The Role of Arsenic-Thiol Interactions in Metalloregulation of the ars Operon*

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The ars operon of the Escherichia coli plasmid R773 that confers arsenical and antimonial resistance is negatively regulated by the ArsR repressor. ArsR residues Cys-32 and Cys-34 were previously identified as involved in induction by arsenite and antimonite, suggesting coordination between As(III) and the two cysteine thiolates. However, in small molecule thiolate-As(III) complexes, arsenic is frequently three-coordinate. A site-directed mutagenesis approach was employed in a search for a third arsenic ligand. ArsR proteins with C32G, C34G, and C32G/C34G substitutions were active repressors, but were not inducible in vivo. In vitro, the altered repressor-ars DNA complexes could not be dissociated by inducers. Alteration of Cys-37 and Ser-43, residues located in or near the putative helix-turn-helix DNA-binding region of the protein, had no effect on the inducibility of the operon. While these results indicated that neither the thiolate of Cys-37 nor the hydroxyl oxygen of Ser-43 is required for induction, they did not eliminate either atom as a potential arsenic ligand. Another approach involved reaction of an alternative inducer, phenylarsine oxide, which can form only two coordinations. Phenylarsine oxide was shown to be as effective as or more effective than arsenite or antimonite in vivo. In vitro, the organic arsenical was more effective than either arsenite or antimonite in dissociating the repressor-promoter complex. Thus, two ArsR–arsenic bonds are sufficient for induction. The interaction of ArsR proteins with As(III) was examined using a phenylarsine oxide affinity resin. ArsR proteins containing any two of the three cysteine residues Cys-32, Cys-34, and Cys-37 bound to the resin. Alteration of any two of the three resulted in loss of binding. Arsenic x-ray absorption spectroscopy of ArsR treated stoichiometrically with arsenite confirmed the average arsenic coordination as AsS3. These results suggest that all three cysteine thiolates are arsenic ligands, but binding to only two, the Cys-32 and Cys-34 thiolates, is required to produce the conformational change that results in release of the repressor from the DNA and induction.

Plasmid R773-mediated resistance to arsenite, arsenate, and antimonite in Escherichia coli is catalyzed by an arsenite extrusion system whose components are encoded by the ars operon (for recent reviews, see Rosen et al. (1995) and Dey and Rosen (1995)). Transcription of the ars operon is regulated by a substrate-responsive repressor, the ArsR protein. As metalloids, As(III) and Sb(III) can potentially interact with proteins as nonmetallic oxyanions do (Quiocho et al., 1987), or they can react as soft metals, forming covalent bonds with cysteine thiolates (Freeman, 1975). Among the proteins of the R773 ars operon, the ArsA ATPase, the catalytic component of the Ars pump, is allosterically activated by As(III)/Sb(III) interaction with three cysteine thiolates (Bhattacharjee et al., 1995). In contrast, there are no essential cysteine residues in ArsB, the membrane sector of the pump, indicating that the substrates of the pump are the oxyanions arsenite and antimonite (Chen et al., 1996).

Using a novel selection for arsR mutants that encoded active repressors that were unable to be induced by arsenicals or antimonials, we previously isolated three hydroxylamine mutants in the codons for Cys-32 and Cys-34, resulting in ArsR proteins C32Y, C32F, and C34Y (Shi et al., 1994). All three altered ArsR proteins still bound specifically to the ars operator, but inducers were considerably less effective in dissociating in vitro ArsR-DNA complexes. In vivo, each mutant arsR gene expressed a reporter gene controlled by the ars promoter, and addition of inducer did not relieve repression. Thus, these altered proteins retained repressor function, but had reduced response to inducer, suggesting that As(III)/Sb(III) can react as soft metals with the thiolates of Cys-32 and Cys-34. Among members of the ArsR family of metalloregulatory proteins, this cysteine pair and adjacent residues are highly conserved; we have proposed that this region forms a portion of the inducer recognition site and that the cysteine pair is required for metal binding (Shi et al., 1994).

How does binding of arsenic result in induction? It is reasonable to consider that coordination of As(III) with the cysteine thiolates results in a conformational change in the repressor that disrupts its interaction with the operator DNA. Both sulfur and oxygen can be arsenic ligands. For example, in the arsenic-glutathione complex, sulfur thiolates from three glutathiones serve as arsenic ligands forming As(GS)3 (Delnomdedieu et al., 1994), and in the arsenite-dithiothreitol complex, As(III) forms soft metal bonds with the two sulfur thiolates and one hydroxyl oxygen (Cruse and James, 1972). In addition to Cys-32 and Cys-34, ArsR contains three other cysteine residues, Cys-37, Cys-108, and Cys-116. The latter two have been shown to be unnecessary for repressor function (Wu and Rosen, 1991). In addition, in the putative helix-turn-helix DNA-binding domain, there are two serine residues, Ser-43 and Ser-48. In this study, the requirement for the sulfur thiolate of Cys-37 and the two serine hydroxyls was explored.

It was also not clear from the previous study whether the loss
of inducer recognition stemmed from the loss of the sulfur thiolates of Cys-32 and Cys-34 or from the introduction of the bulky aromatic rings. In this study, a series of single and double mutants in the codons for these residues were isolated by site-directed mutagenesis, where the residues were altered to residues with smaller side groups. In confirmation with the previous study (Shi et al., 1994), only alterations of Cys-32 and Cys-34 altered inducer recognition. In addition, using both in vivo and in vitro assays, the arsenate analog phenylarsine oxide (PAO)\(^1\) was found to be the most effective inducer of the ars operon. Since PAO can form only two arsenic-thiol bonds with ArsR, dicordinate binding must be sufficient for induction.

Using PAO affinity chromatography as an assay for arsenic-ArsR interaction, Cys-32, Cys-34, and Cys-37 were each found to be required for ArsR binding. These results suggest that the thiolates of Cys-32, Cys-34, and Cys-37 form a tricoordinate As\(\text{S}_3\) site. Arsenic x-ray absorption fine structure (EXAFS) was used to probe directly the coordination of As(III) in ArsR treated stoichiometrically with arsenite. As\(\text{S}_3\) coordination is confirmed in these experiments. Thus, while the Cys-37 thiolate is able to form a bond with As(III), binding to the sulfurs of only Cys-32 and Cys-34 is required to produce the conformational change in ArsR that results in dissociation from the operator DNA and transcriptional derepression.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Media—** Cultures of E. coli strains J M109, J M110, and HMS174(DE3) (Sambrook et al., 1989) bearing the indicated plasmids were grown at 37 °C in LB medium (Sambrook et al., 1989). Ampicillin (125 \(\mu\)g/ml), kanamycin (50 \(\mu\)g/ml), tetracycline (15 \(\mu\)g/ml), or chloramphenicol (20 \(\mu\)g/ml) was added as required. Sodium arsenite, potassium antimonyl tartrate, phenylarsine oxide, or isoprpyl-\(\beta\)-thiogalactopyranoside was added at the indicated concentrations. All chemicals were obtained from commercial sources.

**DNA**, which were as follows: constructed by inserting a 0.73-kb SalI-SalI fragment pair EcoRI-HindIII fragment containing the ars operator/promoter, the arsR gene, and a portion of the arsD gene from plasmid pWSU1 (San Francisco et al., 1990) into the multiple cloning site of vector plasmid pALTER\(^{TM}\)-1 (Promega). The pALTER series of plasmids was derived from pALTER by site-directed mutagenesis of the arsR gene. Each of the plasmids in the pALTER series was derived from the corresponding plasmid of the pALTER series by cloning an EcoRI-HindIII fragment containing arsR into expression vector pT7-5 (Tabor and Richardson, 1985). To create the plasmid pBGD23, the EcoRI-BclI fragment from the corresponding plasmid of the pALTER series was inserted into the EcoRI-BamHI site of vector pMLB1034 (Silhavy et al., 1984). Plasmid pBGD3R1 was constructed by deleting a Dral-Stul fragment from arsR in plasmid pBGD23.

**DNA Manipulation and Sequence Analysis—** Plasmid DNA was prepared with a Wizard DNA purification kit (Promega). Restriction endonucleases, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were from Life Technologies, Inc. Restriction endonuclease digestion, DNA ligation, and transformation were performed as described (Sambrook et al., 1989; Chung et al., 1989). The conditions for polymerase chain reaction were as described previously (Xu et al., 1996). The Sequenase kit (Version 2.0, U.S. Biochemical Corp.) was used for automated DNA sequencing, double-stranded DNA manual sequence as described previously (Shi et al., 1990) into the multiple cloning site of vector plasmid pALTER\(^{TM}\)-1. 1 The abbreviations used are: PAO, phenylarsine oxide; EXAFS, x-ray absorption fine structure; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-(morpholinomethanesulfonic acid); XAS, x-ray absorption spectroscopy.

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Metal-binding Site of the ArsR Protein

FIG. 1. Autoregulation of ArsR proteins. Exponentially growing cultures of E. coli strain JM109 bearing pAlR series plasmids with the indicated arsR mutations were grown for 2 h with (+) or without (-) 10 \( \mu \text{M} \) sodium arsenite. Expression of ArsR proteins was monitored by SDS-PAGE followed by immunoblotting with anti-ArsR serum.

Detection was used to measure the intensity of the AsK+ line with a Canberra 13-element germanium solid-state detector (provided by the National Institutes of Health Biotechnology Research Resource). Vertical defining slits attenuated the incoming photon intensity to assure that the germanium detector did not saturate (<35 kilocycles/channel). Standard EXAFS data reduction protocols were used (Scott, 1985) within the EXAFSPAK software (provided by G. N. George). Multiple scans (seven for ArsR and six for models) were averaged; the pre-edge background was fitted over the 11,822–11,868-eV range with a linear function. A spline was fitted to the pre-edge-subtracted data over the 11,872–12,681-eV range (with spline points at 12,142 and 12,411 eV), and the EXAFS was converted to \( k \)-space using an \( E_k \) taken as the midpoint of the edge jump.

Extraction of structural data used curve fitting of raw EXAFS data based on scattering parameters extracted from the EXAFS data for the model compounds arsonite (three oxygens at 1.85 Å) and arsenite + glutathione (three sulfurs at 2.25 Å in solution) (Delnomdedieu et al., 1994). Such analysis typically yields bond distances with an accuracy of \( \pm 0.02 \) Å.

RESULTS

Mutagenesis of the arsR Gene and Expression of Altered Proteins—Using a direct positive selection for arsR mutants that still encoded active repressors but had lost the ability to respond to inducer, three mutations have been isolated, arsR<sub>C32Y</sub>, arsR<sub>C34Y</sub>, and arsR<sub>C37G</sub> (Shi et al., 1994). To exclude the possibility that the phenotype of these ArsR derivatives resulted from the substitution of the bulky aromatic groups of tyrosine or phenylalanine for the two cysteine thiolates, these two cysteine residues in ArsR were changed to glycine residues, individually or together, by site-directed mutagenesis, producing three altered ArsR proteins: C32G, C34G, and C32G/C34G. Since expression of the arsR gene from its native ars promoter is autoregulated, the ability of these mutant genes to control their own production was determined by immunoblot analysis in the absence and presence of inducer (Fig. 1). In the absence of inducer, there was a low basal production of C32G and C32G/C34G, but no increase upon induction. The level of C34G was approximately the same as the uninduced wild-type basal level even under inducing conditions. These results indicate that the single and double glycine substitutions did not affect the ability of ArsR to repress its own synthesis, but did prevent these proteins from responding to inducer.

Since As(III) can form tricoordinate complexes with sulfur or oxygen atoms, the involvement of a third residue was investigated. Possible residues include cysteine thiolates and oxygens of serine or threonine hydroxyls. In R773 ArsR, there are 5 cysteine, 10 serine, and 2 threonine residues (Fig. 2). Comparison of the R773 repressor with the four other known ArsR homologs demonstrates that Cys-32, Cys-34, Cys-108, Ser-43, and Ser-48 are conserved (Fig. 2). Cys-108 has been shown previously not to be required for ArsR function (Wu and Rosen, 1991). Ser-43 and Ser-48 are within the predicted helix-turn-helix DNA-binding domain of the repressor (Shi et al., 1994); coordination with As(III) could cause a conformational change in that domain, producing dissociation from the DNA. For this reason, the codons for Ser-43 and Ser-48 were altered by site-directed mutagenesis to produce S43A, S43P, S48A, and S48T. The nonconserved Cys-37 was also changed to an alanine residue, and an arsR<sub>C32G/C34G</sub> double mutant was constructed. The single Cys-37 and Ser-43 substitutions inducibly regulated their own synthesis from the ars promoter (Fig. 1). The Ser-48 substitutions resulted in a constitutive phenotype, and the arsR<sub>C32G/C34G</sub> double mutant exhibited inducible phenotypes similar to that of the arsR<sub>C32G</sub> single mutant.

In Vivo Regulatory Properties of arsR Mutants—To investigate the repressive and metalloregulatory activities of the arsR mutants, an arsR<sub>S43A</sub>:lacZ fusion was constructed that produced a chimeric protein in which residue 9 of the lacZ gene from its native E. coli promoter (Fig. 3). In this plasmid, pBGD23, transcription of the gene fusion was under control of the upstream arsR gene. A derivative was constructed in which arsR was deleted by removal of an DraI-Stul fragment, creating plasmid pBGDAR1. Finally, the wild-type arsR of pBGD23 was replaced individually with each mutant arsR gene, creating the pBGD23 plasmid series. Arsenite-inducible \( \beta \)-galactosidase activity was measured in cells expressing each arsR gene (Fig. 3). The metalloregulatory activities of the arsR<sub>C37A</sub> and arsR<sub>S43A</sub> mutants were similar to that of the wild-type repression in the absence of inducer and inducibility with arsenite. \( \beta \)-Galactosidase activity in cells expressing the arsR<sub>C32G</sub> double mutant was slightly higher than the repressed basal level of the wild type, but was only slightly increased in the presence of arsenite. In cells expressing the arsR<sub>C34G</sub> mutant, only basal expression was observed in the presence or absence of inducer. Cells with the arsR<sub>C32G/C34G</sub> or arsR<sub>C32G/C34G</sub> double mutation had the slightly higher basal level of \( \beta \)-galactosidase expression observed with the arsR<sub>C32G</sub> single mutant, but did not respond to inducer. Expression of \( \beta \)-galactosidase in cells with the arsR<sub>S48A</sub> mutation was comparable to that of the arsR deletion in pBGDAR1, corresponding to constitutive expression. These results indicate that glycine substitutions in either Cys-32 or Cys-34 result in active repressors that no longer respond to inducer, consistent with the results of the phenylalanine and tyrosine substitutions (Shi et al., 1994). Thus, the loss of metalalloregulation is most likely due to the loss of the cysteine thiolate rather than to the introduction of bulky aromatic side chains. In contrast, substitution of alanine for Cys-37 or Ser-48 had no effect on the regulatory properties of the arsR gene in vivo. Since alteration of Ser-48 to alanine resulted in constitutive expression, no conclusion on its interaction with inducer could be made.

In Vitro DNA Binding Properties of Altered ArsR Proteins—
ArsR binds to a sequence immediately upstream of the ars promoter and can be dissociated with either As(III) or Sb(III) (Wu and Rosen, 1993). The DNA binding properties of the altered ArsR proteins and their interaction with inducer were determined. A 160-base pair fragment containing the R773 ars promoter was used as target DNA in both assays. This DNA fragment was synthesized by polymerase chain reaction and was 32P-labeled at the 3'-end of either one strand or the other. The affinity of the purified ArsR proteins for this DNA was determined in gel mobility shift assays (Fig. 4). From least-squares fits of the data, the Kd of the wild-type repressor was calculated to be 0.33 μM. For the majority of the altered proteins, there was no significant change in the affinity for the operator, indicating that their DNA binding properties were unchanged by the substitutions. The affinity of C32G was decreased to 0.75 μM, consistent with the increased basal level of in vivo expression observed in arsRC32G mutants. The affinity of S48A was reduced to 1 μM, consistent with the in vivo results and in support of the model in which Ser-48 is within the DNA-binding domain of the repressor.

To demonstrate that the altered proteins bound to the same site on the DNA, the DNase I footprints of the wild-type and C32G/C34G repressors were compared (Fig. 5). The doubly substituted C32G/C34G protein protected the same regions as the wild type, corresponding to nucleotides 261 to 237 on the coding strand and nucleotides 264 to 240 on the noncoding strand. While addition of the inducer arsenite or PAO (see below) dissociated the wild-type repressor from the DNA, C32G/C34G was not dissociated by inducers.

Induction of the ars Operon by Phenylarsine Oxide—PAO is an organic trivalent arsenical that inhibits proteins with critical thiol pairs (Frost and Schwalbe, 1990). PAO has recently been shown to be an effective inducer of the chromosomal ars operon (Xu et al., 1996). A major difference in the chemistry of PAO and sodium arsenite is that the As(III) in arsenite can form coordinate bonds with three ligands in proteins, while PAO forms only two (Fig. 6A). The properties of PAO as an inducer of the R773 ars operon were determined. PAO was found to induce ampicillin resistance in cells carrying plasmid pBLB51 (data not shown), in which expression of an arsB::blaM fusion is controlled by the ars promoter. In in vitro gel mobility shift assays, PAO dissociated the wild-type ArsR repressor at concentrations as low as 0.5 μM (Fig. 6B). PAO did not dissociate the inducer-nonresponsive C32Y repressor from the DNA, indicating that the effect of PAO is specific, involving interaction with the repressor at the in-
The affinity of the repressor for the physiological inducers was determined (Fig. 7). In this assay, 1 μM ArsR was mixed with sufficient DNA to produce 60% protein-DNA complex and 40% free DNA. To dissociate half of the bound repressor from the DNA required 1 μM PAO, compared with 10 μM sodium arsenite or potassium antimonyl tartrate. These results demonstrate that PAO is a better inducer of the ars operon in vitro than inorganic As(III) or Sb(III). Since induction in vivo is multifactorial, reflecting a combination of inducer uptake, transcriptional activation, and effects of inducer on the activity of the reporter gene product, in vitro inducibility may be a better measure of the strength of the inducer-repressor interaction.

The effect of PAO on the DNA binding properties of ArsR proteins with substitutions of cysteine residues was determined (Fig. 8). Neither the C32G nor C34G repressor dissociated from the DNA with any concentration of PAO tested. The C37A repressor exhibited the same response to PAO as the wild type. The doubly substituted C32G/C34G and C32G/C34G proteins gave the same response as the C32G protein (data not shown). Similarly, 0.5 μM PAO prevented the wild-type repressor, but not C32G/C34G, from protecting the DNA fragment from DNase I digestion (Fig. 5, lanes 4, 7, 11, and 14). Thus, for the in vitro response of the repressor to PAO, cysteine thiolates appear to be required at residues 32 and 34, but not 37.

Binding of ArsR Proteins to Immobilized Phenylarsine Oxide—Although the effects of mutation of arsR indicated that Cys-32 and Cys-34 are required for induction, they do not eliminate the possibility that other residues interact with As(III) without producing the conformational change that results in dissociation from DNA. Direct binding studies with $^{77}$AsO$_4^{3-}$ were uninformative due to high background levels of binding (data not shown). To determine residues in ArsR involved in coordination with As(III), interaction with PAO was measured using PAO immobilized on a matrix. Strong binding to immobilized PAO requires a vicinal cysteine pair (Hoffman and Lane, 1992). Wild-type and altered ArsR proteins were applied to the column, which was washed extensively to remove unbound proteins. Proteins were then eluted in steps with...
increasing concentrations of β-mercaptoethanol. The position of elution of ArsR was determined by SDS-PAGE followed by immunoblotting (Fig. 9). Wild-type ArsR protein required 0.6 M β-mercaptoethanol to be dissociated from the PAO column. The singly substituted C32G, C34G, and C37A proteins eluted with 0.4 M β-mercaptoethanol. The PAO binding ability of the doubly substituted C32G/C37G protein was dramatically decreased, requiring only 10 mM β-mercaptoethanol, and the C32G/C34G protein did not bind to the affinity resin. Bovine serum albumin and the ArsC arsenate reductase proteins, neither of which have vicinal cysteine pairs, did not bind to the matrix. The ArsA ATPase eluted with 10 mM β-mercaptoethanol. Although ArsA has three cysteine residues involved in As(III) activation, none are vicinal pairs (Bhattacharjee et al., 1995). These results demonstrate that ArsR is a high affinity As(III)-binding protein. Since substitution of the cysteine thiolates of Cys-32, Cys-34, or Cys-37 individually did not eliminate strong interaction with PAO, each altered protein must still contain a cysteine pair capable of forming two coordinations with the As(III) of PAO. The fact that doubly substituted proteins no longer exhibited strong interaction with PAO indicates that any pair composed of Cys-32, Cys-34, or Cys-37 is sufficient for binding to PAO. The genetic results suggest that binding to only the Cys-32 and Cys-34 pair results in induction. Thus, binding of As(III) to Cys-37 occurs, but is not sufficient to produce induction.

XAS Studies—Wild-type ArsR reacted with stoichiometric arsenite was analyzed by arsenic x-ray absorption spectroscopy. The EXAFS technique provides direct local structural information about the coordination environment of a selected element (Scott, 1985). Fig. 10 shows the AsK edge EXAFS and Fourier transform data for As(III)-loaded ArsR as well as the curve-fitting simulation for three arsenic-sulfur interactions at 2.25 Å (relative Debye-Waller factor, $\Delta\sigma^2 = 0.000$ Å$^2$, compared with the arsenite + glutathione solution as the reference). For comparison, a simulation of the EXAFS and Fourier transform data for hypothetical AsO$_5$S$_2$ coordination based on arsenic-oxygen and arsenic-sulfur interactions extracted from model compound EXAFS data is included. These data show that on average, each As(III) is bonded to three sulfur-containing ligands with arsenic-sulfur bonds of 2.25 Å.

**FIG. 9. PAO-ArsR interaction chromatography.** PAO affinity chromatography was performed as described under "Material and Methods." One mg of each repressor protein was applied to the column, followed by stepwise elution with increasing concentrations of β-mercaptoethanol (β-ME). In each assay, the height of the bar indicates the concentration of β-mercaptoethanol required for elution. BSA, bovine serum albumin.

**FIG. 10. Determination of arsenic ligands in ArsR.** AsK edge EXAFS (top) and Fourier transform (FT) (bottom; $k^2$-weighted, $k = 3.5-13.0$ Å$^{-1}$) data are shown for As(III)-loaded ArsR. Solid lines, experimental data; dashed lines, the best fit simulation with three arsenic-sulfur interactions at 2.25 Å; dotted lines, simulation of hypothetical AsO$_5$S$_2$ coordination (arsenic-oxygen = 1.85 Å; arsenic-sulfur = 2.25 Å).

**DISCUSSION**

ArsR is the arsenic/antimony-responsive repressor of the plasmid R773 ars operon (Wu and Rosen, 1991, 1993). The protein is the best characterized member of the ArsR family of metalloregulatory proteins (Shi et al., 1994). In members of this family, there is the highly conserved sequence ELCVCDL located adjacent to the putative helix-turn-helix DNA-binding domain. Using a direct selection of arsR mutants that lost the ability to respond to inducer but retained repression, we previously isolated by chemical mutagenesis three mutants in the cysteine pair of this conserved sequence (C32Y, C32F, and C34Y) (Shi et al., 1994). These altered proteins retained the ability to bind to the ars promoter, but had reduced metal response, suggesting that the cysteine pair Cys-32 and Cys-34 is a component of the metallosensory domain of ArsR, forming soft metal bonds between trivalent arsenic and the sulfur thiolates. However, it was possible that the phenotypes resulted from the introduction of aromatic side chains rather than from loss of the thiolates. In this study, it was shown that glycine substitutions produced the same effects both in vivo and in vitro as the phenylalanine and tyrosine substitutions, supporting the hypothesis that cysteine thiolates are ligands for As(III) coordination (Rosen et al., 1995).

In addition to Cys-32 and Cys-34, there are three other cysteine residues in the ArsR protein: Cys-37, Cys-108, and Cys-116. The last two have been shown not to be required for ArsR function (Wu and Rosen, 1993). In the present study, Cys-37 was altered mutagenically to alanine, and this substitution did not affect the in vivo regulatory or in vitro DNA binding properties. Additionally, the possible participation of the hydroxyl oxygens of Ser-43 and Ser-48, residues conserved in the five known ArsR proteins, was investigated. Alteration of Ser-43 to alanine or proline had no effect on ArsR function in vivo or in vitro, while alteration of Ser-48 resulted in constitutivity in vivo and decreased affinity of DNA in vitro. Although both residues are in the putative helix-turn-helix DNA-binding
domain (Shi et al., 1994), Ser-43 is located in the turn region, and its substitution may not be affected by introduction of a helix breaker such as proline. The use of phenylarsine oxide has proven additionally instructive. This organic arsenical can form only two coordinations, the third being an arsenic–carbon bond. PAO is not only an effective inducer in vivo; in vitro DNA binding assays, ArsR exhibited 10–100-fold higher affinity for this organic arsenical than for inorganic arsenic or antimonite. Since a third coordination is not possible with PAO, induction must require only two bonds between inducer and ligands in ArsR. The combination of the results of mutagenesis and PAO induction suggests that only the Cys-32 and Cys-34 pair is involved in the interaction with As(III) that results in induction.

However, it was possible that there are other interactions with As(III) not required for induction. While there appear to be two soft metal bonds to the Cys-32 and Cys-34 sulfur thiolates, the existence or nature of a third coordination in arsenite was unknown. In PAO, it is the phenyl ring; in arsenite, posibilities included other cysteine sulfur thiolates, the oxygens of serine or threonine hydroxyls, or the third hydroxyl group of arsenite itself. PAO-protein interactions can be measured using matrix-bound PAO (Huffman and Lane, 1992). Wild-type ArsR bound to the PAO resin with extremely high affinity, characteristic of a protein with a vicinal thiol pair. However, ArsR proteins in which either Cys-32 or Cys-34 was altered to glycine still bound strongly to the PAO resin. The C32G/C34G ArsR proteins in which either Cys-32 or Cys-34 was altered to As(III) and that there are no oxygen ligands, confirming that only cysteine residues, and not serine or threonine, are arsenic ligands. Combining these results with the information from the known structures of the arsenite-dithiothreitol (Cruse and James, 1972) and the arsenite-glutathione (Delnomdedieu et al., 1994) complexes, we propose a model for the arsenic-ArsR complex in which As(III) is bound in a cage formed by the three cysteine thiolates at residues 32, 34, and 37 (Fig. 11). Even though coordination to Cys-37 thiolate occurs, the soft metal bonds formed between arsenic and the sulfurs of Cys-32 and Cys-34 are sufficient to produce a conformational change in the DNA-binding domain that results in dissociation of the repressor from the operator DNA.

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