ArsD Residues Cys\textsuperscript{12}, Cys\textsuperscript{13}, and Cys\textsuperscript{18} Form an As(III)-binding Site Required for Arsenic Metallochaperone Activity\textsuperscript{*}

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The ArsA ATPase is the catalytic subunit of the ArsAB pump encoded by the \textit{arsRDABC} operon of \textit{Escherichia coli} plasmid R773. ArsD is a metallochaperone that delivers As(III) to ArsA, increasing its affinity for As(III), thus conferring resistance to environmental concentrations of arsenic. R773 ArsD is a homodimer with three vicinal cysteine pairs, Cys\textsuperscript{12}-Cys\textsuperscript{13}, Cys\textsuperscript{112}-Cys\textsuperscript{113}, and Cys\textsuperscript{119}-Cys\textsuperscript{120}, in each subunit. Each vicinal pair binds As(III) or Sb(III). Alignment of the primary sequence of homologues of ArsD indicates that only the first vicinal cysteine pair, Cys\textsuperscript{12}-Cys\textsuperscript{13}, and an additional cysteine, Cys\textsuperscript{18}, are conserved. The effect of cysteine-to-alanine substitutions and truncations were examined. By yeast two-hybrid analysis, nearly all of the ArsD mutants were able to interact with wild type ArsD, indicating that the mutations do not interfere with dimerization. ArsD mutants with alanines substituting for Cys\textsuperscript{112}, Cys\textsuperscript{113}, Cys\textsuperscript{119}, or Cys\textsuperscript{120} individually or in pairs or truncations lacking the vicinal pairs retained ability to interact with ArsA and to activate its ATPase activity. Cells expressing these mutants retained ArsD-enhanced As(III) efflux and resistance. In contrast, mutants with substitutions of conserved Cys\textsuperscript{12}, Cys\textsuperscript{13}, or Cys\textsuperscript{18}, individually or in pairs, were unable to activate ArsA or to enhance the activity of the ArsAB pump. We propose that ArsD residues Cys\textsuperscript{12}, Cys\textsuperscript{13}, and Cys\textsuperscript{18}, but not Cys\textsuperscript{112}, Cys\textsuperscript{113}, Cys\textsuperscript{119}, or Cys\textsuperscript{120}, are required for delivery of As(III) to and activation of the ArsAB pump.

Arsenic is a toxic metalloid found in water, soil, and air from both natural and anthropogenic sources. As a consequence of its environmental pervasiveness, arsenic ranks first on the United States Government’s Comprehensive Environmental Response, Compensation, and Liability (Superfund) Act Priority List of Hazardous Substances (\url{www.atslrc.doe.gov/cercla/05list.html}). Health effects associated with exposure to low levels of arsenite include cardiovascular and peripheral vascular disease, neurological disorders, diabetes mellitus, and various forms of cancer (1, 2). In response to its ubiquity in the environment, nearly every organism, from \textit{Escherichia coli} to humans, have arsenic detoxification mechanisms (3). \textit{E. coli} has a chromosomal three-gene \textit{arsRBC} operon that confers moderate resistance to arsenate (As(V)), arsenite (As(III)), and antimonite (Sb(III)). However, some operons have two extra genes, \textit{arsD} and \textit{arsA}, genes as the \textit{arsRDABC} operon in \textit{E. coli} plasmid R773, and cells expressing the \textit{arsRDABC} operon are more resistant to As(V) and As(III) than those expressing the \textit{arsRBC} operon (3, 4). ArsA is an As(III)/Sb(III)-stimulated ATPase, the catalytic subunit of the ArsAB As(III)/Sb(III) extrusion pump (5). ArsD is a homodimer of two 120-residue subunits and has a weak \textit{ars} operon repressor activity (6, 7). Recently ArsR was identified as an arsenic metallochaperone that delivers As(III) to the ArsA ATPase (4). Interaction with ArsD increases the affinity of ArsA for As(III), producing increased efflux and resistance at environmental concentrations of arsenic.

The current state of knowledge of metallochaperone-mediated ion transfer derives largely from studies of copper chaperones (8). Most copper chaperones bind copper ions with multiple cysteine ligands. In the metallochaperone CCS (copper chaperone for superoxide dismutase, four cysteine residues from both the N-terminal CXXC (where X can be any residue) motif and the C-terminal CXXC motif form the metal-binding sites (MBSs))\textsuperscript{2} (9). Yet only the C-terminal CXXC motif appears poised to deliver metal ions to the SOD1 site. Atx1 is a yeast copper chaperone that delivers copper to the transport ATPase Ccc2p in the trans-Golgi network (10). The human homologue of Atx1, Hah1 (Atx1), interacts with the copper-transporting ATP7A and ATP7B pumps, and mutations in these pumps lead to genetic disorders such as Menkes and Wilson diseases (11). These Atx1-like chaperones are characterized by a conserved CXXC motif in N terminus. However, the x-ray structure of Atx1 indicates the presence of two or even three sulfur ligands (12, 13). Two of these ligands probably derive from the CXXC motif, and the third could either be an exogenous thiol or a cysteine from a second Atx1 molecule. Cxx17, the chaperone for cytochrome c oxidase, also binds copper ions with cysteine ligands. It binds three ions with a conserved CXXC motif in which all three cysteines are required to produce active oxidase. CopZ, characterized by a MXCXXC metal-binding motif, transfers copper ions to CopY, a copper-dependent regulatory protein (14). Copper transfer between CopZ and CopY is dependent on electrostatic interactions (15).

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\textsuperscript{2} The abbreviations used are: MBS, metalloid-binding site; LB, Luria broth; SD, synthetic dextrose; MBP, maltose-binding protein; dibromobimane, 4,6-bis(bromomethyl)-3,7-di-methyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione; MOPS, 4-morpholinepropanesulfonic acid.
By extrapolation of the role of thiol-metal ion chemistry in copper chaperones, we considered the possibility that As(III)-thiol interactions might be involved in its chaperone activity. ArsD has three vicinal cysteine pairs, Cys12-Cys13, Cys112-Cys113, and Cys119-Cys120. Cysteine residues 12, 13, and 18 are conserved in all known ArsD homologues. In contrast, the last two vicinal cysteine pairs are found only in some homologues (see Fig. 1). To examine the role of these cysteine residues in ArsD metallochaperone activity, a series of mutants and truncations were created. Analysis of these variants demonstrates that each of the three vicinal pairs forms an independent metalloid-binding site. The data suggest that only the As(III)-binding site formed by the CC4C motif is required for metallochaperone activity.

**MATERIALS AND METHODS**

**TABLE 1**

| Strains/plasmids | Genotype/description | Reference |
|------------------|----------------------|-----------|
| *E. coli* strains |                      |           |
| JM109            | F' traD36 lacIq Δ(lacZMd15) proA Δ883.9 /Δ883.9 (McrA Δ(lac-proAB) thi gyrA96 (Na+) endA1 hsdR17(rK-mK-) recA1 relA1 supE44 | Ref. 16 |
| BL21(DE3)        | hsdS galD(Δlac857) ind1 Sam7 nin5 lacUV5-T7 gene I | Ref. 16 |
| AW3110           | Δars-cam F' IN(rrnD-rrnE) | Ref. 35 |
| *Saccaromyces cerevisiae* strain AH109 | MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, lys2::GAL1, lacIΔM15, HIS3, GAL2::GAL1-TATA, ADE2::URA3::MEL1::MEL1-TATA, lacZ | Clontech |

**Plasmids**

| Plasmids | Description | Reference |
|----------|-------------|-----------|
| pSE380 | Expression vector offering trc promoter, lacO operator, lacIq repressor (Ap’) | Invitrogen |
| pMAL-c2X | A vector for expression of MBP fusion proteins (Ap’) | New England Biolabs |
| pACT2 | GAL4(1–147) DNA-binding domain, TRP1 (Ap’) | Clontech |
| pBAD/Myc-His A | Expression vector with pBAD promoter (Ap’) | Invitrogen |
| pACYC184 | Cloning vector (Cm r) | Ref. 36 |
| pACBAD | 380-bp BclI-EcoRI fragment containing the pBAD promoter of pBAD/Myc-HisA cloned into pACYC184 (Tc’) | Ref. 4 |
| pACT-X series | arsA and arsD genes cloned in pACT2 (Ap’) | Ref. 4 |
| pSE-X series | arsAB or arsB gene in pSE380 (Ap’) | Ref. 4 |
| pMAL-Dx series | MBP-fused arsD derivatives in pMAL-c2X (Ap’) | This study |
| pGDX-Dx series | arsD derivatives in pGDX9 (Ap’) | This study |
| pACBAD-Dx series | arsD derivatives in pACBAD (Tc’) | This study |

**Measurement of Metalloid Binding**—The buffer used for purification of MBP-ArsD was exchanged with a buffer containing 50 mM MOPS-KOH, pH 7, using a Bio-Gel P-6 Micro Bio-Spin column (Bio-Rad). The buffers were degassed by bubbling with argon. Purified protein was incubated at 4°C with indicated concentrations of Sb(III) in the form of potassium antimony tartrate. After 1 h, each sample was passed through a Bio-Gel P-6 column pre-exchanged with the same buffer. Portions (30 μl) were diluted with 2% HNO3, and the quantity of metalloid was measured by inductively coupled mass spectrometry with a PerkinElmer ELAN 9000. Antimony standard solutions in the range of 0.5–10 ppb in 2% HNO3 were obtained from Ultra Scientific, Inc. (North Kingstown, RI).

**Cross-linking Assays**—Cross-linking with dibromobimane, which forms fluorescent adducts with two thiols that are 3–6 Å from each other, was performed as described previously (21). Purified MBP-ArsD derivatives were buffer-exchanged into 50 mM MOPS, 0.2 M NaCl, pH 7.5, using micro-spin gel filtration columns (Bio-Rad). The proteins were quantified and expressed as molar concentration of ArsD monomer. Proteins (24 μM each) were incubated with 0.5 mM dibromobimane for 20 min at room temperature. The samples were analyzed by 8% SDS-PAGE. Formation of fluorescent cross-linked products was visualized at 365 nm and by immunoblotting with anti-serum against ArsD.

**ATPase Assays**—ATPase activity was estimated using a couple assay (22), as described (23). MBP-ArsD derivatives were added at a final concentration of 3 μM into an assay mixture...
containing 5 mM ATP, 1.25 mM phosphoenolpyruvate, 0.25 mM NADH, 10 units of pyruvate kinase and lactate dehydrogenase with or without various concentrations of potassium antimycolic tartrate or sodium arsenite. ArsA was added to a final concentration of 0.3 μM. The mixture was prewarmed to 37 °C, and the reaction was initiated by the addition of 2.5 mM MgCl₂ and measured at 340 nm. The linear steady state rate of ATP hydrolysis was used to calculate specific activity. The reaction volume was 0.2 ml each in 96-well microplates, and the reactions were monitored by microplate reader SPECTRA max 340PC (Molecular Devices).

Yeast Two-hybrid Analysis—A GAL4-based yeast two-hybrid system (24) (Clontech Laboratories, Inc.) was used to determine protein-protein interactions. AH109, a GAL4-activating HIS3 reporter yeast strain, was co-transformed with ars gene-fused pGBT-Dx (BD-ArsDx) and pACT-X (AD-X) series plasmids. The transformed cells were cultured overnight in SD medium at 30 °C and then washed, suspended, and adjusted to an absorbance of 0.600 of 1 in 20 mM Tris-HCl, pH 7.5. Portions of the cell suspensions (1 μl) were inoculated on SD agar plates lacking histidine in serial 10-fold dilutions and incubated at 30 °C for 3 days.

Transport Assays—Transport of As(III) by the ArsB pump was assayed as decreased accumulation in cells (25, 26). E. coli strain AW3110 was co-transformed with pSE-X series and pACBAD-Dx series plasmids. The cultures were grown overnight in LB medium and diluted 50-fold into LB medium containing 5 mM ATP, 1.25 mM phosphoenolpyruvate, 0.25 mM NADH, 10 units of pyruvate kinase and lactate dehydrogenase with or without various concentrations of potassium antimycolic tartrate or sodium arsenite. ArsA was added to a final concentration of 0.3 μM. The mixture was prewarmed to 37 °C, and the reaction was initiated by the addition of 2.5 mM MgCl₂ and measured at 340 nm. The linear steady state rate of ATP hydrolysis was used to calculate specific activity. The reaction volume was 0.2 ml each in 96-well microplates, and the reactions were monitored by microplate reader SPECTRA max 340PC (Molecular Devices).

MBS1 Is Involved in Metalloid Transfer and ArsA Activation—ArsD increases the affinity of the ArsA ATPase for As(III) (4). The ability of ArsD derivatives to activate ArsA was examined (Fig. 3). In the absence of ArsD, ArsA was not able to bind Sb(III), suggesting that Cys₁⁸, in addition to Cys₁² and Cys₁³, may be involved in the formation of MBS1 (Fig. 2B). Although each protein was produced in normal amounts and was fully soluble, the possibility of misfolding cannot be eliminated.

RESULTS

Contribution of Cysteine Residues to ArsD Metalloid-binding Sites—Alignment of the primary sequence of homologues of R. metallidurans ArsD indicates that they all possess a vicinal cysteine pair, Cys₁²-Cys₁³, and a single additional cysteine, Cys₁⁸ (Fig. 1). Two other vicinal cysteine pairs, Cys₁₂-Cys₁³ and Cys₁¹⁹-Cys₁₂⁰, are found in some homologues but not others. Cys₁¹⁹-Cys₁₂⁰ is not required for derepression by As(III) when ArsD functions as a weak transcriptional repressor, although the other two vicinal pairs may be (7, 27).

Binding of Sb(III) to purified MBP-ArsD derivatives was measured as a function of Sb(III) concentration. Sb(III) was used because it binds with higher affinity than As(III), which is difficult to measure using this assay. MBP-fused wild type ArsD bound metalloid with a stoichiometry of six Sb(III) per dimer and an apparent Kₐ of 10⁻⁶ M (Fig. 2A), suggesting that there are three metalloid-binding sites (MBBs) per ArsD monomer. Given the insensitivity of the assay, the actual Kₐ values could be lower. An ArsD derivative, ArsD₁–₁₁⁸C₁₂⁰A, in which Cys₁² and Cys₁₂⁰ were replaced with a six-histidine tag, bound four Sb(III) per dimer, consistent with the C-terminal vicinal pair, Cys₁¹⁹-Cys₁₂⁰, forming two metalloid-binding sites (termed MBS3) per dimer. The truncation ArsD₁–₁₀⁹ in which a stop codon was added after the codon for residue 109, leaving only the first cysteine pair, Cys₁²-Cys₁³, bound two Sb(III) per dimer. This N-terminal vicinal pair is termed MBS1. A derivative, ArsD₁–₁₁⁸C₁₂²A/C₁₂³A, was constructed with the N-terminal cysteine pair changed to alanines and the C-terminal vicinal pair replaced with a six-histidine tag, leaving only the second vicinal pair, Cys₁¹²-Cys₁³. This derivative bound two Sb(III) per dimer; for this reason Cys₁¹²-Cys₁³ was designated MBS2. These results demonstrate that each MBS binds one atom of trivalent metalloid, for a total of three per monomer and six per dimer.

Considering that cysteine pair Cys₁²-Cys₁³ and Cys₁⁸ are conserved in all ArsD homologues identified to date, three derivatives were constructed: ArsD₁–₁₀⁹C₁₂²A, ArsD₁–₁₀⁹C₁₂³A, and ArsD₁–₁₀⁹C₁²₂A. Each variant lacked MBS2 and MBS3 and retained only two of the three conserved cysteine residues. None of the three purified proteins was able to bind Sb(III), suggesting that Cys₁⁸, in addition to Cys₁² and Cys₁³, may be involved in the formation of MBS1 (Fig. 2B). Although each protein was produced in normal amounts and was fully soluble, the possibility of misfolding cannot be eliminated.

MBS1 Is Involved in Metalloid Transfer and ArsA Activation—ArsD increases the affinity of the ArsA ATPase for As(III) (4). The ability of ArsD derivatives to activate ArsA was examined (Fig. 3). In the absence of ArsD₁–₁₁⁸, a truncated ArsD lacking MBS₁, the half-maximal concentration of As(III) was 10 μM (Fig. 3A), similar to that of wild type ArsD (4). In the absence of ArsD₁–₁₀⁹, a truncated ArsD lacking both the Cys₁¹²-Cys₁³ and Cys₁¹⁹-Cys₁₂⁰ vicinal pairs, the half-maximal concentration of As(III) was 25 μM. These data suggest that neither MBS2 nor MBS3 is required for ArsD to activate ArsA. In contrast, an ArsD derivative lacking MBS1, ArsD₁–₁₁⁸C₁₂²A/C₁₂³A, was not able to activate ArsA. In the presence of this derivative, the concentration of As(III) required for half-maximal stimulation of ATPase activity was 1.5 mM, similar to that of ArsA in the absence of ArsD. These data are consistent with participation of MBS1, but not MBS2 or MBS3, in increasing the affinity of ArsA for metalloid.

The effects of single cysteine substitutions in MBS1 on the ability of ArsD to activate ArsA were examined (Fig. 3B). Derivatives ArsD₁–₁₁⁸C₁₂²A, ArsD₁–₁₁⁸C₁₂³A, and ArsD₁–₁₁⁸C₁²₂A, each having a cysteine-to-alanine substitution in MBS1 but retaining MBS2, were unable to activate ArsA. Cysteine-to-serine substitutions gave similar results (data not shown). Because metalloids alone augment the ATPase activity of ArsA, it seems plausible that Cys₁², Cys₁³, and Cys₁⁸ are directly responsible for delivering As(III) to ArsA to enhance its affinity. In other words, the affinity for As(III) in the ArsD-ArsA complex would be largely determined by ArsD rather than by ArsA.
| ARSENIC-BINDING SITES IN AN ARSENIC CHAPERONE |
|---------------------------------------------|
| **R773**                                   |
| R6                                           |
| pmH12                                       |
| pKw301                                      |
| R46                                         |
| Shekanella                                  |
| Alcaligenes                                 |
| Methylococcus                               |
| Azoxys                                      |
| Dechloromonas                               |
| Rhodoferax                                  |
| prE1                                        |
| pBD2                                        |
| Magnetosporillum                            |
| Rhodosporillum                              |
| Burkholderlia                              |
| Rhodopirellula                             |
| plLi100                                     |
| pSSP1                                       |
| Mycobacterium                               |
| Syntrophomonas                              |
| Phyllobacterium                             |
| Acidithiobacillus                           |
| Psychromonas                                |
| Alkalichromatium                           |
| Geobacter                                   |
| pchi10987                                  |
| Bacillus                                    |
| Bacteroides                                |
| Desulfotobacterium                         |
| pNRC100                                    |
| Alkaliphilus                                |

| **R773**                                   |
| R6                                           |
| pmH12                                       |
| pKw301                                      |
| R46                                         |
| Shekanella                                  |
| Alcaligenes                                 |
| Methylococcus                               |
| Azoxys                                      |
| Dechloromonas                               |
| Rhodoferax                                  |
| prE1                                        |
| pBD2                                        |
| Magnetosporillum                            |
| Rhodosporillum                              |
| Burkholderlia                              |
| Rhodopirellula                             |
| plLi100                                     |
| pSSP1                                       |
| Mycobacterium                               |
| Syntrophomonas                              |
| Phyllobacterium                             |
| Acidithiobacillus                           |
| Psychromonas                                |
| Alkalichromatium                           |
| Geobacter                                   |
| pchi10987                                  |
| Bacillus                                    |
| Bacteroides                                |
| Desulfotobacterium                         |
| pNRC100                                    |
| Alkaliphilus                                |

| **112/113**                                  |
| **119/120**                                 |

| **R773**                                   |
| R6                                           |
| pmH12                                       |
| pKw301                                      |
| R46                                         |
| Shekanella                                  |
| Alcaligenes                                 |
| Methylococcus                               |
| Azoxys                                      |
| Dechloromonas                               |
| Rhodoferax                                  |
| prE1                                        |
| pBD2                                        |
| Magnetosporillum                            |
| Rhodosporillum                              |
| Burkholderlia                              |
| Rhodopirellula                             |
| plLi100                                     |
| pSSP1                                       |
| Mycobacterium                               |
| Syntrophomonas                              |
| Phyllobacterium                             |
| Acidithiobacillus                           |
| Psychromonas                                |
| Alkalichromatium                           |
| Geobacter                                   |
| pchi10987                                  |
| Bacillus                                    |
| Bacteroides                                |
| Desulfotobacterium                         |
| pNRC100                                    |
| Alkaliphilus                                |

| **112/113**                                  |
| **119/120**                                 |
Arsenic-binding Sites in an Arsenic Chaperone

Metalloid-binding sites in ArsD. Purified MBP-ArsD was incubated at 4 °C with the indicated concentrations of potassium antimonyl tartrate. Sb(III)–protein complexes were obtained as described under "Materials and Methods," and the molar ratios were calculated. A, ArsD has three MBSs. Sb(III) binding was assayed with wild type ArsD, which has Cys12-Cys13 (MBS1), Cys13-Cys18 (MBS2), and Cys18-Cys19 (MBS3) (AAPG01000022); Alkalilimnicola ehrlichei (AE017195); Bacillus sp. CDB3 (CP000309); Desulfitobacterium hafniense (Y51 (AP008230)); Halobacterium sp. RP62A (CP000022); Acidithiobacillus caldus plasmid pSSP1 (CP000069); Mycobacterium flavescens PYR (AAPA01000005); Acidiphilium multivorans (AAPH01000002); Psychromonas sp. CNPT3 (AAGP01000021); Dechloromonas aromatica RCB (CP000089); Methylococcus capsulatus (CP0000267); Halobacterium sp. Y51 (AP008230); Acidithiobacillus thiooxidans T118 (CP000069); Rhodococcus eurhythmus strain CM13 (CP00000024); Termobacterium thermophylum strain BAA (CP000069); Geobacter metallireducens strain Ru4 (AAPA01000005); and Bacillus cereus plasmid pEG2 (AAPA01000051).

The Effect of Elimination of ArsD As(III)-binding Sites and Truncations on Protein-Protein Interactions—The ability of the six cysteine-to-alanine ArsD mutants and two truncated derivatives to dimerize was examined by yeast two-hybrid analysis using SigmaPlot 9.0, with the error bars representing the standard error.

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Arsenic-binding Sites in an Arsenic Chaperone

Effect of Elimination of ArsD As(III)-binding Sites on Arsenic Accumulation and Resistance—The ability of ArsD derivatives with cysteine-to-alanine substitutions to enhance the activity of the ArsAB pump in vitro was examined. Although cells expressing 

\[ \text{arsD}_{1–118,C12A,C13A} \] 

which encodes an ArsD derivative having only MBS1, in \textit{trans} with \textit{arsAB} accumulated As(III) to nearly the same extent as those expressing wild type \textit{arsDAB}, cells expressing mutants lacking \textit{MBS1} (\textit{arsD}_{C12A,C13A}) or \textit{arsD}_{C18A} in \textit{trans} with \textit{arsAB} accumulated slightly more As(III) than cells with only \textit{arsAB} (Fig. 5A). These data are consistent with our proposal that Cys\textsuperscript{12}, Cys\textsuperscript{13}, and Cys\textsuperscript{18} are involved in functional interaction of ArsD with the ArsAB efflux pump.

We also examined whether the same \textit{arsD} mutants affected resistance conferred by the ArsAB pump. Cells expressing an \textit{arsD} mutant having only \textit{MBS1} (\textit{arsD}_{1–118,C12A,C13A}) in \textit{trans} with \textit{arsAB} had only a modest reduction in arsenite resistance compared with cells expressing wild type \textit{arsDAB}. In contrast, cells expressing the mutants lacking \textit{MBS1} (\textit{arsD}_{C12A,C13A} or \textit{arsD}_{C18A}) were no more resistant than cells with only \textit{arsAB} (Fig. 5B). These results strongly support our hypothesis that the MBS1, including the Cys\textsuperscript{12}-Cys\textsuperscript{13} pair and Cys\textsuperscript{18}, but not MBS2 or MBS3, is required for activation of the ArsAB pump.

(see Fig. 5A). All of the mutants and the C-terminal truncation \textit{arsD}_{1–109}, which lacks the last 11 residues, were able to interact with wild type ArsD, indicating that these derivatives are stably produced in yeast and able to form dimers. Although the interaction with the mutant C12A/C13A ArsD seemed somewhat weaker than the others, yeast two-hybrid results are not necessarily quantitative. The truncated derivative lacking the first 13 residues (\textit{arsD}_{1–120}) was unable to interact with wild type ArsD, suggesting the involvement of those residues either in ArsD dimerization or protein stability.

The effect of the loss of MBS1, MBS2, or MBS3 on ArsD-ArsA interactions was also examined (Fig. 4B). The ability of three ArsD derivatives retaining MBS1 but lacking MBS2 and MBS3 was analyzed: \textit{arsD}_{1–109}, in which both MBS2 and MBS3 were deleted; \textit{arsD}_{1–118,C12A}, lacking MBS2 and one cysteine residue in MBS2 and \textit{arsD}_{1–118,C12A,C13A}, lacking MBS3; and both cysteine residues in MBS2. All three mutants were able to interact with ArsA. In contrast, mutants or truncations lacking Cys\textsuperscript{12}, Cys\textsuperscript{13}, and/or Cys\textsuperscript{18} were unable to interact, including \textit{arsD}_{1–118,C12A}, \textit{arsD}_{1–118,C13A}, \textit{arsD}_{1–118,C12A,C13A}, and a truncated derivative lacking the first 13 residues (\textit{arsD}_{14–120}). Cysteine-to-serine substitutions in MBS1 showed similar results as the cysteine-to-alanine substitutions (data not shown). Although it is possible that the loss of interaction is a result of misfolding, it seems unlikely that the loss in every one of these derivatives could be due to misfolding, and it is more reasonable to conclude that it is due to a loss of interaction. When 0.1 mM sodium arsenite was added to the medium, the yeast cells grew more slowly, but the overall results were similar to those without metalloids (data not shown). These results suggest that cysteine residues 12, 13, and 18 are involved in interaction with ArsA.

Further truncations of ArsD that lack the first 2, 4, 7, or 11 residues or the last 27, 43, 55, or 77 residues were constructed to establish which regions of ArsD are responsible for dimerization or interaction with ArsA. The truncations were examined for ability to interact with wild type ArsD by yeast two-hybrid analysis. C-terminally truncated ArsD derivatives with as few as 43 N-terminal residues (\textit{arsD}_{1–109}, \textit{arsD}_{1–93}, \textit{arsD}_{1–77}, \textit{arsD}_{1–69}, and \textit{arsD}_{1–43}) were able to interact with both wild type ArsD and ArsA (Fig. 6C). In contrast, truncated ArsD derivatives lacking 7 or more residues from the N terminus (\textit{arsD}_{8–120}, \textit{arsD}_{12–120}, and \textit{arsD}_{14–120}) were unable to interact either with wild type ArsD or with ArsA, indicating the involvement of the N terminus of ArsD in protein-protein interaction. These data are consistent with our previous suggestion that the ArsD dimer interacts with ArsA (4).
ArsD Cys12, Cys13, and Cys18 in MBS1 Are Cross-linked by Dibromobimane—The proximity of three cysteine thiolates in MBS1 was examined by reaction of MBP-ArsD with the homobifunctional fluorogenic cross-linking reagent dibromobimane (Fig. 6). As expected, MBP-ArsD1–109,C12A/C13A/C18A, lacking all three MBSs, did not react with dibromobimane (Fig. 6, lane 7). MBP-ArsD1–109, having MBS1, formed a highly fluorescent monomer band and a small amount of cross-linked dimer (Fig. 6, lane 1). Fluorescence in the monomer band could be due in part to formation of cross-links between cysteine residues and in part to a slow, nonspecific displacement of the second bromine by water following reaction of the first with cysteine. The cross-linked dimer could form only by reaction of one cysteine in each monomers with a dibromobimane molecule. If only one cysteine residue in MBS1 was changed to an alanine, those derivatives (MBP-ArsD1–109,C13A/C18A, and MBP-ArsD1–109,C18A) still formed a small amount dimer (Fig. 6, lanes 2–4). A derivative with only Cys12 (MBP-ArsD1–109,C13A/C18A) formed fluorescent dimers (Fig. 6, lane 6). Similarly, MBP-ArsD1–109,C12A/C13A (containing only Cys18) formed fluorescent dimers (Fig. 6, lanes 5). These results suggest that the thiol groups of Cys12, Cys13, and Cys18 are probably 3–6 Å of each other in each monomer and perhaps also near the corresponding cysteine thiolates from the other subunit.

DISCUSSION

Understanding the organization of the metalloid-binding sites in the ArsD metallochaperone is crucial to a molecular description of the metalloid transfer process. Previous studies suggested from the results of indirect assays that the three vicinal cysteine pairs in ArsD form three independent metalloid-binding sites, MBS1, MBS2, and MBS3, per monomer (27, 28). In this study, binding of Sb(III) or As(III) to ArsD was directly...
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Proposed mechanism of arsenic transfer between ArsD and ArsA. Step 1, ArsD binds As(III) by exchange of the three hydroxyls of As(OH)₃ for the thiols of MBS1 residues Cys¹¹³, Cys¹²⁰, and Cys¹⁸. Step 2, exchange of one thiol ligand in MBS1 of ArsD for one thiol ligand in MBS1 of ArsA. Step 3, As(III) exchanges a second thiol ligand in ArsD for a second in ArsA. Step 4, As(III) is transferred to MBS1 in ArsA by a final ligand exchange, inducing a conformational change in ArsA that increases the rate of ATP hydrolysis. The model should not be taken to specify an order of ligand exchange in the transfer reaction.

measured. Wild type ArsD binds three metalloid atoms per monomer (or six per dimer). Elimination of any of the three vicinal cysteine pairs, Cys¹²⁻Cys¹³⁻Cys¹¹³⁻Cys¹¹⁸⁻Cys¹²⁰, in turn, by mutagenesis or deletion, eliminated MBS1, MBS2, or MBS3, respectively, and a derivative having none of the cysteine pairs bound no metalloid. A cysteine-to-alanine substitution of Cys₁₈, which is conserved in all ArsD homologues identified to date, eliminated binding at MBS1, indicating that MBS1 requires Cys¹₈ in addition to Cys¹₂ and Cys¹₃.

MBS3 is not well conserved in ArsD homologues, and no phenotype has been associated with deletion of the C-terminal region containing MBS3. MBS2 has been associated with the weak transcriptional activity of ArsD (27), but substitutions or deletions of the cysteines in MBS2 do not eliminate the metalchaperone activity of ArsD. In contrast, MBS1 appear to be required for metalchaperone activity.

ArsD interacts with ArsA, the catalytic subunit of the ArsA/B translocating ATPase (4). ArsA is a pseudodimer, with two homologous halves, A1 and A2, each of which contains a nucleotide-binding domain (29). ArsA ATPase activity is activated by binding of As(III) or Sb(III) (23). In the structure three metalloid-binding sites are observed that are formed from residues contributed from both halves at the A₁–A₂ interface (29). Each metalloid is coordinated with one residue in A₁ and another in A₂, forming a “molecular glue” that brings the two halves of ArsA into contact with each other. Because each nucleotide-binding domain has residues from the opposite half of the protein, the formation of this A₁–A₂ interface is proposed to complete the nucleotide-binding domains, activating catalysis (30). Although Sb(III) is observed in all three ArsA MBSs in the crystal structure, only MBS₁, containing Cys¹¹³ and Cys¹²², is a high affinity binding site; the other two may be low affinity sites (31). Although Sb(III) is bound between Cys¹¹³ and Cys¹²² in the crystal structure, Cys¹²² is actually closer to Cys¹⁷² than Cys¹¹³, and recent data suggest that Cys¹⁷² can participate in MBS1 at some point during the reaction.³ Thus, we now consider ArsA MBS1 to be an S₃ site consisting of those three thiolates.

Although the details of metalloid transfer from ArsD to ArsA are presently unknown, the mechanism of copper transfer from copper chaperones to their respective partner proteins is instructive. Pufahl et al. (13) first proposed a chemical exchange reaction from transfer of copper from the yeast chaperone Atx1p to the N-terminal domain of the Cu(I)-translocating P-type ATPase Ccc2p. In this transfer, Cu(I) is initially two-coordinately bound to the two cysteine thiolates of the MTCXXC domain in Atx1p. It forms a transient three-coordinately bound complex with one of the cysteine thiolates of one of the MTCXXC domains of Ccc2p, followed by exchange of the Cu(I) from one of the thiolates in Atx1p to the second thiolate in the Ccc2p domain. This three-coordinate complex then dissociates to form free Atx1p and two-coordinate bound Ccc2p-Cu(I). Similar mechanisms have been proposed for the human Hah1 copper chaperone and the Menkes and Wilson disease proteins (32) and the yeast CCS chaperone and its partner superoxide dismutase (33). In the structure Hah1 is observed as a crystallographic dimer in which Cu(I) is bound four-coordinate to the two MTCXXC cysteine residues. This suggests that Hah1 and its yeast homologue Atx1p form heterodimers with the N-terminal domains of their respective copper pump partners and that transfer would occur by chemical exchange at their interfaces. Docking analysis of Atx1p and Ccc2p supports this mechanism (34). Similarly, the yeast CCS forms a heterodimer with superoxide dismutase and is proposed to use a similar chemical exchange mechanism between cysteine thiolates in the two proteins (33).

³ X. Ruan, H. Bhattacharjee, and B. P. Rosen, manuscript in preparation.
analysis with N-terminal truncations suggests that a core region in ArsD from residues 5–43, including MBS1, is involved in both dimerization and interaction with ArsA. Metalloid bound to MBS1 in ArsD is transferred to MBS1 in ArsA, which is then extruded out of the cell by ArsB. Although not shown in the model, it is possible that ArsA MBS2 and MBS3 participate in sequential transfer of metalloid to ArsB. Even though the two monomers can be cross-linked to some degree with dibromobimane, it is not clear whether ArsD MBS1 is molecularly. For that reason, the model shows only one monomer of ArsD. ArsA is shown in the ATP-bound form because the two monomers can be cross-linked to some degree with dibromobimane, it is not clear whether ArsD MBS1 is composed of cysteines from a single subunit or is formed intermolecularly. For that reason, the model shows only one monomer of ArsD. ArsA is shown in the ATP-bound form because nucleotide is required for metalloid transfer from ArsD to ArsA (4). The model implies that the chemical exchange occurs in three discrete steps, although the order is not known. The model provides a basis for future experiments.

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