Cys-113 and Cys-422 Form a High Affinity Metalloid Binding Site in the ArsA ATPase

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The arsRDABC operon of Escherichia coli plasmid R773 encodes the ArsAB extrusion pump for the trivalent metalloids As(III) and Sb(III). ArsA, the catalytic subunit has two homologous halves, A1 and A2. Each half has a consensus signal transduction domain that physically connects the nucleotide-binding domain to the metalloid-binding domain. The relation between metalloid binding by ArsA and transport through ArsB is unclear. In this study, direct metalloid binding to ArsA was examined. The results show that ArsA binds a single Sb(III) with high affinity only in the presence of Mg2+-nucleotide. Mutation of the codons for Cys-113 and Cys-422 eliminated Sb(III) binding to purified ArsA. C113A/C422A ArsA has basal ATPase activity similar to that of the wild type but lacks metalloid-stimulated activity. Accumulation of metalloid was assayed in intact cells, where reduced uptake results from active extrusion by the ArsAB pump. Cells expressing the arsA C113A/C422A genes had an intermediate level of metalloid resistance and accumulation between those expressing only arsB alone and those expressing wild type arsAB genes. The results indicate that, whereas metalloid stimulation of ArsA activity enhances the ability of the pump to reduce the intracellular concentration of metalloid, high affinity binding of metalloid by ArsA is not obligatory for transport or resistance. Yet, in mixed populations of cells bearing either arsAB or arsA C113A/C422A growing in subtoxic concentrations of arsenite, cells bearing wild type arsAB replaced cells with mutant arsA C113A/C422A in less than 1 week, showing that the metalloid binding site confers an evolutionary advantage.

The arsRDABC operon of the clinically isolated Escherichia coli plasmid R773 encodes the ArsAB ATPase, a metalloid pump that confers resistance by actively extruding As(III) or Sb(III) from cells (1). The 63-kDa ArsA is a metalloid-stimulated ATPase that comprises the catalytic subunit of the pump (2, 3). ArsA has two homologous halves, A1 and A2, connected by a short linker. Each half has a consensus nucleotide-binding domain (NBD) (4). At the absence or presence of the pump substrate, Sb(III) or As(III), both NBD1 and NBD2 hydrolyze ATP, with steady state hydrolysis dominated by the activity of NBD1 (4). The two NBDs are located at the interface between A1 and A2, in close proximity to each other (5). Over 20 Å distant from the NBDs is a metalloid-binding domain (MBD), where three Sb(III) or As(III) are bound at the A1-A2 interface (Fig. 1). One Sb(III) is connected to Cys-113 from A1 and Cys-422 from A2 (Site 1), a second to Cys-172 from A1 and His-453 from A2 (Site 2), and the third to His-148 from A1 and Ser-420 from A2 (Site 3). Thus, the three metalloid atoms act as molecular glue to bring the A1 and A2 halves of ArsA together, an event that is linked to activation of ATP hydrolysis. ArsA has two signature sequences that serve as signal transduction domains (STDs), D1445TAPTGH1448TIRLL in A1 (STD1) and D447TAPTGH448TLLL in A2 (STD2), which corresponds to the Switch II region of many other nucleotide-binding proteins and have been proposed to be involved in transmission of the energy of ATP hydrolysis to metalloid transport (6). Asp-142 and Asp-447 are Mg2+-ligands in NBD1 and NBD2, respectively, and His-148 and His-453 are Sb(III) ligands in the MBD (5, 7).

The relation between binding of trivalent metalloid to ArsA and its transport through the ArsAB pump is not clear. Is the metalloid bound at the MBD a transport substrate or is it an allosteric activator of the pump? A C113A/C422A mutation introduced into ArsA was found to eliminate high affinity metalloid binding to ArsA. The C113A/C422A ArsA had basal ATPase activity similar to that of the wild type but lacked metalloid-stimulated activity. However, cells expressing the mutant arsA C113A/C422A pump were able to extrude metalloid with higher efficiency than ArsB alone, exhibiting intermediate resistance between cells with wild type ArsAB and cells with only ArsB. These results indicate that the basal activity of the ArsAB pump is sufficient for ATP-driven efflux of metalloid and that the MBD is an allosteric site, with metalloid binding increasing pump activity. Finally, cells with wild type ArsAB were more successful than cells with the mutant arsA C113A/C422A in growth in mixed cultures. We propose that in the low concentrations of metalloid ubiquitously present in the environment, the allosteric site evolved to increase the fitness of the organism for growth.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—The E. coli strains and plasmids used in this study are listed in Table 1. Cells were grown at 37 °C in Luria-Bertani (LB) medium (8) with either ampicillin (Ap) (125 µg/ml) or tetracycline (Tc) (12.5 µg/ml), as required. For arsenite resistance assays, overnight cultures were diluted 100-fold into fresh LB medium containing the indicated concentrations of sodium arsenite. After 6 h of growth at 37 °C, the absorbance at 600 nm was measured. Growth in mixed cultures was performed as follows. Cells of E. coli strain AW3110 bearing either pAlter1-ArsAB (Ap’/Tc’) or pAlter1-ArsA C113A/C422A B (Ap’/Tc’), were grown overnight in LB medium at 37 °C. The cultures were mixed at a 1:1 ratio, diluted 1:1000 in fresh LB medium containing 125 µg/ml ampicillin and 4 µM sodium arsenite, and grown at 37 °C overnight. Each morning for 6 days the cultures were diluted 1:1000 into the same medium and grown overnight. Viable counts following serial dilutions were determined on LB plates containing 125 µg/ml ampicillin to obtain a total cell count or 12.5 µg/ml tetracycline to determine the fraction of cells bearing only pAlter1–ArsA C113A/C422A B.

DNA Manipulation—Plasmid DNA was purified using the Wizard® Plus Miniprep DNA Purification System (Promega). DNA restriction — Plasmid DNA was purified using the Wizard® Plus Miniprep DNA Purification System (Promega). DNA restriction
Metalloid Binding to the ArsA ATPase

Oligonucleotide-directed Mutagenesis—Mutations in the arsA gene were introduced by site-directed mutagenesis using the Altered Sites II in Vitro Mutagenesis System (Promega). The arsA gene in plasmid pTZ4H6, which had previously been mutated to remove the four native tryptophan codons and to add six histidine codons at the 3’-end, was used as the template to produce the T461W-H6 mutant (for simplicity, T461W). The C113A/C422A substitution was introduced into T461W, using bovine serum albumin as a standard.

Measurement of Metalloid Binding.—The buffer used for purification of ArsA was exchanged with a buffer containing 50 mM MOPS-KOH, pH 7.5 (Buffer A). ATPase activity was measured using an NADH-coupled assay (10, 11) with 5 mM ATP and 2.5 mM MgCl2, unless otherwise noted. The concentration of purified ArsA was determined using a Bio-Rad Protein Assay with a 340 PC microplate reader (Molecular Devices), noting the concentration of metalloid measured by inductively coupled mass spectrometry with a PerkinElmer ELAN 9000.
standard solutions in the range of 0.5–10 ppb in 2% HNO₃ were obtained from Ultra Scientific, Inc. (North Kingstown, RI).

**Limited Trypsin Digestion**—Limited trypsin digestion was performed at room temperature in Buffer A containing 0.25 mM EDTA and 1 mg/ml ArsA at an ArsA to trypsin ratio of 500:1 (w/w) (12). ArsA was incubated with 5 mM ATP and/or 0.5 mM Sb(III), as indicated. Digestion was initiated by addition of N-ε-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Sigma). Proteolysis was terminated at the indicated times by addition of a 5-fold excess of soybean trypsin inhibitor. Samples were analyzed by SDS-PAGE on 12% acrylamide gels and stained with Coomassie Blue (13).

**Fluorescence Measurements**—Fluorescence measurements were performed on an Aminco Bowman-2 luminescence spectrometer with a built-in magnetic stirrer at room temperature. The bandwidths for emission and excitation monochromators were 4 nm. Tryptophan fluorescence was monitored with an excitation wavelength of 295 nm, and an emission wavelength of 334 nm for T461W, 337 nm for W159, and 331 nm for M446W ArsA (6, 9). The fluorescence of Buffer A alone was subtracted from each spectrum. The concentration of ArsA was 1.25 mM, unless otherwise noted. Nucleotides, MgCl₂, and potassium antimonyl tartrate were added as indicated.

**Transport Assays**—Transport of arsenite by the ArsAB pump was assayed as decreased accumulation in cells (14, 15). Cells bearing plasmids were grown to an optical density of 1 at 37 °C with aeration in LB medium. The cells were harvested, washed, and suspended in a buffer consisting of 75 mM HEPES-KOH, pH 7.5, containing 0.15 M KCl and 1 mM MgCl₂ (Buffer B) and suspended at a density of 10¹⁰ cells/ml. To initiate the assay, 10 μM sodium arsenite, final concentration, was added to 1 ml of cells. Portions (0.1 ml) were withdrawn at the indicated times, filtered through 0.45-μm pore diameter nitrocellulose filters (Whatman), and washed with 15 ml of the Buffer B, all at room temperature. The filters were dried and digested with 0.3 ml of 70% HNO₃ (EM Science) at 70 °C.

**FIGURE 2. Stoichiometry of Sb(III) binding to ArsA.** A, effect of nucleotides on the binding of Sb(III) to ArsA. 10 μM ArsA was incubated with 2.5 mM MgCl₂, 2 mM nucleotide, and 100 μM antimonite. ATP, ATP₅S, ADP, no nucleotide, and 100 μM bovine serum albumin (BSA) incubated with 2.5 mM MgCl₂ and 100 μM antimonite are indicated under the bars. The error bars represent the standard deviations (n = 4). B, antimonite binding to wild type (WT) and C113A/C422A ArsA. ArsA (10 μM) was incubated with 2.5 mM MgCl₂, 2 mM ATP₅S, and increasing amounts of potassium antimonyl tartrate. The lines represent best fit of the data using SigmaPlot 9.0 from five experiments for wild type ArsA and two experiments for the C113A/C422A ArsA. C, kinetic analysis of Sb(III) binding to either the wild type or C113A/C422A ArsA. Data were analyzed with the Scatchard equation: B = n·Kₛ·P/F, where B represents the concentration of Sb(III) bound to ArsA, and F represents the concentration of free Sb(III). Kₛ and n are the dissociation constant and the maximum number of Sb(III) bound per ArsA molecule, respectively.

**FIGURE 3. ATPase activity of ArsA.** ATPase activity of either the wild type (WT) or C113A/C422A ArsA was determined in the presence of antimonite (A) or arsenite (B). The lines represent best fit of the data using SigmaPlot 9.0. The error bars represent the standard deviations (n = 3).
for 20 min, allowed to cool to room temperature, and diluted with 7 ml of high pressure liquid chromatography grade water (Sigma) to produce a final concentration of HNO₃ of ~2.6%. Arsenic was quantified by inductively coupled mass spectrometry.

**RESULTS**

**Binding of Sb(III) to ArsA** — The 2.3-Å structure of the ArsA ATPase revealed a novel and complex MBD containing three atoms of Sb (5). Each Sb atom is bound three-coordinately with two protein ligands and one unidentified non-protein ligand. One Sb was bound to A1 residue Cys-113 and A2 residue Cys-422. Because this binding site, which we term Site 1, includes two thiol ligands, it is expected to be a tight binding site. A second Sb was bound to A1 residue His-148 and A2 residue Ser-420. This site, termed Site 3, uses an imidazole nitrogen and serine hydroxyl as ligands. Because Sb(III) is a relatively soft metal, it would be expected to bind to this site with low affinity. The third Sb was bound to A1 residue Cys-172 and A2 residue His-453. With only a single thiol, this site, termed Site 2, would be expected to be a metalloid binding site with intermediate affinity between the other two sites.

High affinity binding of Sb(III) to purified ArsA was measured by rapid gel filtration. Binding of As(III) could not be measured using this assay because of low affinity for this metalloid (cf. Fig. 3B). In the absence of nucleotide, only 0.1 mol of Sb(III) was bound per mol of ArsA (Fig. 2A). The same amount of Sb(III) binding was observed with bovine serum albumin, a protein of similar size to ArsA, indicating nonspecific interaction. Binding increased with addition of MgATP or MgADP, with binding of 1 mol of Sb(III) per mol of ArsA with the nonhydrolyzable nucleotide (Fig. 2A). Reciprocally, binding of nucleotide is also enhanced by metalloid binding (4). However, it should be pointed out that the concentrations of ADP and ATP in the cytosol of *E. coli* are sufficient to saturate ArsA at all times, so in vivo binding of metalloid would be independent of nucleotide.

Binding to purified ArsA was measured as a function of Sb(III) concentration. In the absence of Mg²⁺-nucleotide, only background binding was observed. In the presence of saturating MgATP₅S, metalloid binding was saturable (Fig. 2B), with a stoichiometry of one Sb(III) per ArsA and an apparent Kₐ of ~10⁻⁵ M (Fig. 2C). In the presence of ATP₅S but in the absence of Mg²⁺, wild type ArsA still bound Sb(III) in a 1:1 ratio, but the apparent affinity was reduced by an order of magnitude (data not shown). In contrast, the C113A/C422A derivative bound only background levels of Sb(III) (Fig. 2, B and C), demonstrating that loss of Site 1 eliminates high affinity metalloid binding.

**C113A/C422A ArsA Lacks Metalloid-stimulated ATPase Activity** — The ability of Sb(III) to activate ArsA was examined in wild type and C113A/C422A ArsA (Fig. 3). The wild type enzyme exhibited a basal ATPase activity of ~100 nmol mg⁻¹ min⁻¹. Both metalloids stimulated ATP hydrolysis but to different extents. Sb(III)-stimulated ATPase activity 8-fold (Fig. 3A), with half-maximal stimulation at 8 μM, a value similar to the Kₐ for binding of Sb(III) (Fig. 2C). As(III) stimulated activity 3-fold (Fig. 3B), with half-maximal stimulation at 340 μM. C113A/C422A ArsA had basal activity similar to that of the wild type but lacked metalloid-stimulated activity. These results show a clear relationship between binding of metalloid to the high affinity binding Site 1 and metalloid stimulation of catalytic activity.
Metalloid Binding to the ArsA ATPase

**Effect of Elimination of MBD Site 1 on Metalloid-associated Conformational Changes in ArsA**—Loss of metalloid stimulation of the catalytic activity implies loss of conformational coupling between the MBD and NBDs. Because these are separated by at least 20 Å, interaction between these two sites requires substantial conformational changes. Limited trypsin digestion has been used as a sensitive tool to assess these ligand-induced changes under noncatalytic conditions (12). In the absence of ligands, wild type ArsA was rapidly cleaved by trypsin to one or more polypeptides of 30 kDa (Fig. 4A). Addition of Sb(III) had little effect (Fig. 4A), which reflects the fact that high affinity Sb(III) binding occurs only in the presence of nucleotide (cf. Fig. 2). In the presence of ATP, the initial cleavage by trypsin produced a 50-kDa species with subsequent cleavage to smaller polypeptides (Fig. 4B). The rate of cleavage was decreased synergistically when both ATP and Sb(III) were present together (Fig. 4B), showing that the enzyme adopts a trypsin-resistant conformation when both the MBD and NBD are filled. The pattern of trypsin sensitivity in C113A/C422A ArsA was similar in the absence of Sb(III) (Fig. 4, C and D), indicating that the overall structure of the mutant protein is not grossly different from wild type. Importantly, there was one major difference: addition of Sb(III) had no effect on the mutant protein in the presence of ATP (Fig. 4D). Thus, the lack of MBD Site 1 does not affect nucleotide-dependent conformational changes but does eliminate the metalloid-dependent effect.

**Effect of MBD Site 1 on NBD2**—Another single tryptophan derivative, M446W, has been useful for reporting events associated during nucleotide binding (9). The fluorescence of Trp-446, which is located between NBD2 and the first residue of the A2 STD (Fig. 1), has been shown to report conformational changes associated with ligand binding, with basal ATP hydrolysis, and with metalloid-stimulated ATPase activity (6, 9). Trp-159, which is located near the C-terminal end of STD1, reports slow conformational changes during basal hydrolysis and rapid conformational changes during metalloid-activated ATP hydrolysis (6). This fluorescence enhancement reports predominantly NBD1 activity in both the basal and activated states because NBD1 hydrolyzes ATP considerably faster than NBD2 under both sets of conditions (4). Moreover, the rate of fluorescence enhancement in the unactivated state is equivalent to the steady-state rate of basal hydrolysis (16). The effect of ligands on the intrinsic fluorescence of W159 ArsA was compared with a W159 derivative of C113A/C422A ArsA (Fig. 5). Both proteins exhibited slow enhancement of fluorescence in the presence of MgATP but not MgADP. Addition of Sb(III), which produces activated ATP hydrolysis, elicited a large and rapid quenching of fluorescence in the W159 enzyme (Fig. 5A) that was absent in the MBD Site 1 mutant (Fig. 5B). This is consistent with occupancy of MBD Site 1 by metalloid being required for activation of NBD1. When the rate of fluorescence enhancement was measured as a function of ATP concentration in W159 (Fig. 6A) or W159/C113A/C422A (Fig. 6B) ArsAs, no significant differences were observed. If Sb(III) is added to W159 ArsA prior to MgATP, no enhancement of intrinsic fluorescence is observed (17). In contrast, addition of Sb(III) to W159/C113A/C422A ArsA prior to MgATP did not prevent the nucleotide-dependent enhancement of fluorescence (Fig. 6C). The Site 1 mutant and its parent had similar affinity for ATP during basal hydrolysis, but Sb(III) did not increase the affinity of the mutant (Fig. 6D). Thus, MBD Site 1 appears to be required for activation of ATP hydrolysis but not for basal hydrolysis by NBD1.

**Effect of Elimination of MBD Site 1 on NBD1**—The fluorescence of several single tryptophan-containing ArsA derivatives has been shown to report conformational changes associated with ligand binding, with basal ATP hydrolysis, and with metalloid-stimulated ATPase activity (6, 9). Trp-159, which is located near the C-terminal end of STD1, reports slow conformational changes during basal hydrolysis and rapid conformational changes during metalloid-activated ATP hydrolysis (6). This fluorescence enhancement reports predominantly NBD1 activity in both the basal and activated states because NBD1 hydrolyzes ATP considerably faster than NBD2 under both sets of conditions (4). Moreover, the rate of fluorescence enhancement in the unactivated state is equivalent to the steady-state rate of basal hydrolysis (16). The effect of ligands on the intrinsic fluorescence of W159 ArsA was compared with a W159 derivative of C113A/C422A ArsA (Fig. 5). Both proteins exhibited slow enhancement of fluorescence in the presence of MgATP but not MgADP. Addition of Sb(III), which produces activated ATP hydrolysis, elicited a large and rapid quenching of fluorescence in the W159 enzyme (Fig. 5A) that was absent in the MBD Site 1 mutant (Fig. 5B). This is consistent with occupancy of MBD Site 1 by metalloid being required for activation of NBD1. When the rate of fluorescence enhancement was measured as a function of ATP concentration in W159 (Fig. 6A) or W159/C113A/C422A (Fig. 6B) ArsAs, no significant differences were observed. If Sb(III) is added to W159 ArsA prior to MgATP, no enhancement of intrinsic fluorescence is observed (17). In contrast, addition of Sb(III) to W159/C113A/C422A ArsA prior to MgATP did not prevent the nucleotide-dependent enhancement of fluorescence (Fig. 6C). The Site 1 mutant and its parent had similar affinity for ATP during basal hydrolysis, but Sb(III) did not increase the affinity of the mutant (Fig. 6D). Thus, MBD Site 1 appears to be required for activation of ATP hydrolysis but not for basal hydrolysis by NBD1.

**Effect of Trp-461 Reports Binding of Metalloid to MBD Site 1**—A new single tryptophan derivative, T461W, was constructed in an otherwise wild type ArsA background as a probe of metalloid binding to MBD Site 1. The intent was to alter Tyr-464, the A2 residue corresponding to

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**FIGURE 5.** Effect of ligands on the intrinsic fluorescence of W159 ArsA proteins. Fluorescence of W159 ArsA (A) and W159/C113A/C422A ArsA (B). At the arrows, 1 mM each, final concentration, of nucleotide, MgCl2 and potassium antimonyl tartrate were added sequentially. The excitation and emission wavelengths were 295 and 337 nm, respectively. WT, wild type.
Trp-159, to a tryptophan that could report similar conformational changes in A2. However, the fluorescence of a Y464W single tryptophan derivative was unresponsive to ligands (data not shown). Residues 462–479 are not visible in the crystal structure, indicating that they are in a mobile region of the protein. The closest residue to Tyr-464 visible in the structure is Thr-461. This residue occupies a position in A2 roughly corresponding to that of Trp-159 in A1, with both residues located on the outer surface of the A1-A2 interface near the MBD. T461W ArsA denatured with 6 M guanidine HCl (Fig. 8A, curve 5) had a fluorescence emission spectrum similar to an equimolar concentration of free tryptophan (curve 6), with an emission maximum at 353 nm. Native T461W exhibited a substantial blue shift and fluorescence enhancement, with an emission maximum at 334 nm (curve 1). Addition of saturating MgATP, MgADP, MgATPγS (only MgADP is shown in Fig. 8B), yielding a concentration of ~10 μM for the half-maximal response for each of the three nucleotides (Fig. 8C). This value is comparable with the $K_d$ value obtained by direct binding assays (Fig. 2C). The fluorescence titrations exhibited an apparent sigmoidicity that was absent in the direct metal binding titrations, but the significance of this response is not known. Perhaps the two weak binding sites contribute to the overall fluorescence response, a possibility that is being investigated. The fluorescence properties of a single T461W/C113A/C422A derivative were compared with T461W ArsA (Fig. 9). In the presence of either MgADP or MgATP, Sb(III) produced substantial fluorescence quenching in T461W ArsA (Fig. 9A). In contrast, there was little change in fluorescence with the T461W/C113A/C422A ArsA. Thus the synergistic response of Trp-461 to nucleotide and metalloid requires metalloid binding at Site 1 of the MBD.
Effect of Elimination of MBD Site 1 on Metalloid Resistance and Accumulation—
The effect of mutagenesis of Site 1 on the ability of \( \text{arsA} \) to confer resistance to arsenite in vivo was examined (Fig. 10A). Cells of \( \text{E. coli} \) JM109 were As(III) sensitive. Cells expressing \( \text{arsB} \) exhibited low level resistance. Cells expressing wild type \( \text{arsAB} \) genes were highly resistant to arsenite compared with cells expressing \( \text{arsB} \) alone. Cells expressing the mutated \( \text{arsAC}_{\text{113A/422AB}} \) genes exhibited an intermediate level of resistance.

This result suggests that the loss of Site 1 and the inability to activate \( \text{ArsA} \) reduces but does not eliminate the ability of the \( \text{ArsAB} \) pump to extrude metalloid. To examine that possibility, the steady state level of As(III) accumulation was measured in cells expressing wild type and mutant \( \text{arsA} \) (Fig. 10B). The steady state level of As(III) in cells expressing \( \text{arsB} \) with vector was reduced in cells expressing \( \text{arsB} \) alone. The intracellular level of As(III) in cells expressing wild type \( \text{arsAB} \) was considerably lower than cells with \( \text{arsB} \) alone. Cells with the mutated \( \text{arsAC}_{\text{113A/422AB}} \) genes had a steady state level of intracellular As(III) that was lower than cells with \( \text{arsB} \) but statistically higher than cells expressing the wild type pump. Thus, it appears that Site 1 is not obligatory for \( \text{ArsAB} \) function but increases the efficiency of the pump.

Effect of Mutations on As(III) Accumulation—

The increase in resistance conferred by wild type \( \text{arsA} \) compared with the mutant lacking MBD Site 1 is rather modest, with the greatest differences observed at concentrations of arsenite exceeding those found in all but the most highly contaminated corners of the world. Is high affinity metalloid binding at the MBD sufficient to confer an evolutionary advantage on the host organism? To examine this question, cells of \( \text{E. coli} \) strain AW3110 bearing either wild type
arsAB or arsA_{C113A/C422A} were allowed to compete with each other in a mixed culture for growth in the presence of 4 μM sodium arsenite. Whereas this is a subtoxic concentration, it is equivalent to 300 ppb, which is about the midrange of arsenic found in water from highly contaminated wells in Bangladesh (18). Each day the culture was diluted 1000-fold, and the relative amounts of cells with the wild type and mutant pumps were determined (Fig. 11). By 4 days of growth, cells with arsAB had largely replaced those with only arsA_{C113A/C422A}. We propose that the MBD provides a competitive advantage to cells expressing the wild type ArsAB As(III)/Sb(III)-translocating ATPase for growth under environmental conditions where arsenic is commonly found.

DISCUSSION

ArsA is the catalytic subunit of the ArsAB pump, extruding As(III) or Sb(III) from cells, thus conferring resistance (19). The catalytic activity of ArsA is stimulated by binding of metalloid substrate of the pump. This raises two questions: 1) where is metalloid bound, and 2) how does it activate catalysis? We have recently shown that Sb(III) increases the affinity for nucleotide in both NBD1 and NBD2 (4). In the structure of ArsA crystallized in the presence of MgADP and Sb(III), the two NBDs are shown to be composed of residues from both A1 and A2 (5). We propose that the two NBDs are incomplete unless metalloid is bound, where the incomplete sites have low affinity for nucleotide, and the complete sites have higher affinity. Thus, the metalloids are the “molecular glue” that hold the two halves of ArsA together, completing the NBDs to activate catalysis. Whereas no structure in the absence of ligands is available, the results of trypsin accessibility are instructive (Fig. 4). Trypsin sensitivity provides an indication of the solvent accessible surface area of a protein. ArsA has 53 trypsin sites, 21 in A1, one in the linker that connects the two halves, and 31 in A2. In the absence of ligands, ArsA is quite sensitive to trypsin, which indicates that the enzyme is in an “open state” in which much of its surface is accessible to protease under conditions of basal hydrolysis. In a relatively short period of time, the 63-kDa protein is proteolyzed to a 30-kDa fragment. Jia and Kaur (20) have shown that this trypsin product is primarily derived from A1, one in the linker that connects the two halves, and 31 in A2. In the absence of ligands, ArsA is quite sensitive to trypsin, which indicates that the enzyme is in an “open state” in which much of its surface is accessible to protease under conditions of basal hydrolysis.
Metalloid Binding to the ArsA ATPase

Understanding the nature of the metalloid-binding domain is crucial to a molecular description of the catalytic cycle. Transient kinetic measurements of the nucleotide-dependent fluorescence signals of W159 in ArsA produced a triphasic fit, with three different affinities for Sb(III): tight, intermediate, and weak that most likely reflect the affinities of the three metalloid binding sites (17). Consistent with that stopped-flow analysis, the crystal structure of ArsA revealed three atoms of Sb in the MBD bound in three distinct types of sites (5). Each site has two ArsA residues, one in A1 and the other in A2, stabilizing the A1-A2 interface. Because trivalent metalloids are thiophilic soft metals, Site 1 is a reasonable candidate for the tight binding site because it has two sulfur ligands, Cys-113 and Cys-422. Individual substitution of either Cys-113 or Cys-422 with serine is composed of Cys-113 and Cys-422 with serine greatly reducing metalloid stimulation of ArsA activity, supporting the participation of these two residues in the high affinity metalloid binding site of ArsA increases the ability of cells to compete in an arsenic-containing environment. Competition assays between cells with arsAB and arsA<sub>C113A/C422A</sub> were performed as described under "Experimental Procedures." Mixed cultures of cells of E. coli strain AW3110 bearing either pArsAB or pArsA<sub>C113A/C422A</sub> were allowed to grow with daily dilutions into fresh medium. By 4 to 6 days, cells with pArsA<sub>C113A/C422A</sub> were lost from the population, as determined from the fraction of Tc<sup>+</sup> colonies. The data are the average of two separate assays.

Different affinities for Sb(III): tight, intermediate, and weak that most likely reflect the affinities of the three metalloid binding sites (17). Consistent with that stopped-flow analysis, the crystal structure of ArsA revealed three atoms of Sb in the MBD bound in three distinct types of sites (5). Each site has two ArsA residues, one in A1 and the other in A2, stabilizing the A1-A2 interface. Because trivalent metalloids are thiophilic soft metals, Site 1 is a reasonable candidate for the tight binding site because it has two sulfur ligands, Cys-113 and Cys-422. Individual substitution of either Cys-113 or Cys-422 with serine is composed of Cys-113 and Cys-422 with serine greatly reducing metalloid stimulation of ArsA activity, supporting the participation of these two residues in the high affinity metalloid binding site of ArsA increases the ability of cells to compete in an arsenic-containing environment. Competition assays between cells with arsAB and arsA<sub>C113A/C422A</sub> were performed as described under "Experimental Procedures." Mixed cultures of cells of E. coli strain AW3110 bearing either pArsAB or pArsA<sub>C113A/C422A</sub> were allowed to grow with daily dilutions into fresh medium. By 4 to 6 days, cells with pArsA<sub>C113A/C422A</sub> were lost from the population, as determined from the fraction of Tc<sup>+</sup> colonies. The data are the average of two separate assays.

Different affinities for Sb(III): tight, intermediate, and weak that most likely reflect the affinities of the three metalloid binding sites (17). Consistent with that stopped-flow analysis, the crystal structure of ArsA revealed three atoms of Sb in the MBD bound in three distinct types of sites (5). Each site has two ArsA residues, one in A1 and the other in A2, stabilizing the A1-A2 interface. Because trivalent metalloids are thiophilic soft metals, Site 1 is a reasonable candidate for the tight binding site because it has two sulfur ligands, Cys-113 and Cys-422. Individual substitution of either Cys-113 or Cys-422 with serine is composed of Cys-113 and Cys-422 with serine greatly reducing metalloid stimulation of ArsA activity, supporting the participation of these two residues in the high affinity metalloid binding site of ArsA increases the ability of cells to compete in an arsenic-containing environment. Competition assays between cells with arsAB and arsA<sub>C113A/C422A</sub> were performed as described under "Experimental Procedures." Mixed cultures of cells of E. coli strain AW3110 bearing either pArsAB or pArsA<sub>C113A/C422A</sub> were allowed to grow with daily dilutions into fresh medium. By 4 to 6 days, cells with pArsA<sub>C113A/C422A</sub> were lost from the population, as determined from the fraction of Tc<sup>+</sup> colonies. The data are the average of two separate assays.
Metalloid Binding to the ArsA ATPase

reports slow hydrolysis of MgATP in the absence of metalloid with an equally slow rate of fluorescence enhancement (6). Because basal hydrolysis results from NBD1 catalysis (4), Trp-159 fluorescence reports events in NBD1. Addition of Sb(III) to wild type ArsA rapidly reverses the fluorescence enhancement with a corresponding red shift in the emission maximum. These results indicate that there is a conformational change in the vicinity of Trp-159 into a less solvent-exposed environment during basal hydrolysis and a more solvent-exposed environment during activated hydrolysis. In the crystal structure, which corresponds to a conformation during activated hydrolysis, Trp-159 is observed on the surface of ArsA. In the ArsA derivative lacking MBD Site 1, the enhancement associated with the basal rate of hydrolysis is unaffected, but the metalloid-dependent quenching is absent. These results are consistent with binding of metalloid at Site 1 activating catalysis in NBD1.

A second single tryptophan derivative containing only Trp-446, which is located adjacent to NBD2, reports binding of MgADP and MgATP in the absence of metalloid with a slight enhancement of fluorescence (6). This nucleotide-dependent enhancement of Trp-446 fluorescence was unaffected by mutagenesis of Site 1 (Fig. 7B). When Sb(III) was added to ArsA containing bound MgADP, there was a rapid quenching of fluorescence (Fig. 7A) that corresponds to an increase in affinity for nucleotide (4). In contrast, when Sb(III) was added to the Trp-446 ArsA with bound MgATP, there was a slow quenching that corresponds to the rate of formation of ADP by NBD2. This reflects the fact that the two NBDs are not equivalent, and NBD2 is always slower than NBD1, even in the activated state (4). Thus, loss of Site 1 does not affect binding of nucleotide or basal catalysis in NBD2 but does prevent activation of catalysis in NBD2. Overall, the data indicate that Site 1 is required for activation of catalysis in both NBD1 and NBD2 by metalloid.

A fundamental issue is the role of the MBD in metalloid transport and resistance. One possibility is that the MBD is a transport site in which the bound metalloid atom is inserted into the translocation channel of ArsB as a result of ATP hydrolysis. The results of mutagenesis argue against obligatory binding at Site 1 for physiological function. Cells expressing the \( \text{ars}_{\text{C113A/C422A}} \) genes had reduced transport and resistance compared with cells expressing wild type \( \text{arsAB} \), even though purified C113A/C422A ArsA lost high affinity metalloid binding. We cannot rule out the possibility that the metalloid bound at Site 1 is a transport substrate but that alternate pathways, perhaps through Sites 2 or 3, assume that role in the mutant. A more sensible inference is that Site 1 is an allosteric site that enhances the activity of the pump without contributing its bound metalloid to ArsB. Finally, why did the MBD evolve? Clearly cells with the plasmid-encoded ArsAB pump are considerably more resistant to arsenite than cells that express only chromosomal ArsB (15, 21). But is the presence of Site 1, which confers only a small growth advantage, sufficient to confer a competitive advantage over cells expressing an ArsA lacking the Cys-113 and Cys-422? When strains with wild type and mutant pumps were allowed to compete with each other in media containing a subtoxic concentration of arsenite, those with the wild type ArsA easily outgrew the mutants. Thus, the ability to activate ArsA by binding of metalloid to the MBD gives increased fitness for growth in containing concentrations of arsenic found in drinking water in many parts of the world (18).

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