bacteriol.* (in press).
O'Halloran, T. V. (1989) *Met. Ions Biol. Syst. 25.*
O'Halloran, T. V., & Walsh, C. T. (1987) *Science 235*, 211.
O'Halloran, T. V., Frantz, B., Shin, M. K., Ralston, D. M., & Wright, J. G. (1989) *Cell 56*, 119.
Ross, W., Park, S. J., & Summers, A. O. (1989) *J. Bacteriol.* (in press).
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Shewchuk, L. M., Verdine, G. V., & Walsh, C. T. (1989a) *Biochemistry 28*, 2331.
Shewchuk, L. M., Helmann, J. D., Ross, W., Park, S. J., Summers, A. O., & Walsh, C. T. (1989b) *Biochemistry 28*, 2340.
Silver, S., & Misra, T. K. (1988) *Annu. Rev. Microbiol. 42*, 717.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074.
Walsh, C. T., Distefano, M. D., Moore, M. J., Shewchuk, L. M., & Verdine, G. V. (1988) *FASEB J. 2*, 124.

G Protein–Effector Coupling: Interactions of Recombinant Inhibitory γ Subunit with Transducin and Phosphodiesterase†

Irene Griswold-Prenner, Narendra Tuteja,‡ Debora B. Farber, and Bernard K.-K. Fung*
Jules Stein Eye Institute, University of California School of Medicine, Los Angeles, California 90024
Received April 4, 1989; Revised Manuscript Received May 19, 1989

ABSTRACT: A bacterial expression vector for the inhibitory γ subunit of retinal rod phosphodiesterase has been constructed by inserting a mouse γ cDNA into pUC19. *Escherichia coli* 222 transformed with this plasmid produces a 12-kDa recombinant protein consisting of 18 additional amino acids attached to the amino terminus of γ. The fusion protein, designated β-gal-γ, has been refolded into an active form in formic acid and partially purified by gel filtration chromatography. Despite a large extended sequence at the amino terminus, β-gal-γ is able to inhibit the activity of trypsin-activated phosphodiesterase, bind tightly to the catalytic $\alpha\beta$ subunits, and interact with the α subunit of transducin in a nucleotide-dependent manner. The availability of large quantities of active bacterial γ, together with the ability to change its primary structure by site-directed mutagenesis, promises to provide considerable new information on the interaction between transducin and phosphodiesterase, as well as insights into the molecular mechanism of G protein–effector coupling.

One of the best studied biological processes involving G protein interaction with an effector enzyme is visual excitation of vertebrate retinal rods, where the direct activation of a cGMP-specific phosphodiesterase (PDE)[1] by transducin has been conclusively demonstrated (Chabre, 1985; Fung, 1986; Stryer, 1986; Hurley, 1987; Liebman et al., 1987). In this system, photolyzed rhodopsin catalyzes the exchange of GTP for GDP bound to transducin (Fung & Stryer, 1980). The transducin–GTP complex, in turn, stimulates the PDE activity (Fung et al., 1981), leading to a transient reduction of the intracellular level of cGMP (Yee & Liebman, 1978; Woodruff & Bownds, 1979; Blazynski & Cohen, 1986; Cote et al., 1986) and the closure of many cGMP-sensitive cation channels (Fesenko et al., 1985; Yau & Nakatani, 1985). As a result, the influx of Na^+ through the plasma membrane of the rod outer segment (ROS) decreases (Hagins et al., 1970; Baylor et al., 1979) and the rod hyperpolarizes (Tomita, 1970).

cGMP-specific PDE, the key enzyme involved in the regulation of intracellular cGMP concentration of ROS, is a peripheral membrane protein consisting of α (M_r = 90 000), β (M_r = 86 000), and γ (M_r = 10 000) polypeptides (Baehr et al., 1979). In this multimeric form, the catalytic activity associated with the $\alpha\beta$ subunits is inhibited by γ (Hurley & Stryer, 1982). The inhibition can be relieved by limited tryptic digestion, which selectively destroys the γ subunit (Hurley & Stryer, 1982), or alternatively, by interaction with the GTP-bound form of T_α (Fung et al., 1981; Wensel & Stryer, 1986). A clue that T_α may stimulate the phosphodiesterase activity by binding to the inhibitory γ subunit was first noted by

† This work was supported by grants from the National Eye Institute (EY05895 and EY0265) and by the Jules and Doris Stein Research to Prevent Blindness Professorship (to B.K.-K.F.) from Research to Prevent Blindness, Inc.
* Address correspondence to this author.
‡ Present address: International Center for Genetic Engineering and Biotechnology, Padriciano 99, I-34012 Trieste, Italy.

[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); mAb, monoclonal antibody; PDE, retinal cGMP phosphodiesterase; T_α, α subunit of transducin; α, β, and γ, subunits of phosphodiesterase; β-gal-γ, β-galactosidase-γ fusion protein.

0006-2960/89/0428-6145$01.50/0