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Mutations in the ArsA ATPase that restore interaction with the ArsD metallochaperone

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Abstract

The ArsA ATPase is the catalytic subunit of the ArsAB As(III) efflux pump. It receives trivalent As(III) from the intracellular metallochaperone ArsD. The interaction of ArsA and ArsD allows for resistance to As(III) at environmental concentrations. A quadruple mutant in the *arsD* gene encoding a K2A/K37A/K62A/K104A ArsD is unable to interact with ArsA. An error-prone mutagenesis approach was used to generate random mutations in the *arsA* gene that restored interaction with the quadruple *arsD* mutant in yeast two-hybrid assays. A number of *arsA* genes with multiple mutations were isolated. These were analyzed in more detail by separation into single *arsA* mutants. Three such mutants encoding Q56R, F120I and D137V ArsA were able to restore interaction with the quadruple ArsD mutant in yeast two-hybrid assays. Each of the three single ArsA mutants also interacted with wild type ArsD. Only the Q56R ArsA derivative exhibited significant metalloid-stimulated ATPase activity *in vitro*. Purified Q56R ArsA was stimulated by wild type ArsD and to a lesser degree by the quadruple ArsD derivative. The F120I and D137V ArsAs did not show metalloid-stimulated ATPase activity. Structural models generated by *in silico* docking suggest that an electrostatic interface favors reversible interaction between ArsA and ArsD. We predict that mutations in ArsA propagate changes in hydrogen bonding and salt bridges to the ArsA-ArsD interface that affect their interactions.

Keywords

Arsenite; ArsA; ArsD; Metallochaperone; Resistance; Transport ATPase

Introduction

Arsenic is a ubiquitous environmental toxic substance and enters the environment from both geochemical and anthropogenic sources. The Agency for Toxic Substances and Disease Registry puts arsenic at the top of its Substance Priority List (<http://www.atsdr.cdc.gov/spl/>) because this metalloid poses the most significant potential threat to human health due to its

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toxicity and potential for human exposure. In humans, exposure to arsenic is associated with a number of diseases, including several forms of cancer, cardiovascular disease, peripheral neuropathies and diabetes mellitus (Abernathy et al. 1999; Beane Freeman et al. 2004). As a consequence of continual exposure to arsenic, nearly every organism, from *Escherichia coli* to humans, has evolved pathways for arsenic detoxification (Zhu et al. 2014). The *arsRDABC* operon of *E. coli* plasmid R773 encodes five genes for arsenic detoxification (Chen et al. 1986; San Francisco et al. 1990). The ArsAB complex is an efflux pump that confers resistance to arsenicals and antimonials. ArsA is the catalytic subunit of the pump that hydrolyzes ATP in the presence of As(III) or Sb(III) (Hsu and Rosen 1989). ATP hydrolysis is coupled to extrusion of As(III) or Sb(III) through ArsB, which serves both as a membrane anchor for ArsA and as the substrate-conducting pathway (Tisa and Rosen 1990). ArsD is an As(III) metallochaperone that interacts with ArsA to increase arsenic resistance (Lin et al. 2006). Purified ArsD binds As(III) and transfers it to ArsA, stimulating its ATPase activity (Lin et al. 2006; Yang et al. 2010). The apo structure of ArsA (Zhou et al. 2000) and ArsD (Ye et al. 2010) have each been determined by x-ray crystallography, and structural models of the As(III)-bound forms of both protein and the ArsD-ArsA complex have been constructed (Ye et al. 2010).

A yeast two-hybrid genetic mapping approach identified seventeen residues of ArsD that either enhance or reduce interaction with ArsA (Yang et al. 2011). These residues included Cys12, Cys13 and Cys18, which form the As(III) binding site, and a series of N-terminal residues that form a spine along the surface of the protein. Independently, an *in silico* docking model (Ye et al. 2010) identified many of the same residues obtained from the genetic mapping studies, suggesting that ArsD and ArsA interact at an interface between the two proteins (Yang et al. 2011).

ArsD has six lysine residues at positions 2, 37, 60, 62, 90, and 104. The quadruple lysine mutant K2A/K37A/K62A/K104A of ArsD (4KA ArsD) did not interact with wild type ArsA in yeast two hybrid studies and also was not able to stimulate ATPase activity in wild type ArsA (Yang et al. 2011). The metalloid binding ability of 4KA ArsD was determined to be similar as the wild type (Yang et al. 2011). In contrast, the quadruple K2A/K37R/K62R/K104A ArsD derivative stimulated ArsA activity nearly as well as wild type ArsD. In the 100 closest ArsD homologues in the NCBI database, residue 37 is either lysine or arginine, and residue 62 is always lysine (Yang et al. 2011). These observations suggested that alanine substitutions in ArsD abolish its interaction with ArsA, but arginine substitutions are permitted in Lys37 and Lys62.

As mentioned above, ArsD has a dual role: first, transfer of metalloids from ArsD to ArsA and second, to enhance the catalytic activity of ArsA. Although extensive research have been carried out on both the structural details of ArsA and ArsD and their biochemical roles, limited studies have been performed to understand and identify residues of ArsA that either directly interact with ArsD or indirectly facilitate interaction. In this study, the interaction between ArsA and ArsD was further examined by screening for suppressor mutants of ArsA that interact with 4KA ArsD.

Materials and methods

Reagents

3-Amino-1, 2, 4-triazole (3-AT) was purchased from MP Biomedicals (Solon, OH). 5-Fluoroorotic Acid (5-FOA) was purchased from Thermo Scientific (Pittsburgh, PA). Yeast Minimal Media-SD Base and Amino Acid Dropout Mixes (-Leu/-Trp and -His/-Leu/-Trp) were purchased from Clontech (Mountain View, CA). Unless otherwise mentioned, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Strains, plasmids and media

E. coli strain JM109 was used for molecular cloning (Table 1). *E. coli* strain BL21(DE3) was used for protein expression and purification. The *arsA* gene with a C-terminal six-histidine tag was cloned into the expression vector pET-28a as pETy-dAhB (Lin et al. 2006). Two forms of ArsD were used in this study. The gene for full-length wild type *arsD* was used in yeast two-hybrid analyses. All biochemical assays were performed with a fully active ArsD that is C-terminally truncated at residue 109 with a six-histidine tag (termed simply ArsD in this study). The gene for this ArsD was cloned in the expression vector pET28a as pET28a-ArsD109. *E. coli* cells were grown at 37 °C in Luria-Bertani (LB) medium (Green et al. 2012). Ampicillin (100 µg/ml), kanamycin (40 µg/ml) and 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were added as required. Yeast cells were grown in complete yeast extract-peptone-dextrose (YPD) or minimal synthetic dextrose (SD) media with the appropriate supplements at 30 °C (Adams et al. 1998). Growth in liquid culture was estimated from the optical density at 600 nm.

DNA manipulation and mutagenesis

Plasmid extraction, DNA restriction endonuclease analysis, ligation and other general molecular biological procedures were performed as described (Green et al. 2012). Transformation of *E. coli* cells was carried out using a MicroPulser Electroporator (Bio-Rad, Hercules, CA). Transformation of yeast cells was performed using Fast Yeast Transformation kit from G-Biosciences (St. Louis, MO). DNA purification kits were purchased from Qiagen (Valencia, CA). Either a Qiaprep Spin Miniprep kit or a Qiaquick Gel Extraction kit was used to prepare plasmid DNA for restriction enzyme digestion, sequencing, and recovering DNA fragments from agarose gels. Single mutants of *arsA* were made in pACT2-ArsA construct by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis Kit from Agilent Technologies (Santa Clara, CA). The sequence of new plasmid constructs was confirmed by DNA sequencing using Sequetech DNA Sequencing Services (Mountain View, CA).

Generation of a random mutated library of PCR fragments

Random mutagenesis of *arsA* was performed using error-prone (ep)-PCR employing biased nucleotide composition of the PCR buffer, increasing the concentration of Mg²⁺, and addition of Mn²⁺ to the reaction mixture (Cadwell and Joyce 1992). The ep-PCR reaction mixture (50 µl) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 7 mM MgCl₂, 0.5 mM MnCl₂, 0.2 mM each of dATP and dGTP, 1 mM each of dCTP and dTTP, 25 pmol each of

the oligonucleotide primers (forward, CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC; reverse, AGG TTA CAT GGC CAA GAT TGA AAC TTA GAG GAG), 80 ng of template DNA, 2.5 U of *Taq* DNA polymerase (Life Technologies). PCR condition was 1 cycle of 96 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 100 s, and, finally, 1 cycle of 72 °C for 10 min. The ep-PCR product was purified by agarose gel and quantified by absorption at 260 nm.

Yeast two-hybrid analysis

Plasmids pGBT9 and pACT2 were used as *S. cerevisiae*/*E. coli* shuttle vectors for yeast two-hybrid assay (Lin et al. 2006). The yeast strain, AH109 (Fields and Song 1989) was used for yeast two-hybrid assays which is a GAL4-based yeast two-hybrid system. AH109 was used to analyze protein-protein interaction or for selection of mutants retaining capability of interaction or showing stronger interaction in the presence of 3-AT. The plasmid pGBT9 was modified to pBD-k by inserting kanamycin resistance gene through *Aat*II site (Yang et al. 2011). To determine protein-protein interaction by growth of serial dilutions, the transformed cells were cultured overnight in SD medium at 30 °C, centrifuged at 10,000 g, and the pellets were washed with 20 mM Tris-HCl (pH 7.5) and adjusted to an OD600 of 1. Portions of the cell suspension (1 µl) were inoculated in serial 10-fold dilutions on SD agar plates lacking histidine or with 3-AT at the indicated concentration. The plates were incubated at 30 °C for 3 days.

Sequencing of *arsA* mutant genes in yeast colonies

Each yeast colony was grown in SD medium. The plasmids were isolated from yeast by Zymoprep™ Yeast Plasmid Miniprep II kit (Zymo Research Corporation, Irvine, CA), transformed to *E. coli* JM109 and grown on LB plates with ampicillin. The plasmids were extracted from *E. coli* JM109, and *arsA* mutations in the pAD vector were sequenced with the pACT2 sequencing primers GAL4A3 (ACT TGC GGG GTT TTT CAG) and GAL4Act (TAC CAC TAC AAT GGA TG).

Protein expression and purification

Cells bearing the indicated plasmids were grown in LB medium overnight at 37 °C and then diluted 50-fold into 1 L of the same medium. Proteins were expressed by induction with 0.3 mM isopropyl-β-D-thiogalactopyranoside at an OD600 of 0.6–0.8 for 3 h. ArsA with a six histidine tag at the C-terminus was purified from cells of strain BL21(DE3) expressing pETy-dAhB plasmid, as described (Lin et al. 2006). ArsD and its derivatives with a six histidine tag at the N-terminus were purified similarly. Purified proteins were stored at –80 °C until use, and their concentrations were determined from the absorption at 280 nm (Gill and von Hippel 1989).

ATPase activity assays

ATPase activity was estimated using a coupled assay (Vogel and Steinhart 1976), as described previously (Hsu and Rosen 1989). The assay mixture contained in 200 µl: 50 mM MOPS-KOH, pH 7.5, 0.25 mM Na₂EDTA, 5 mM ATP, 1.25 mM phosphoenolpyruvate, 0.25 mM NADH, 2 units of pyruvate kinase (Sigma) and lactate dehydrogenase (Sigma), 0.3

μM ArsA, 3 μM ArsD, and increasing concentrations of sodium arsenite, all pre-warmed to 37 °C. The reaction was initiated by the addition of 2.5 mM MgCl_2 and followed by monitoring the decrease in absorbance at 340 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). The pathlength correction feature of the microplate reader was used to automatically normalize absorbance values to a 1 cm pathlength. The linear steady state rates were used to calculate the specific activity. Each assay was repeated at least three times with two separate batches of purified protein. The data were analyzed using SigmaPlot 12.0.

Measurement of metalloid binding

The buffer used for purification of ArsA was exchanged with a buffer containing 50 mM MOPS-KOH, pH 7.5, 0.25 mM EDTA using a Micro Bio-Spin P-6 gel column (Bio-Rad, Hercules, CA). Purified ArsA (10 μM) was incubated at 4 °C with 2.5 mM MgCl_2 , 2 mM $\text{ATP}\gamma\text{S}$, and 100 μM antimonite. After 1 h, each sample was passed through a Micro Bio-Spin P-6 gel column equilibrated with the same buffer. The concentration of protein in the flow-through was quantified from the absorption at 280 nm (Gill and von Hippel 1989). An aliquot of the flow-through (50 μl) was diluted with 2% HNO_3 , and the quantity of metalloid was measured by inductively coupled plasma-mass spectrometry (ICP-MS) with a PerkinElmer ELAN DRC-e. Antimony standard solutions in the range of 0–50 ppb in 2% HNO_3 were obtained from Ultra Scientific (North Kingstown, RI). From these data the molar ratio of Sb(III) to protein was calculated.

Model building and docking analysis

The model for the open form of ArsA was generated as described earlier (Ye et al. 2010). ArsD was modeled based on its crystal structure (PDB ID: 3MWH), and its missing active site loop (residues 10 to 19) was built using the *MODELLER* program (Sali and Blundell 1993). The ArsA and ArsD models were energy minimized individually before being docked by the *NAMD/VMD* program (Humphrey et al. 1996; Phillips et al. 2005). Docking was done by the online server *HexServer* (<http://hexserver.loria.fr>) (Macindoe et al. 2010). Hydrogen bonds and interactions (G^{diss} values) were calculated by the *PDBePISA* server (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (Krissinel and Henrick 2007). Domain rotation was performed using Chimera (<http://www.cgl.ucsf.edu/chimera>) (Pettersen et al. 2004). Structural models were rendered using PYMOL (<http://www.pymol.org>) (DeLano 2001).

Results

ArsA ATPase mutants exhibiting stronger interaction with ArsD

The interaction of ArsA ATPase and ArsD metallochaperone has been demonstrated earlier (Lin et al. 2006) using the yeast two-hybrid analysis (Fields and Song 1989). The *arsD* gene was cloned into the C-terminal DNA-binding domain (BD) as *bait*, and the *arsA* gene was cloned into the C-terminal activation domain (AD) as *prey* of the split *GAL4* transcriptional activator. Interaction of the two proteins resulted in expression of the *HIS3* reporter gene, allowing growth of yeast cells on media lacking histidine (Lin et al. 2006) (Fig. 1). Yeast

two-hybrid showed that the quadruple ArsD mutant K2A/K37A/K62A/K104A (4KA ArsD) does not interact with wild type ArsA (Yang et al. 2011) (Fig. 1).

In this study, random mutagenesis of *arsA* was performed by error-prone polymerase chain reaction (ep-PCR) induced with Mn^{2+} (Cadwell and Joyce 1992) using a pair of primers homologous to the flanking regions of the *arsA* gene in plasmid pAD-WT ArsA, in which *arsA* was fused to the sequence for the GAL4 DNA-activation domain. The purified ep-PCR product was mixed with plasmid pAD-ArsA linearized with *Bam*HI and *Eco*RI, co-transformed with pBD-4KA ArsD into *Saccharomyces cerevisiae* strain AH109 and selected on SD (-His/-Leu/-Trp) plates. By this method the ep-PCR fragments integrate into pAD-ArsA *in vivo* through homologous recombination (Hua et al. 1997). Twenty-two colonies were isolated on SD (-His/-Leu/-Trp) plates that showed interaction with 4KA ArsD. The *arsA* gene from each colony was sequenced. Of the 22 colonies, some were duplicates, and 15 were unique (Table 2). Of those 15, only one clone contained a single mutation (D137V), while the other clones contained multiple mutations in the *arsA* gene. Some substitutions such as Q56R, T115K, F120L and K404R were found in more than one mutant. Conservative substitutions were found at residue 120; Phe120 altered to either Ile or Leu. Using a combination of X-ray crystallography and *in silico* modeling and docking studies, 20 residues of ArsA were predicted to be within 4 Å of 30 ArsD residues, and they may form the contact regions between the two proteins (Ye et al. 2010). Based on the *in silico* predictions (Ye et al. 2010) and also that some of the mutations occurred more than once, seven mutations (Q56R, V90F, T115K, F120I, V190I, K404R, and E506G) were reintroduced individually by site-directed mutagenesis into the *arsA* gene to examine whether the single ArsA mutants could interact with 4KA ArsD. Other multiple mutations in the *arsA* gene have not yet been analyzed. From the results of yeast two-hybrid analysis, three single ArsA mutants (Q56R, F120I and D137V) exhibited interaction with 4KA ArsD (Fig. 1). These ArsA mutants also interacted with wild type ArsD (Fig. 1).

The repressed transactivator two-hybrid system has been used to select for mutants with increased affinity for a binding partner (Hirst et al. 2001; Joshi et al. 2007; Yang et al. 2011). The *HIS3* gene encodes imidazole glycerol-phosphate dehydratase, catalyzing the sixth step in histidine biosynthesis. Histidine synthesis by this enzyme is competitively inhibited by 3-AT (Klopotowski and Wiater 1965). If 3-AT is added to the medium, yeast cells must express higher levels of His3p to grow in the absence of histidine. AH109 yeast cells co-transformed with pBD-k-4KA (ArsD mutant K2A/K37A/K62A/K104A inserted into GAL4 binding domain in plasmid pGBT9) and pAD-AX (each of the single ArsA mutants (Q56R, F120I and D137V) fused to the GAL4 activation domain sequence in plasmid pACT2) could not grow on SD plates containing 10 mM 3-AT but lacking histidine, leucine and tryptophan. In contrast, AH109 yeast cells co-transformed with pBD-k-D (wild type ArsD) and pAD-AX (single ArsA mutants Q56R, F120I and D137V) were able to grow in the presence of up to 90 mM 3-AT (Fig. 1). These results indicate that the single ArsA mutants have increased interaction with wild type ArsD but not with 4KA ArsD.

Analysis of altered ArsAs

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)-stimulated ATPase (Hsu and Rosen 1989; Rosen et al. 1988). Each altered ArsA was soluble in the cytosol and purified to >95% homogeneity using nickel affinity chromatography (Green et al. 2012). There was no difference between the wild type and altered proteins in mobility on sodium dodecyl sulfate - polyacrylamide gel electrophoresis, nor was abnormal degradation of the altered proteins observed (data not shown), indicating that the altered proteins folded relatively normally.

ATPase activity of ArsA mutants

The ATPase activity of ArsA is stimulated by As(III) (Hsu and Rosen 1989; Rosen et al. 1988). In the absence of metalloid, the wild type showed a basal ATPase activity of 25 nanomoles of ATP hydrolyzed/min/mg of protein. In the presence of As(III), the wild type ArsA showed a 5.5-fold stimulation of ATPase activity over its basal rate. Addition of ArsD to the assay mix increases the affinity of ArsA for As(III), i.e. decreases the concentration of As(III) required for half-maximal stimulation of ArsA ATPase activity, thereby increasing its ATPase activity at even lower concentrations of As(III) (Lin et al. 2006). When wild type ArsD was added to the ATPase assay, the apparent affinity of ArsA for As(III) increased 8.5-fold (Fig. 2 and Table 3). Addition of 4KA ArsD to the assay decreased the half maximal concentration for As(III) by 1.6-fold. Q56R ArsA showed a similar basal ATPase activity as the wild type, which was stimulated 2.8-fold in the presence of As(III). Addition of either wild type or 4 KA ArsD to the assay mix increased the affinity for As(III) by 9-fold and 2-fold, respectively. These results indicate that, although 4KA ArsD does not stimulate ArsA ATPase activity to the same degree as the wild type, it has a small but significant effect on the affinity of ArsA for As(III).

F120I and D137V ArsA showed a basal ATPase rate of 80 and 60 nanomoles of ATP hydrolyzed/min/mg of protein, respectively. The ATPase activity of either proteins were not stimulated by As(III); nor were any changes observed upon addition of either wild type or 4KA ArsD. Note that both F120I and D137V ArsA show a basal ATPase rate that is similar to the As(III)-stimulated rate of Q56R ArsA.

Binding of Sb(III) to altered ArsAs

To directly examine the binding of metalloid to ArsA, Sb(III) was used because it is bound with higher affinity than As(III) (Ruan et al. 2006). ArsA has a single high affinity metalloid binding domain (Bhattacharjee et al. 1995; Bhattacharjee and Rosen 1996; Ruan et al. 2006; Ruan et al. 2008). Each Sb atom is bound three-coordinately with Cys113, Cys172, and Cys422. Binding of Sb(III) to altered ArsAs was measured by rapid gel filtration. In the presence of saturating Mg^{2+} and the nonhydrolyzable nucleotide ATP γ S, a stoichiometry of one mole of Sb(III) per mole of protein was observed for both wild type and Q56R ArsA, whereas a stoichiometry of 0.8 mole of Sb(III) per mole of protein was observed for F120I and D137V ArsA (Fig. 3). We do not consider this difference in Sb(III) binding as significant. Overall, these results indicate that while F120I and D137V ArsA do not show

metalloid-stimulated ATPase activity, their affinity for metalloid is unaffected by the mutations.

Docking analysis of the interaction between Q56R ArsA and ArsD

ArsA undergoes a number of conformational transitions during the catalytic cycle, an open form in the absence of ATP and As(III), and a closed form when both are present (Ruan et al. 2008; Zhou and Rosen 1997). The X-ray crystal structure of ArsA has been solved in the closed form with different nucleotides and metalloid (Zhou et al. 2000; Zhou et al. 2001). However, since metalloid transfer requires ArsA to be undergoing catalysis (Yang et al. 2010), it is likely that ArsD interacts initially with an open form of ArsA. A *S. cerevisiae* homologue of ArsA, Arr4p or Get3 (Auld et al. 2006; Shen et al. 2003) is involved in targeting tail-anchored proteins in the endoplasmic reticulum (Denic et al. 2013). The crystal structures of Get3 from different yeast strains were solved in the open (nucleotide free) and closed (ADP-AlF₄⁻) conformations (Bozkurt et al. 2009; Mateja et al. 2009). An open model of the Q56R ArsA based on the open Get3 structure was generated, as described earlier (Ye et al. 2010). A model of the metalated form of ArsD was constructed based on its crystal structure (PDB ID: 3MWH) with the missing active site loop (residues 10-19) built using MODELLER (Sali and Blundell 1993) and subjected to energy minimization using VMD/ NAMD program (Humphrey et al. 1996; Phillips et al. 2005). The ArsA and ArsD structures were docked by the online protein docking server, HexServer (<http://hexserver.loria.fr/>), which produced a ranked list of 100 docking predictions. Since biochemical analysis indicates that the three cysteines of ArsD and the three cysteines of ArsA must be in proximity for transfer (Lin et al. 2007), and also that Q56R ArsA interacts strongly with wild type ArsD, the single model most consistent with these parameters was selected (Fig. 4A). The docking analysis predicts 40 Q56R ArsA residues are within 4 Å of 39 residues of wild type ArsD (Table 4). Many of these ArsA residues are positively charged (e.g. Arg56, Arg86 and Arg556) or negatively charged (e.g., Glu75, Asp77, Glu515 and Glu552). These residues are close enough to form charge pairs with residues in ArsD. For example, ArsA residues Glu75 and Arg86 are close enough to ArsD residues Lys37 and Asp21, respectively, to form 3 salt bridges. Similarly, the *in silico* modeling of Q56R ArsA and 4 KA ArsD complex predicts 13-hydrogen bonds and 2-salt bridges between ArsA and ArsD. Our results are consistent with an arginine substitution at ArsA residue 56 altering the interaction with ArsD by changes in hydrogen bonding and salt bridges that propagate to the interface between the two proteins.

Discussion

The ArsD metallochaperone delivers As(III) to ArsA ATPase, the catalytic subunit of ArsAB pump. Interaction with ArsD increases the affinity of ArsA for As(III), conferring resistance to environmental concentrations of arsenic (Lin et al. 2006). Genetic, biochemical and crystallographic data indicate that ArsD residues Cys12, Cys13, and Cys18 are involved in the transfer of As(III) to ArsA (Lin et al. 2007; Yang et al. 2010; Ye et al. 2010). ArsA has two homologous halves, ArsA1 and ArsA2, each with a nucleotide binding domain, NBD1 (residue 1-296) and NBD2 (residue 305-583) connected by a short linker (Zhou et al. 2000). Two cysteine residues from NBD1 (Cys113 and Cys172) and a single cysteine

(Cys422) from NBD2, form a high-affinity metalloid binding site (Bhattacharjee and Rosen 1996; Ruan et al. 2008). ArsA undergoes a number of conformational transitions during the catalytic cycle, an open form in the absence of ATP and As(III), and a closed form when both are present (Ruan et al. 2008; Zhou et al. 2000; Zhou et al. 2001; Zhou and Rosen 1997). Biochemical analysis suggests that the three cysteines of ArsD must be in proximity to the three cysteines of ArsA for metalloid transfer (Lin et al. 2007). However, since metalloid transfer requires ArsA to be undergoing catalysis (Yang et al. 2010), it is likely that ArsD initially interacts with an open form of ArsA.

ArsD mutant K2A/K37A/K62A/K104A (4KA ArsD) does not interact with wild type ArsA in a two-hybrid assay (Yang et al. 2011). Three suppressor mutations in ArsA (Q56R, F120I, and D137V) were isolated that exhibits increased interaction with wild type ArsD than 4KA ArsD (Fig. 1). Each of these three residues in ArsA (Gln56, Phe120, and Asp137) is located in NBD1. Gln56 is situated in between the H3-S3 loop and exposed to solvent. Phe120 is in the middle of H6 helix and surrounded by hydrophobic residues. Asp137 is in the H7-S5 loop and exposed to solvent.

A combination of *in silico* modeling and docking was used earlier to examine the interaction of ArsD metallochaperone with ArsA ATPase (Ye et al. 2010). The model depicted ArsD to fit into the cavity of the open form of ArsA (Fig. 4B). This model predicted that ArsA residues His148, Arg151, Gly157, Ala158, Ser160, Ser161, Glu168, Ala170, Tyr394, Thr401, Lys402, Lys404, Glu405, Asp417, His453, Leu457, Leu458, Ala460, Thr461, and Arg516 are within 4 Å of the ArsD interface (Ye et al. 2010). However, our two-hybrid screening for suppressor mutants with increased ArsA-ArsD interaction did not pick up any of the ArsA residues indicated above, except Lys404 (Table 2). Interestingly, the single ArsA mutant K404R did not interact with either the wild type or 4KA ArsD (data not shown). This model also cannot explain the increased interaction of Q56R ArsA with ArsD. As shown in Fig. 4B, Gln56 or its replacement is exposed to solvent and distant from the ArsA-ArsD interface.

We now propose an alternate model for ArsA-ArsD interaction (Fig. 4A). While in the previous model, ArsD goes in a ‘frontal-attack’ mode with the open form of ArsA (Fig. 4B), in the new model, ArsD interacts such that its cysteine (Cys12, Cys13, and Cys 18) bearing loop is lowered into the active site of ArsA from its top (Fig 4A). In this model, wild type ArsA-ArsD complex shows 16-intermolecular hydrogen bonds and 4-salt bridges, with a G^{diss} of 11.1 kcal/mol. The Q56R ArsA-wild type ArsD complex exhibits 28-intermolecular hydrogen bonds and 3-salt bridges, with a G^{diss} of 16.0 kcal/mol. Thus, the alteration of Gln56 to arginine builds increased interaction with wild type ArsD, which supports the two-hybrid data. The Q56R ArsA-4 KA ArsD complex shows 13-intermolecular hydrogen bonds and 2-salt bridges, and a G^{diss} of 7.8 kcal/mol. The alteration of four lysine residues in ArsD attenuates the positive charge, which in turn rearranges the surface charge on the protein surface and affects its interaction with ArsA. However, if we determine the free energy of assembly dissociation using the earlier model (Ye et al. 2010) (Fig. 4B), the G^{diss} values for wild type ArsA and ArsD, Q56R ArsA-wild type ArsD, and Q56R ArsA-4 KA ArsD complex are 15.4, 15.4, and 14.9 kcal/mol, respectively, predicting no differences in interaction between the altered ArsA and ArsD.

Thus, the new *in-silico* model (Fig. 4A) supports our experimental data of Q56R ArsA strongly interacting with wild-type ArsD and weakly with 4 KA ArsD. The new model also accommodates most of the ArsD residues that were shown to interact with ArsA by two-hybrid analysis (Yang et al. 2011). A side-by-side analysis comparing the two hybrid data (Table 2) and *in silico* docking predictions (Table 4) suggests that ArsA residues Gly51, Ser55, Arg88, Val90, Val101 and Ser560 might play a role in protein-protein interaction. However, the effect of any of this single-residue alteration on ArsA-ArsD interaction was not examined.

It should be noted that the new model (Fig. 4A) predicts that the sulfur atoms of ArsA residues Cys113 and Cys172 are approximately 12 Å distant from Cys12 and Cys13 of ArsD. It is reasonable to assume that the complex undergoes multiple conformational changes that bring those residues in proximity to each other during other steps in the catalytic cycle.

Neither model explains the increased interaction between F120I or D137V ArsA with the wild type or 4 KA ArsD. Both proteins show a basal ATPase activity that is not stimulated by As(III). Both proteins bound Sb(III), albeit at a slightly lower stoichiometry than either the wild type or Q56R ArsA. This suggests that neither the nucleotide nor metalloid binding site of F120I and D137V ArsA are affected by the mutation. However, both showed a higher basal rate that is similar as the activated rate for Q56R ArsA. We interpret this observation as the conformation of F120I and D137V are permanently locked in the metalloid bound form of the protein. Why do these mutants interact strongly with ArsD? An Asp137 to Val substitution is a major alteration of charge on the protein surface and probably creates a 'sticky' surface. We predict that a Phe120 to Ile change affects the surface hydrophobicity of the protein that result in increased hydrophobic interactions with either wild type or 4KA ArsD, forming abortive complexes. Identification of suppressor mutants that exhibit stronger interaction with ArsD provides an opportunity to perform co-crystallization experiments that would lead to characterization of the interface at the atomic level.

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FIG. 1. Yeast two-hybrid analysis of ArsA mutants exhibiting increased interaction with ArsD
S. cerevisiae strain AH109 bearing pAD-A (wild-type ArsA) or pAD-AX (single ArsA mutants Q56R, F120I or D137V) and either pBD-ArsD or pBD-4KArsD plasmids was grown in SD medium overnight and inoculated with 10-fold serial dilutions on SD agar plates lacking leucine, tryptophan or histidine and supplemented with the indicated concentrations of 3-AT. The plates were incubated at 30 °C for 3 days and scored for growth.

FIG. 2. Activation of ArsA ATPase activity by ArsD

ArsA ATPase activity was assayed at the indicated concentrations of sodium arsenite in the presence or absence of either wild type or 4 KA ArsD, as described under “Materials and Methods.” The basal ATPase activity was subtracted from the total activity. The curves were fitted using SigmaPlot 12.0, with the error bars representing the standard error. *Left panel*, Wild-type ArsA; *Right panel*, Q56R ArsA. (●), no ArsD; (○), wild type ArsD; (▼), 4 KA ArsD.

FIG. 3. ArsA mutants bind antimonite

The antimony binding ability of wild-type and altered ArsA was assayed as described under “Materials and Methods.” After incubation of 10 μ M ArsA and 100 μ M potassium antimonite at 4 °C in the presence of ATP γ S and Mg²⁺, free Sb(III) was removed by gel filtration, and the Sb(III):ArsA molar ratio determined. BSA was used as a negative control. The error bars represent the standard deviation from three assays.

FIG. 4. *In silico* docking analysis of ArsA-ArsD complex

(A) Cartoon diagram of the interaction between Q56R ArsA and wild type ArsD. ArsD attaches to the back of ArsA with the cysteine rich loop (Cys12, Cys13, and Cys18) of ArsD (cyan) hanging down the metalloid binding domain of the open form of ArsA (ArsA1 in orange and ArsA2 in split pea). R56, F120 and D137 in ArsA are represented by magenta ball and sticks. The active site thiolates in both ArsA and ArsD are represented by yellow ball and sticks. (B) Cartoon diagram of the interaction of Q56R ArsA and wild type ArsD based on an earlier model (Ye et al. 2010). ArsD (cyan) is proposed to fit into the cavity of the open form of ArsA (ArsA1 in orange and ArsA2 in split pea).

Table 1**Strains and plasmids**

Strains/Plasmids	Genotype/description	Reference
<u><i>E. coli</i> strains</u>		
JM109	e14 ⁻ (McrA ⁻) <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1</i> (<i>lac-proAB</i>) [F' <i>traD36 proAB lacI^q Z M15</i>]	(Green et al. 2012)
BL21(DE3)	<i>fhuA2 [lon] ompT gal (λ DE3) [dcm] hsdS λ DE3 = λ sBamHI</i> <i>EcoRI-B int::</i> (<i>lacI::PlacUV5::T7 gene1</i>) <i>i21 nin5</i>	(Green et al. 2012)
<u><i>S. cerevisiae</i> strains</u>		
AH109	<i>MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4⁻, gal80⁻, LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ</i>	Clontech
<u>Plasmids</u>		
pACT2	GAL4 ₍₇₆₈₋₈₈₁₎ activation domain, <i>LEU2</i> (Ap ^r)	Clontech
pGBT9	GAL4 ₍₁₋₁₄₇₎ DNA-binding domain, <i>TRP1</i> (Ap ^r)	Clontech
pAD-A	<i>arsA</i> gene cloned in pACT2 (Ap ^r)	(Lin et al. 2006)
pBD-k	Kanamycin resistance gene PCR amplified from pET28a and inserted into pGBT9 at AatII site (Kn ^r)	(Yang et al. 2011)
pBD-k-D	Kanamycin resistance gene PCR amplified from pET28a and inserted into pBD-D at AatII site (Kn ^r)	(Yang et al. 2011)
pBD-k-4KA	DNA fragment encoding ArsD mutation K2A/K37A/K62A/K104A inserted into pBD-k (Kn ^r) at the EcoRI and BamHI sites.	(Yang et al. 2011)
pAD-AX	Single mutations (X) Q56R, V90F, T115K, F120I, V190I, K404R, and E506G separately introduced by site-directed mutagenesis into pAD-A	This study
pETy-dAhB	<i>arsA</i> cloned into pET28a with six histidine tags at the C-terminus	(Lin et al. 2006)
pETy-dAxB	Single mutations (X) Q56R, F120I, D137V separately introduced by site directed mutagenesis into pETy-dAhB	This study
pET28a	Expression vector for his-tagged proteins controlled by T7 promoter (Kn ^r)	Novagen
pET28a-ArsD109	<i>arsD</i> ₁₋₁₀₉ truncation inserted into pET28a at EcoRI and SalI sites (Kn ^r)	(Yang et al. 2011)
pET28a-ArsD109 _{K2A/K37A/K62A/K104A}	Mutations K2A/K37A/K62A/K104A constructed by site directed mutagenesis into pET28a-ArsD109 (Kn ^r)	(Yang et al. 2011)

Table 2

ArsA mutants exhibiting interaction with 4KA ArsD

ArsA mutations	Times isolated
E34K/G51C/S55T/R88I/V90F/V101L	1
E34K/G51C/R88I/F120L/K404R	1
Q56R/T115K/Q182L/S210T/N229D/A345G/T497M	1
Q56R/T115K/Q182L/S210T/N229D/T497M	1
L71S/V190I	1
V96A/D121E	3
F120L/E168G/M354V/N374I/S376I	1
F120L/L201P/V435A/S560R	1
F120I/K404R	1
D137V	5
F162I/Q307R	2
E168G/M354V/N374I/K402M	1
Q182R/E258V/T451S/E506G	1
Y188N/Y305C/E415G/D447G	1
Y188N/E415G/D447G	1

Table 3

ArsA ATPase kinetics in the presence or absence of either wild type or 4KA ArsD

ArsA	ArsD ^I	<u>V_{max} (nmol of ATP hydrolyzed/min/mg of protein)</u>		As(III) [C] _{50% activation} (μM)
		-As(III)	+As(III)	
Wild type	none	25 ± 5	137 ± 10	382 ± 48
	Wild type	35 ± 2	117 ± 6	45 ± 7
	4KA ArsD	35 ± 2	117 ± 10	231 ± 40
Q56R	none	32 ± 2	83 ± 4	205 ± 20
	Wild type	40 ± 2	87 ± 4	23 ± 4
	4KA ArsD	40 ± 2	75 ± 5	109 ± 16
F120I	none	75 ± 2	75 ± 2	-
	Wild type	80 ± 2	80 ± 2	-
	4KA ArsD	82 ± 3	82 ± 3	-
D137V	none	55 ± 2	55 ± 2	-
	Wild type	57 ± 2	57 ± 2	-
	4KA ArsD	59 ± 2	59 ± 2	-

^I ArsD was added in a 10-fold molar excess over ArsA

Table 4Q56R ArsA and wild-type ArsD residues predicted from *in-silico* docking to be within 4 Å of each other

Q56R ArsA residues	Wild-type ArsD residues
Pro46	Arg45, Gln51
Ala47	Phe46
Ser48	Glu44
Gly51	Gln42
Gln52	Met5
Ser55	Gln42
Arg56	Gln38, Cys39, Gly40, Gln42
Thr57	Gly40, Val41, Gln42, Glu44
Ile58	Lys37, Gln38
Gly59	Gln34
Asn60	Gln34, Gln38
Thr61	Gln38
Gln63	Gln38
Glu75	Lys37, Glu44
Asp77	Gln34
Gln79	Arg45, Asn47, Gln51
Ala80	Gln34
Gln83	Ser30, Arg45
Gln84	Ser30, Thr31
Arg86	Asp21, Val22
Ser103	Asp21
Asn106	Asp21
Glu107	Thr20, Asp21, Val22
Asn281	Gln57
Met282	Gln57
Thr501	Pro52, Met53
Leu505	Cys13
Ala508	Ser14, Thr15
Asn509	Cys13, Ser14
Gln511	Thr15
Ala512	Thr15, Gly16
Glu515	Gly16
Gln545	Glu59, Lys62
Gln546	Val56, Gln57
Pro549	Phe55, Val56, Lys62
Gln550	Pro52, Val56
Glu552	Ile65, Glu66
Arg556	Ile65, Glu66, Ala67, Ser68, Gly69, Ala70, Glu71
Gln557	Pro9, Ala10, Ala70, Glu71

Q56R ArsA residues	Wild-type ArsD residues
His558	Thr15