Spatial Proximity of Cys\textsuperscript{113}, Cys\textsuperscript{172}, and Cys\textsuperscript{422} in the Metalloactivation Domain of the ArsA ATPase*

(Received for publication, April 17, 1996, and in revised form, June 6, 1996)

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ArsA ATPase activity is allosterically activated by salts of the semimetal arsenic or antimony. Activation is associated with the presence of three cysteine residues in ArsA: Cys\textsuperscript{113}, Cys\textsuperscript{172}, and Cys\textsuperscript{422}. To determine the distance between cysteine residues, wild type ArsA and ArsA proteins with cysteine to serine substitutions were treated with the bifunctional alkylating agent dibromobimane, which reacts with thiol pairs within 3–6 Å of each other to form a fluorescent adduct. ArsA proteins in which single cysteine residues were altered by site-directed mutagenesis still formed fluorescent adducts. Proteins in which two of the three cysteine residues were substituted did not form fluorescent adducts. These results demonstrate that Cys\textsuperscript{113}, Cys\textsuperscript{172}, and Cys\textsuperscript{422} are in close proximity of each other. We propose a model in which As(III) or Sb(III) interacts with these three cysteines in a trigonal pyramidal geometry, forming a novel soft metal-thiol cage.

Plasmid-mediated resistance to the metalloid salts arsenite and antimonite has been observed in both Gram-negative and Gram-positive bacteria (Rosen et al., 1995). In the Gram-negative bacterium Escherichia coli, the conjugative R-factor R773 confers resistance by coding for an ATP-coupled arsenite pump that actively extrudes the metalloid oxyanions out of the cell, thereby reducing their intracellular concentration to subtoxic levels (Rosen et al., 1995). The pump is composed of ArsA, a 63-kDa peripheral membrane protein that is the catalytic subunit of the pump, and ArsB, a 45-kDa integral membrane protein that serves both as the membrane anchor for ArsA and as the anion-conducting pathway. Under physiological conditions, ArsA is a part of a complex with the ArsB protein in the inner membrane of E. coli. When expressed at high levels, ArsA is found predominantly as a soluble protein in the cytosol (Rosen et al., 1988). Soluble ArsA has been purified and shown to be an As(III)- or Sb(III)-stimulated ATPase. The 583-amino acid ArsA has distinct binding sites for its substrate, ATP, and effecter, As(III) or Sb(III) (Hsu and Rosen, 1989).

Genetic evidence suggests that allosteric activation occurs via the formation of a three-coordinate complex of As(III) or Sb(III) with the cysteine thiolates of residues 113, 172, and 422 (Bhattacharjee et al., 1995). Although these three residues are located distant from each other in the primary sequence, these results imply that they come close together in the tertiary structure to interact with either As(III) or Sb(III). From the crystal structure of known As-thiol and Sb-thiol compounds (Sowerby, 1994), we would predict that the cysteine thiolates should be 3–4 Å from each other. The homobifunctional cross-linker 4,6-bis(bromomethyl)-3,7-dimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione (dibromobimane (bBBr)) was used to map distance between pair of cysteines in ArsA. bBBr has two equivalent bromomethyl groups that can cross-link a thiol pair located within 3–6 Å of each other (Mornet et al., 1985). bBBr is nonfluorescent in solution but becomes fluorescent when both of its alkylating groups have reacted (Haugland, 1994). This property allows bBBr to be used as a molecular ruler to map distance between cysteine residues.

In this report we demonstrate that ArsA proteins with any one cysteine residue group altered by mutagenesis still formed fluorescent adducts. Proteins lacking any two of the three cysteines at residue 113, 172, or 422 did not form fluorescent adducts. The results demonstrate that Cys\textsuperscript{113}, Cys\textsuperscript{172}, and Cys\textsuperscript{422} are located within a distance of 3–6 Å from each other. A model is proposed where As(III) or Sb(III) interacts with the three cysteine thiolates in a trigonal pyramidal geometry, forming a soft metal-thiol cage at the metalloactivation domain of the enzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—The strains and plasmids used in this study are given in Table I. Cultures of E. coli strains JM109 or JM110 (Sambrook et al., 1989) bearing the indicated plasmids were grown at 37 °C in LB medium (Sambrook et al., 1989). Ampicillin (125 μg/ml) or tetracycline (10 μg/ml) was added as required. Sodium arsenite, potassium antimonyl tartrate, or isopropyl β-D-thiogalactopyranoside was added at the indicated concentrations. All chemicals were obtained from commercial sources.

Oligonucleotide-directed Mutagenesis—Mutations in the sequence of the araC gene were introduced by site-directed mutagenesis using the Altered Sites™ in vitro Mutagenesis System (Promega), as described earlier (Bhattacharjee et al., 1995). Plasmid pALTER-AB, containing the araC and araB genes in the multiple cloning site of vector pALTER-1 (Promega) was used as the template (Bhattacharjee et al., 1995). The mutagenic oligonucleotides used and the respective changes (underlined) introduced were as follows: C26S, CGTGGCGGAGGAAATAGA; C113S, CTCTGTTGTGGATGCACCTGA; C172S, TGGGCCGAGAGAGCACTTG; C422S, TTCCCACGGGAAGGGTAGC. Oligonucleotides were synthesized in the Macromolecular Core Facility of Wayne State University School of Medicine. The identity of the mutations was confirmed by DNA sequencing of the entire araC gene of each mutant.

DNA Manipulation and Sequence Analysis—Plasmid DNA was prepared using a Wizard DNA purification kit (Promega). Plasmid isolation, DNA restriction endonuclease analysis, ligigation, and transformation were performed as described (Sambrook et al., 1989; Chung et al., 1989). For DNA digestion with ClaI, appropriate plasmids were introduced into E. coli strain JM110. Restriction enzymes and nucleic acid-
modifying enzymes were obtained from Life Technologies, Inc. except for Seek387L, which was purchased from Takara Biomedicals. The Sequenase kit (version 2.0, U.S. Biochemical Corp.) was used for double-stranded DNA manual sequencing, as described previously (Bhattacharjee et al., 1995). For automated DNA sequencing, double-stranded DNA was isolated with a QIAGEN plasmid kit and sequenced using an ALFExpress system and Cy5 labeled sequence kit (Pharmacia Biotech Inc.).

**Purification and Assay of ArsA—** Altered ArsA proteins were purified from cultures of *E. coli* strain JM109 bearing pALTER-1 derivatives containing the mutated *arsA* genes. Cultures were grown at 37 °C overnight with aeration in 0.25 liters of LB medium containing 0.125 mg/ml of ampicillin. The cultures were diluted into 2.5 liters of pre-warmed LB medium containing 0.125 mg/ml of ampicillin. At an A600 of 0.6–0.8, production of the mutant protein was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. ArsA was purified essentially as described (Hsu and Rosen, 1989) and stored at ~70 °C until use. Each ArsA was judged to be ~95% homogeneous by Coomassie Blue staining of samples separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The concentration of ArsA in purified preparations was determined using a modification of the method of Lowry et al. (1951) or from the absorption at 280 nm using a molar extinction coefficient of 33,480 (Rosen et al., 1988).

**Polyacrylamide Gel Electrophoresis and Immunological Blotting—** Cultures of cells containing the appropriate plasmids were grown in LB medium at 37 °C to an A600 of 0.6, at which point expression of the genes was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside. ArsA was purified essentially as described (Hsu and Rosen, 1989).

**Inactivation of ArsA by Dibromobimane—** Altered ArsA proteins were purified from cultures of *E. coli* strain JM109 bearing pALTER-1 derivatives containing the mutated *arsA* genes. Cultures were grown at 37 °C overnight with aeration in 0.25 liters of LB medium containing 0.125 mg/ml of ampicillin. The cultures were diluted into 2.5 liters of pre-warmed LB medium containing 0.125 mg/ml of ampicillin. At an A600 of 0.6–0.8, production of the mutant protein was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. ArsA was purified essentially as described (Hsu and Rosen, 1989) and stored at ~70 °C until use. Each ArsA was judged to be ~95% homogeneous by Coomassie Blue staining of samples separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The concentration of ArsA in purified preparations was determined using a modification of the method of Lowry et al. (1951) or from the absorption at 280 nm using a molar extinction coefficient of 33,480 (Rosen et al., 1988). ATPase activity was assayed using the coupled assay of Vogel and Steinhart (1976), as described previously (Hsu and Rosen, 1989).

**Reaction of ArsA with bBBr—** bBBr was purchased from Molecular Probes, Inc. bBBr was dissolved in dimethyl formamide and used as a 10 mM stock solution. Before reaction of ArsA with bBBr, dithiotreitol was removed using a spin column (Penesky, 1977). ArsA (10 μM) was incubated at room temperature with a 2-fold molar excess of bBBr in 50 mM MOPS-KOH buffer, pH 7.5, containing 0.25 mM disodium ethylenediaminetetraacetate (buffer A). For activity measurements, portions of the reaction mixture were removed at intervals, the reaction was quenched by the addition of 20 μl dithiothreitol, and residual activity was determined.

**Titration of ArsA with 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB)—** The reactivity of the sulphydryl groups in the native and denatured enzyme was determined by reaction with DTNB. ArsA (1–2 × 10−5 M in buffer A) was reacted with DTNB, and the reaction was monitored by the increase in absorbance at 412 nm. The total number of cysteine residues was determined by denaturing the enzyme with a solution of 6 M guanidine HCl in buffer A prior to reaction with DTNB. The concentration of sulphydryl groups was calculated using ε412 = 14,150 M−1 cm−1 (Riddles et al., 1979).

**RESULTS**

**Inactivation of ArsA by Dibromobimane—** The effect of bBBr on the Sb(III)-stimulated ATPase activity of wild type ArsA was examined. ArsA was rapidly and completely inactivated by a 2-fold molar excess of bBBr. A plot of 1/(V/Vt) against time
was linear, consistent with second-order reaction kinetics (Fig. 1), where \( V_t \) is the rate at various times of inactivation by bBBr, and \( V_i \) is the rate at zero time. Partial protection was observed when the protein was preincubated with either ATP or Sb(III). The \( t_{1/2} \) of inactivation by bBBr alone was 2.1 min, whereas the half-time of inactivation reaction increased to 2.6 min and 3.5 min when preincubated with either antimone or ATP, respectively. When both ATP and antimone were added together, synergistic protection was observed, with \( t_{1/2} \) increasing to 16.2 min. These results indicate that together antimone and ATP synergistically induce a conformational change that decreases the accessibility of the cysteine thiolates to bBBr. The protection experiments were done in the absence of MgCl\(_2\); thus, the observed effects occurred in the absence of ATP hydrolysis.

To examine the correlation between loss in activity and the number of cysteines modified, the C26S ArsA was treated with bBBr. We had shown earlier that Cys\(^{26} \) is not involved in either activation or catalysis and that the C26S enzyme is fully active (Bhattacharjee et al., 1995). For this reason C26S was used as the control. C26S was treated with a 2-fold molar excess of bBBr, and the rate of inactivation was determined. The number of cysteines modified by bBBr was determined by removing portions at intervals and extracting bBBr with chloroform. The protein was then denatured with 6 M guanidine hydrochloride, and the concentration of free thiol was determined with DTNB. C26S was completely inactivated following modification of two of the three cysteines (Fig. 2). The bBBr modification reaction of C26S exhibited biphasic kinetics, where one of the cysteines reacted rapidly, with more than 50% of the activity lost in the first min. The other cysteine reacted more slowly, following which activity was completely lost. The kinetics of inactivation suggest rapid displacement of the bromine of bBBr by a thiol, followed by slow displacement of the second bromine with another thiol. C26S modified with bBBr did not form significant amounts of dimers or higher order oligomers as determined by SDS-PAGE (data not shown), suggesting the absence of intermolecular cross-linking. The monomer was highly fluorescent on SDS gels (data not shown), indicating that any two of the three available cysteines can be cross-linked within a monomer.

### Table I

| Strain/plasmid | Genotype/description | Reference |
|----------------|----------------------|-----------|
| JM 109         | recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1 (lac-proAB) F\(^{rtraD36 proAB- lacY galK galT araTonA thr-1} \Delta(lac-proAB) B\(^{ftraD36 proAB- lacY galK galT araTonA thr-1} \Delta(lac-proAB) | Sambrook et al., 1989 |
| JM 110         | dam dcm supE44 hsdR17 thy leu proA1 lacY galK galT araTonA thr-1 | Sambrook et al., 1989 |
| Plasmid        |                      |           |
| pAlterAB (arsAB) | Site-directed mutagenesis of arsA Cys\(^{26} \) codon to serine codon | Bhattacharjee et al., 1995 |
| pC26S (arsA\(_{C26S} \)) | Site-directed mutagenesis of arsA Cys\(^{113} \) codon to serine codon | Bhattacharjee et al., 1995 |
| pC172S (arsA\(_{C172S} \)) | Site-directed mutagenesis of arsA Cys\(^{172} \) codon to serine codon | Bhattacharjee et al., 1995 |
| pC422S (arsA\(_{C422S} \)) | Site-directed mutagenesis of arsA Cys\(^{422} \) codon to serine codon | Bhattacharjee et al., 1995 |
| pC26S/C113S (arsA\(_{C26S/C113S} \)) | Site-directed mutagenesis of arsA Cys\(^{26} \) and Cys\(^{113} \) codons to serine codons | This study |
| pC26S/C172S (arsA\(_{C26S/C172S} \)) | 2.8-kilobase pair Hind III-KpnI fragment containing arsA and arsB genes cloned into the multiple cloning site of pALTER-1 vector (Promega) | Bhattacharjee et al., 1994 |
| pC26S/C113S/C172S (arsA\(_{C26S/C113S/C172S} \)) | 2037-bp SphI-KpnI fragment from pC26S cloned into See8387-KpnI-deleted pC26S | This study |
| pC113S/C172S (arsA\(_{C113S/C172S} \)) | 2505-bp Aval fragment from pC113S cloned into Aval-deleted pC172S | This study |
| pC113S/C422S (arsA\(_{C113S/C422S} \)) | 1757-bp ClaI fragment from pC113S cloned into ClaI-deleted pC422S | This study |
| pC172S/C422S (arsA\(_{C172S/C422S} \)) | 2505-bp Aval fragment from pC26S/C113S/C422S cloned into Aval-deleted pC172S | This study |
| pC26S/C113S/C422S (arsA\(_{C26S/C113S/C422S} \)) | 2053-bp Sse8387-EcoRI fragment from pC422S cloned into See8387-EcoRI-deleted pC422S | This study |
| pC26S/C172S/C422S (arsA\(_{C26S/C172S/C422S} \)) | 2037-bp Sse8387-KpnI fragment from pC422S cloned into See8387-KpnI-deleted pC26S/C172S | This study |
| pC113S/C172S/C422S (arsA\(_{C113S/C172S/C422S} \)) | 2050-bp Aval fragment from pC26S/C113S/C172S cloned into Aval-deleted pC113S/C172S | This study |
| pC26S/C113S/C172S/C422S (arsA\(_{C26S/C113S/C172S/C422S} \)) | 2050-bp Aval fragment from pC26S/C113S/C172S/C422S cloned into Aval-deleted pC113S/C172S | This study |

### Strains and plasmids

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| JM 110         | dam dcm supE44 hsdR17 thy leu proA1 lacY galK galT araTonA thr-1 | Sambrook et al., 1989 |

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| JM 110         | dam dcm supE44 hsdR17 thy leu proA1 lacY galK galT araTonA thr-1 | Sambrook et al., 1989 |
expression of the \( \text{LB} \) medium containing varying concentrations of sodium arsenite. Expression wild type and mutant \( \text{ars} \) plasmids were diluted 100-fold into fresh LB medium containing varying concentrations of sodium arsenite. Expression of the \( \text{ars} \) genes was induced with 0.1 mM isopropyl-\( \beta \)-D-thiogalactopyranoside, and growth was measured after 6 h of growth at 37 °C. Cells shown in A had the following plasmids: \( \text{pALTER-AB (arsAB)} \) \( \bullet \), \( \text{pC26S/C113S (arsA}_{26S/C113S} \) \( \bullet \), \( \text{pC26S/C172S (arsA}_{26S/C172S} \) \( \bullet \), \( \text{pC26S/C422S (arsA}_{26S/C422S} \) \( \bullet \), \( \text{pC113S/C172S (arsA}_{113S/C172S} \) \( \bullet \), \( \text{pC113S/C422S (arsA}_{113S/C422S} \) \( \bullet \), \( \text{pC26S/C113S/C172S (arsA}_{26S/C113S/C172S} \) \( \bullet \), \( \text{pC26S/C113S/C422S (arsA}_{26S/C113S/C422S} \) \( \bullet \), \( \text{pC26S/C172S/C422S (arsA}_{26S/C172S/C422S} \) \( \bullet \), \( \text{pC26S/C113S/C172S/C422S (arsA}_{26S/C113S/C172S/C422S} \) \( \bullet \), \( \text{pC113S/C172S/C422S (arsA}_{113S/C172S/C422S} \) \( \bullet \), \( \text{pC26S/C113S/C172S/C422S (arsA}_{26S/C113S/C172S/C422S} \) \( \bullet \), vector plasmid \( \text{pALTER-1} \) \( \bullet \), cells shown in B had the following plasmids: \( \text{pALTERAB (arsAB)} \) \( \bullet \), \( \text{pC26S/C113S/C172S (arsA}_{26S/C113S/C172S} \) \( \bullet \), \( \text{pC26S/C113S/C422S (arsA}_{26S/C113S/C422S} \) \( \bullet \), \( \text{pC26S/C172S/C422S (arsA}_{26S/C172S/C422S} \) \( \bullet \), \( \text{pC113S/C172S/C422S (arsA}_{113S/C172S/C422S} \) \( \bullet \), \( \text{pC26S/C113S/C172S/C422S (arsA}_{26S/C113S/C172S/C422S} \) \( \bullet \), \( \text{pC26S/C113S/C172S/C422S (arsA}_{26S/C113S/C172S/C422S} \) \( \bullet \), vector plasmid \( \text{pALTER-1} \) \( \bullet \).

Limited Trypsin Digestion of the Altered ArsA Proteins—Accessibility to trypsin has been used as a measure of the structure of ArsA and of the conformational changes induced by binding of nucleotide and metalloid effector (Hsu and Rosen, 1989). When wild type ArsA was exposed to trypsin in the absence of ligands, the protein was digested to a 30-kDa species (Fig. 4A). In the absence of ligands the accessibility of C26S/ C113S to trypsin was essentially identical to the wild type (Fig. 4B). ATP afforded the same protection for C26S/C113S as the wild type (Fig. 4, C and D), indicating that binding of ATP produced a similar conformational change in both. While the addition of antimonite had no effect on the rate of proteolysis (Fig. 4A), the addition of both ATP and antimonite produced synergistic protection from trypsin (Fig. 4C), as had been shown previously (Hsu and Rosen, 1989). In contrast, the combination of ATP and antimonite did not provide additional protection over that afforded by ATP alone with the C26S/ C113S ArsA (Fig. 4D). The other combinations of double, triple, and quadruple serine-substituted ArsAs showed the same pattern of trypsin accessibility as C26S/C113S (data not shown). The loss of this cooperative effect of ligands in the proteins lacking any of the three cysteine residues at position 113, 172, or 422 indicates decreased binding of the metalloid effector in these serine-substituted proteins.

ATPase Activity of Altered ArsAs—The purified proteins were analyzed for their ability to catalyze metalloid-activated ATP hydrolysis. Of the six double serine-substituted ArsAs, only C26S/C113S, C26S/C172S, and C26S/C422S showed Sb(III)-stimulated ATPase activity (Table II). Each altered protein had a \( K_m \) for ATP that was within the same order of magnitude as that of the wild type enzyme, while the concentration of antimonite required for half-maximal activation differed considerably. Both C26S/C113S and C26S/C172S exhibited approximately a 20-fold increase in the concentration of antimonite required for half-maximal activation, and the C26S/ C422S enzyme exhibited more than a 200-fold increase. The results with arsenite as an activator were consistent with the results with antimonite; C26S/C113S and C26S/C172S both exhibited nearly a 10-fold increase in the concentration of arsenite required for half-maximal activation, whereas C26S/ C422S required about 20-fold more than the wild type enzyme (data not shown). The intrinsic rates of hydrolysis of the altered enzymes were slightly less than the wild type, but the major effect of replacement of the cysteine residues was on the Sb(III)-activated rates (Table II). The Sb(III)-stimulated \( V_{max} \) of the C26S/C172S enzyme was near that of the wild type. Enzymes with any two of the three essential cysteines altered simultaneously to serines had no measurable Sb(III)-stimulated ATPase activity, including the double-substituted C113S/ C172S, C113S/C422S, and C172S/C422S; triple-substituted C26S/C113S/C172S, C26S/C113S/C422S, C26S/C172S/C422S, and C113S/C172S/C422S, and cysteine-free C26S/C113S/ C172S/C422S enzymes.

Dibromobimane Modification of ArsA—The increase in fluorescence of bBBr on cross-linking of spatially proximate thiols was used for distance mapping of cysteines in ArsA. If the cysteine triad of Cys \(^{113}\), Cys \(^{172}\), and Cys \(^{422}\) are indeed each As(III) or Sb(III) ligands, they should be close enough to each other to form fluorescent adducts with bBBr. C26S, which still has the cysteine triad, reacted with bBBr to form a fluorescent species with an absorption maximum at 395 nm and emission maximum at 470 nm (Fig. 5, curve 1). Mutations into the codons of each of the three cysteines in the triad were introduced into the \( \text{ars}_{26S} \) gene, and the gene products with only two of the three cysteines remaining became highly fluorescent following bBBr modification, as illustrated with C26S/C172S (Fig. 5, curve 2). C26S/C113S and C26S/C422S gave superimposable results (data not shown). In contrast, removal of two of the three cysteines of the triad resulted in ArsA proteins with substantially reduced levels of fluorescence (Fig. 5, curves 3–5), and the cysteine-free ArsA had almost no increase in fluorescence over base line following reaction with bBBr (Fig. 5, curve 6).

Steady state experiments with bBBr have a relatively high background level of fluorescence. This is due in part to decomposition of bBBr in the presence of light and in part to a slow, nonspecific displacement of the second bromine by water following reaction of the first with cysteine. bBBr can also react with other nucleophilic residues to produce fluorescence. Since cysteine thiolates react much more rapidly with bBBr than do other nucleophilic residues, the rate of bBBr reaction with ArsA was examined (Table III). Single serine-substituted ArsAs C26S, C113S, C172S, and C422S and the doubly substituted ArsAs C26S/C113S, C26S/C172S, and C26S/C422S reacted with bBBr at rates only slightly slower than wild type. C113S/C172S was 3-fold less reactive, whereas C113S/C422S and C172S/C422S were 7-fold less reactive compared with wild type. Triple serine substitutions C26S/C113S/C172S, C26S/ C113S/C422S, and C26S/C172S/C422S were on the average 9-fold less reactive. Both C113S/C172S/C422S and the cysteine-free ArsA had more than a 60-fold decrease in reactivity. The near complete absence of steady state fluorescence in the cysteine-free protein (Fig. 5) and the low rate of modification of the triple serine-substituted ArsAs by bBBr clearly indicate that bBBr is specific for thiols in ArsA and that other functional groups are not modified. Moreover, the kinetics of the bBBr reaction show that at least two of the three cysteines at resi-

![Fig. 3. Resistance to arsenite in cells expressing wild type and mutant \text{ars} genes.](image-url)
dues 113, 172, and 422 are required for rapid cross-linking by the bifunctional reagent bBBr.

**DISCUSSION**

From the results of mutagenesis of cysteine codons in arsA we demonstrated that Cys113, Cys172, and Cys422 are required for allosteric activation of the ArsA ATPase (Bhattacharjee et al., 1995). We proposed that activation by arsenite or antimonite occurs through soft metal-thiol interaction of As(III) or Sb(III) with the thiolate of those three cysteine residues. Although the three cysteine residues are located far apart in the primary sequence, they must come close enough in the tertiary structure to interact with the soft metals. From X-ray crystal data of As(III) or Sb(III) complexed to dithiols, the length of an As–S bond and Sb–S bond are 2.23 and 2.45 Å, respectively, while S–As–S and S–Sb–S angles are 92.7 and 84.8°, respectively (Sowerby, 1994). Moreover, using a combination of site-directed mutagenesis and arsenic X-ray absorption spectroscopy, we have recently shown that the ArsR repressor binds As(III) to the thiolates of the cysteine triad of Cys32, Cys34, and Cys37 with As–S bonds of 2.25 Å (Shi et al., 1996). With the knowledge of these examples we propose a model for interaction of ArsA with As(III) or Sb(III) in which the thiolates of Cys113, Cys172, and Cys422 are coordinated to the metalloid in a trigonal pyramidal geometry (Fig. 6). This model predicts that the thiolates should be within 3.1–3.5 Å of each other. To test this hypothesis, dibromobimane was used as a molecular ruler. bBBr rapidly inactivated the ATPase activity of ArsA, and
synergistic protection from bBBr inactivation was observed when both the nucleotide substrate and metalloidal effector were present (Fig. 1). Since bBBr reacts preferentially with cysteines, the protection experiment suggested that it is reacting with the cysteines located in the metalloactivation domain. Inactivation required modification of only two of the three essential cysteines. One cysteine reacted rapidly, whereas the other reacted more slowly (Fig. 2). This might reflect modification of a cysteine by one bromomethyl group of bBBr, followed by reaction of the remaining bromomethyl group with an adjacent cysteine.

Dibromobimane is nonfluorescent until it cross-links two adjacent nucleophiles located within 3–6 Å of each other. The thioles of the cysteine triad would therefore be expected to react with bBBr to form fluorescent adducts. ArsA reacted with bBBr became highly fluorescent, and nearly all of the fluorescence was found in the 63-kDa band on SDS-PAGE, indicating intramolecular cross-linking of cysteines. To determine which cysteines reacted with bBBr, mutations were introduced to create proteins in which two, three, or four cysteines were altered to serines, greatly decreasing resistance to arsenite and antimonite (Fig. 3). The gene products were not grossly altered in structure. The rate of production of tryptic peptides was the same in the wild type and altered ArsAs, showing that surface accessibility of arginine and/or lysine residues was not modified. Binding of ATP produces a conformational change that decreases the rate of trypsin digestion (Hsu and Rosen, 1989).

None of the serine substitutions affected ATP protection, suggesting that the mutations did not reduce substrate binding (Fig. 4). The synergistic protection afforded by ATP and Sb(III) reflects a conformational change produced by effector binding. This cooperative protection was lost in each of the proteins in which any of the three cysteines of the triad were replaced by serine residues (C113S/C172S, C113S/C422S, and C172S/C422S). Only the C26S/C113S, C26S/C172S, and C26S/C422S enzymes retained any catalytic activity. The $K_m$ value for ATP for each was within an order of magnitude of the wild type enzyme. In contrast, the concentration of semimetal oxyanion required for activation was increased by 1–2 orders of magnitude, suggesting that As(III) or Sb(III) cannot interact with ArsA when any two cysteine thiolates of the triad are missing.

The fluorescence of the ArsAs was monitored after bBBr modification. Substitution of serine for Cys265 had little effect on the steady state level of fluorescence (data not shown). C26S/C113S, in which Cys113 and Cys422 remain, produced fluorescent products similar to C26S. Thus, only two cysteines of the triad are required for bBBr cross-linking. C172S/C422S showed a significant lowering in the steady state level of fluorescence and a 7-fold decrease in the rate of modification, demonstrating that Cys113 and Cys422 are located within 3–6 Å of each other in the tertiary structure of ArsA. C113S/C422S reacted similarly with bBBr; thus, Cys113 and Cys422 must likewise be spatially proximate. C26S/C422S, in which Cys113 and Cys422 remain, was fluorescent after modification with bBBr, indicating that Cys113 and Cys422 are close enough to each other to cross-link. These data clearly demonstrate that Cys113, Cys172, and Cys422 are located within a distance of 3–6 Å from each other in the tertiary structure of ArsA, supporting the hypothesis that their thiolates are liganded to As(III) or Sb(III), forming a novel soft metal-thiol structure (Fig. 6). Interestingly, C113S/C172S, which retains Cys265 and Cys422 produced a fluorescent product with bBBr with a rate of reaction only 3-fold less than wild type ArsA (Table III). This is compared with a 7-fold decrease in reactivity in proteins with Cys265 and either Cys113 or Cys172. This may indicate that Cys265, which is not involved in metalloactivation, may be within 6 Å of Cys422 but further from Cys113 and Cys172.

What is the relationship of metalloid binding and allosteric activation? The catalytically competent form of purified ArsA is a dimer, and the concentration of dimer in solution increases in the presence of Sb(III) or As(III) (Hsu et al., 1991). This suggests that the metalloid binds to the conformation of the protein that preferentially self-associates. Thus, by mass action, effector binding increases the concentration of the active species. Why is Sb(III) nearly 10-fold more effective than As(III) as an activator? Although the overall geometry of the Sb-thiol structure should be similar to that of the As-thiol, the bond angles and distances would be different (Fig. 6). The As–S distance is predicted to be 2.23 Å, with S–S distances of 3.2 Å, while the Sb–S distance would be 2.45 Å, with S–S distances of 3.5 Å. The activator binding site may be slightly too large to accommodate As(III) without strain. The possibility that the metal binding specificity of this site could be altered mutagenically raises interesting possibilities for protein engineering.

Acknowledgment—We thank Michael Vish for technical assistance.

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