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ArsD: an As(III) metallochaperone for the ArsAB As(III)-translocating ATPase

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Abstract The toxic metalloid arsenic is widely disseminated in the environment and causes a variety of health and environment problems. As an adaptation to arsenic-contaminated environments, organisms have developed resistance systems. Many *ars* operons contain only three genes, *arsRBC*. Five gene *ars* operons have two additional genes, *arsD* and *arsA*, and these two genes are usually adjacent to each other. ArsA from *Escherichia coli* plasmid R773 is an ATPase that is the catalytic subunit of the ArsAB As(III) extrusion pump. ArsD was recently identified as an arsenic chaperone to the ArsAB pump, transferring the trivalent metalloids As(III) and Sb(III) to the ArsA subunit of the pump. This increases the affinity of ArsA for As(III), resulting in increased rates if extrusion and resistance to environmentally relevant concentrations of arsenite. ArsD is a homodimer with three vicinal cysteine pairs, Cys12–Cys13, Cys112–Cys113 and Cys119–Cys120, in each subunit. Each vicinal pair binds one As(III) or Sb(III). ArsD mutants with alanines substituting for Cys112, Cys113, Cys119 or Cys120, individually or in pairs or truncations lacking the vicinal pairs, retained ability to interact with ArsA, to activate its ATPase activity. Cells expressing these mutants retained ArsD-enhanced As(III) efflux and resistance. In contrast, mutants with substitutions of conserved Cys12, Cys13 or Cys18, individually or in pairs, were unable to activate ArsA or to enhance the activity of the ArsAB pump. It is proposed that ArsD residues Cys12, Cys13 and Cys18, but not Cys112, Cys113, Cys119 or Cys120, are required for delivery of As(III) to and activation of the ArsAB pump.

Keywords Arsenic · Arsenite · Antimony · Resistance pump · ArsD · ArsA

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Introduction

Arsenic is one of the most common toxins found in the environment, introduced from both natural and anthropogenic sources. Because of its ubiquity, arsenic ranks first on the Superfund List (http://www.atsdr.cdc.gov/cercla/05list.html). Exposure to arsenic is a causative agent of cardiovascular and peripheral vascular disease, neurological disorders, diabetes mellitus and various forms of cancer (Abernathy et al. 2003; Beane Freeman et al. 2004). As a consequence of its pervasiveness, nearly every organism, from *E. coli* to humans, has mechanisms for arsenic detoxification (Bhattacharjee and Rosen 2007). In bacteria, the genes for arsenic detoxification are usually encoded by arsenic resistance (*ars*) operons. Many *ars* operons have only three genes, *arsRBC*, where ArsR is an As(III)-responsive transcriptional repressor (Xu and Rosen 1999), ArsB is a $As(OH)_3/H^+$ antiporter that extrudes As(III), conferring resistance (Meng et al. 2004), and ArsC is an arsenate reductase that converts As(V) to As(III), the substrate of ArsB, hence extending the range of resistance to include As(V) (Mukhopadhyay and Rosen 2002). Some *ars* operons have two additional genes, a*rsD* and *arsA*, such as the *arsRDABC* operon in *E. coli* plasmid R773, and cells expressing the *arsRDABC* operon are more resistant to As

(V) and As(III) than those expressing *arsRBC* operons because ArsA forms a complex with ArsB that catalyzes ATP-driven As(III)/Sb(III) efflux.

The 583-amino acid ArsA has two homologous halves, A1 and A2, connected by a short linker. Each half has a consensus nucleotide-binding domain (NBD) at the A1-A2 interface (Zhou et al. 2000). A metalloid binding domain (MBD) that binds three As(III) or Sb(III) is located about 20 Å from the NBDs (Zhou et al. 2000, 2001; Fig. 1). Each metalloid is bound by two ArsA residues. The highest affinity site is composed of Cys-113 and Cys-422 (Ruan et al. 2006), and a third cysteine, Cys-172, can participate in high affinity binding (X. Ruan, H. Battacharjee and B. P. Rosen, unpublished). Binding of As(III) or Sb(III) brings the two halves of ArsA together, activating ATP hydrolysis. ArsA has two signature sequences that serve as signal transduction domains (STDs), D^{142}TAPTGH^{148}TIRLL in A1 (STD1) and D^{447}TAPTGH^{453}TLLLL in A2 (STD2), that 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 (Zhou and Rosen 1997). Asp142 and Asp447 are Mg^{2+} ligands in NBD1 and NBD2, respectively, and His148 and His453 are Sb(III) ligands in the MBD (Zhou et al. 2000).

ArsD is a homodimer of two 120-residue subunits (Chen and Rosen 1997). Three cysteine residues, Cys12, Cys13 and Cys18, are conserved in all ArsD homologues. While ArsD exhibits weak repressor activity (Chen and Rosen 1997; Wu and Rosen 1993), its primary function has recently been shown to be as an arsenic metallochaperone that delivers As(III) to the ArsA ATPase (Lin et al. 2006). Interaction with ArsD increases the affinity of ArsA for As (III), producing increased efflux and resistance at environmental concentrations of arsenic.

While no arsenic chaperones had been identified prior to our studies, chaperones had been identified for transition metal ions such as copper and iron (Field et al. 2002). Metallochaperone-mediated ion transfer is best illustrated by the copper chaperones (Rosenzweig 2002). For example, Atx1, a yeast copper chaperone, delivers copper to the transport ATPase Ccc2p in the trans-Golgi network (Lin et al. 1997). The human homolog, Hah1, is a copper chaperone for the copper pumps ATP7A and ATP7B, and mutations in these pumps produce Menkes and Wilson Diseases (Walker et al. 2002). These chaperones bind metal using cysteine thiolates as ligands, and transfer the metals to thiols in their partner proteins by an exchange of ligands. This review, will focus on the interactions of ArsD and ArsA and on the transfer of As(III) from the three conserved cysteines of ArsD to the three cysteines of the metalloid binding domain of ArsA.

Genomics of arsenic detoxification

Nearly every sequenced bacterial genome contains an *ars* operon. Although the majority is three-gene *arsRBC*

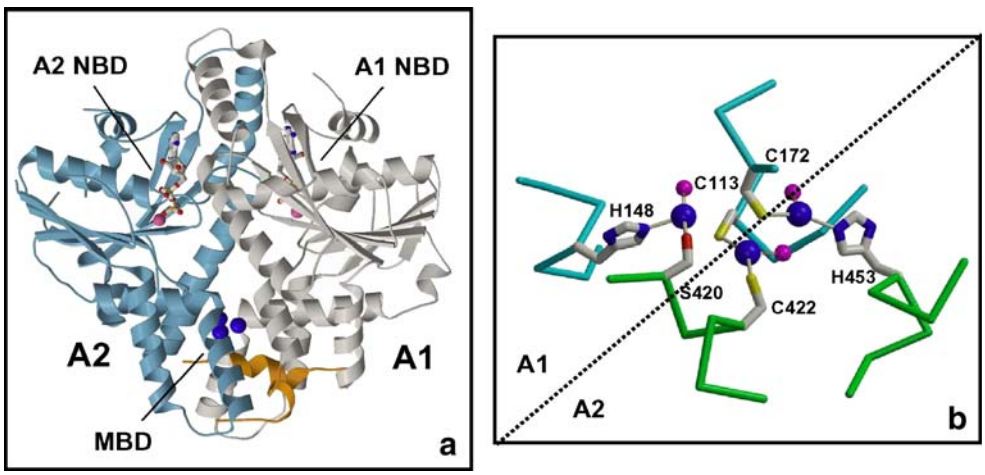

Fig. 1 Structure of R773 ArsA ATPase. **a** The overall structure of ArsA is shown as a *ribbon diagram*. Mg^{2+} ADP is bound to each of the two NBDs in the A1 and A2 halves of ArsA, while three Sb(III) is bound at the single MBD. **b** The metalloid binding domain composed of three three-coordinate As(III)/Sb(III) binding sites. Each site has two protein ligands, one from A1 and one from A2, plus a non-protein ligand that appears to be a chloride in the crystal structure but could be a hydroxyl in the native protein. The high affinity site is composed of Cys-113 from A1 and Cys-422 from A2. The other two sites are composed of His-148 from A1 and Ser-420 from A2 and Cys-172 from A1 and His-453 from A2. The two histidines come from the signal transduction domains ($D_{142/447}$TAPTGH$_{148/453}$) that connect the two nucleotide binding domains, NBD1 and NBD2, to the MBD. Binding of metalloid acts like molecular glue to hold A1 and A2 in a conformation in which residues from both contribute to the formation of NBD1 and NBD2. Thus metalloid binding increases the affinity of NBD1 and NBD2 and activates catalysis

operons, to date, there are more than fifty bacterial and archaeal arsenic resistance operons and gene clusters that contain *arsA* and *arsD* genes. It is striking that *arsA* and *arsD* genes are always found together in *ars* operons. The order of the genes in those operons may differ from each other, but the *arsD* gene nearly always precedes an *arsA* gene. The linkage of these two genes suggests first, that ArsD and ArsA co-evolved before their association with ArsB, second, that the *arsDA* genes moved laterally into an *ars* operon as a unit, and third, that ArsD has a biochemical function related to ArsA in arsenic detoxification.

Genetic evidence for physical interaction between ArsD and ArsA

Yeast two-hybrid analysis demonstrates that ArsD and ArsA physically and specifically interact (Lin et al. 2006). Among the four soluble proteins encoded by the R773 *arsRDABC* operon, ArsA was found to interact with ArsD but not with the ArsR repressor or the ArsC arsenate reductase; ArsD interacted with ArsA and with itself, which would be expected since ArsD is a homodimer (Chen and Rosen 1997), but not with ArsR or ArsC; ArsR, which is a homodimer, also interacts with itself, but not with the other proteins. Since there is no arsenic or antimony in yeast cells, ArsD and ArsA must interact in the absence of metalloid. However, when Cys12, Cys13 or Cys18, the residues in ArsD that form the metalloid binding site (described in more detail below), were mutated to alanines or serines, little interaction was detected (Lin et al. 2007). Since these cysteines are required for metalloid binding, it seems unlikely that there are disulfide bonds formed between the cysteines in the two proteins. The serine-substituted ArsD mutants neither activate ArsA ATPase activity nor bound Sb(III) in this mutated site (Y. F. Lin and B. P. Rosen, unpublished), consistent with the idea that it is the metalloid-bound form of ArsD that activates ArsA. These data suggest that ArsD interacts with ArsA with high affinity when metalloid is bound but with low affinity in the absence of metalloid such as in the yeast cytosol in the two-hybrid assays.

Direct physical interaction between ArsD and ArsA was observed by chemical crosslinking with dibromobimane (dBBr), a fluorogenic, homobifunctional thiol-specific crosslinking reagent that becomes highly fluorescent when its two alkylating groups react with cysteine residues within 3 to 6 Å of each other (Kosower et al. 1980). When the mixture of ArsD and ArsA was reacted with dBBr, a crosslinked species was detected that reacted with both anti-ArsA and anti-ArsD antibodies and migrated as a band with an apparent mass of approximately 90 kDa, the predicted mass of an ArsD dimer crosslinked to a monomer of ArsA. The amount of the ArsD-ArsA crosslinked product was increased by addition of MgATP. These results suggest first that ArsD and ArsA interact at their cysteine-rich metalloid binding sites and second that ArsD interacts with nucleotide-bound form of ArsA.

Cell biology of ArsD function

That *arsD* and *arsA* genes are nearly always found together in *ars* operons implies co-evolution for a common function, most likely arsenic detoxification. Cells co-expressing *arsDAB* were more resistant to high concentrations of As (III) compared to cells expressing only *arsAB*, consistent with interaction of ArsD with ArsA increasing the efficiency of the ArsAB pump (Lin et al. 2006). The concentration of arsenic in such growth experiments is usually in the millimolar range, which might be found in a volcanic area such as Yellowstone National Park, but such concentrations are much greater than would be found under most environmental conditions. To examine whether the presence of a functional *arsD* gene conferred an growth advantage to cells at lower levels of arsenic, two batches of cells of an *E. coli* strain in which the chromosomal *ars* operon had been deleted were mixed together and grown at equal amount in the presence of 10 μM sodium arsenite, a concentration present in many deep tube wells in Bangladesh. One batch of cells had a plasmid with only the *arsAB* genes, and the other had the *arsDAB* genes. After a little more than a week, the cells with *arsDAB* had largely replaced those with only *arsAB*, demonstrating that ArsD provides a competitive advantage for growth in soil or water with moderate amounts of arsenic contamination.

Examination of As(III) accumulation in intact cells revealed that ArsD enhances the ability of the pump to extrude As(III) and reduces the intracellular arsenic concentration to subtoxic levels. Cells expressing only *arsB* were able to lower the intracellular level of As(III) compared to a strain with no *ars*, reflecting the ability of ArsB to catalyze $As(OH)_3/H^+$ exchange (Meng et al. 2004). When *arsA* and *arsB* were co-expressed, the cells have more efficient As(III) extrusion than with *arsB* alone (Dey and Rosen 1995). When *arsD* was co-expressed with *arsAB*, the cells exhibited a further reduction in As(III) accumulation (Lin et al. 2006). These lines of evidence support the idea that ArsD increases the efficiency of the ArsAB pump. The competitive advantage provided by direct interaction of ArsD with ArsA provides a driving force for the co-evolution of the two genes.

Biochemistry of ArsD function

The hallmark of metallochaperones is delivery of metal ions directly to target proteins (Rosenzweig 2002). The ability of ArsD to transfer Sb(III) to ArsA was shown by mixing Sb (III)-loaded ArsD with ArsA, separating the two proteins and determining how much Sb(III) was bound to ArsA. In the presence (but not the absence) of magnesium and a nucleotide, ArsA was able to abstract Sb(III) from ArsD. The effect of nucleotides on Sb(III) transfer was MgATP > MgATPγS > MgADP, indicating that nucleotide enhances transfer, but hydrolysis is not required.

Metalloid transfer studies of the transfer of As(III) or Sb(III) from ArsD to ArsA are consistent with a mechanism in which this metallochaperone accelerates the rate of transfer to its partner. However, for ArsD and ArsA, this transfer process is thermodynamically unfavorable because ArsD has higher affinity for metalloid than does ArsA. However, if the metalloid bound to the ArsAB pump is pumped out of the cell, mass action will provide the directionality of the reaction. This suggests that ArsD might not only serve a role in protecting the cell from free metalloid but also in loading ArsA for metalloid extrusion. Indeed, because ArsD has higher affinity for metalloid than ArsA, it can 'scavenge' the cytosol for free metalloid for delivery to ArsA, allowing the ArsAB pump to confer resistance at significantly lower concentrations of As(III). The K_m of ArsB as a secondary carrier is 0.14 mM (Kuroda et al. 1997), but natural waters range in concentration of total inorganic arsenic from 7 nM to 70 μM, and concentrations of arsenic in drinking water in worst arsenic-contaminated wells in West Bengal and Bangladesh are approximately 40 μM (Smedley and Kinniburgh 2002). By lowering the concentration of substrate at which the pump functions efficiently, ArsD and ArsA provide cells with a mechanism to respond to environmental concentrations of metalloid.

ArsD not only delivers metalloids to ArsA but also enhances its ATPase activity at low metalloid concentrations. In the presence of ArsD, the apparent affinity of ArsA for As(III) is largely increased but the V_{max} remains unchanged in ATPase reactions (Lin et al. 2006). ArsD makes the enzyme more effective at low concentrations of metalloid, a property expected for a metallochaperone. As (III) or Sb(III) activates the ATPase activity of ArsA, so that the increase in affinity of ArsA for metalloids in the presence of ArsD can simply be attributed to the higher affinity of ArsD in the ArsD-ArsA complex, that is, ArsD allows for activation of ArsA ATPase at lower metalloid concentrations. This explains how cells expressing *arsDAB* can rapidly replace cells expressing only *arsAB* at a subtoxic concentration of As(III).

In summary, the increase in ArsA efficiency resulting from interaction with ArsD leads in vitro to augmented activity of the ArsAB extrusion pump, to greater resistance in organisms with the *arsDAB* genes, and finally to increased fitness for growth in the low but ubiquitous levels of environmental arsenic.

Metalloid binding sites in ArsD

R773 ArsD has three vicinal cysteine pairs, Cys12–Cys13, Cys112–Cys113 and Cys119–Cys120. Alignment of the primary sequence of homologues of R733 ArsD indicates that the cysteine pair Cys12–Cys13 and a single additional cysteine, Cys18 are conserved in homologues. Two other cysteine pairs, Cys112–Cys113 and Cys119–Cys120, are found in some homologues but not others. A series of cysteine mutants and truncations of ArsD were constructed, purified and studied for the metalloid binding (Lin et al. 2007). Mutants or truncations with only a single vicinal cysteine pair can still bind to a phenylarsine oxide affinity column, which indicates indirectly that each cysteine pair can form an independent metalloid binding site (Sun et al. 2001). In more recent experiments, binding of Sb(III) was measured directly (Lin et al. 2007). Wild-type R773 ArsD binds three Sb(III) per monomer. If two of the three cysteine pairs were changed to alanine or serine residues, or if the C-terminal vicinal pairs were removed by construction of truncated versions, variants with only a single vicinal pair were found to bind one Sb(III) per monomer, showing directly that each cysteine pair forms an independent metalloid binding site. If the conserved cysteine Cys18 was mutated, the mutant with the only first cysteine pair, Cys12–Cys13, was unable to bind Sb (III). This suggests that the cysteine pair Cys12–Cys13 and Cys18 form a three-coordinate thiolate metalloid binding site, which has been termed MBS1. The nature of this site has been confirmed by extended X-ray absorption fine structure spectroscopy (EXAFS; J. Yang, T. Stemmler and B. P. Rosen, unpublished). The metalloid binding sites formed by cysteine pairs Cys112–Cys113 and Cys119–Cys120 are designated MBS2 and MBS3, respectively. All three binding sites have similar affinity to Sb(III), approximately 1 μM.

A truncated ArsD lacking both MBS2 and MBS3 ($ArsD_{1–109}$), still interacted with ArsA in the yeast-two-hybrid assay and had the same stimulatory effect on ArsA ATPase activity as wild-type ArsD (Lin et al. 2007). In contrast, an ArsD derivative lacking MBS1 but retaining MBS2 and MBS3 ($ArsD_{1–118,C12A/C13A}$) was unable to interact with ArsA in yeast-two-hybrid assay and did not stimulate ArsA activity. These results suggest that MBS1, but not MBS2 or MBS3, participate in metalloid transfer

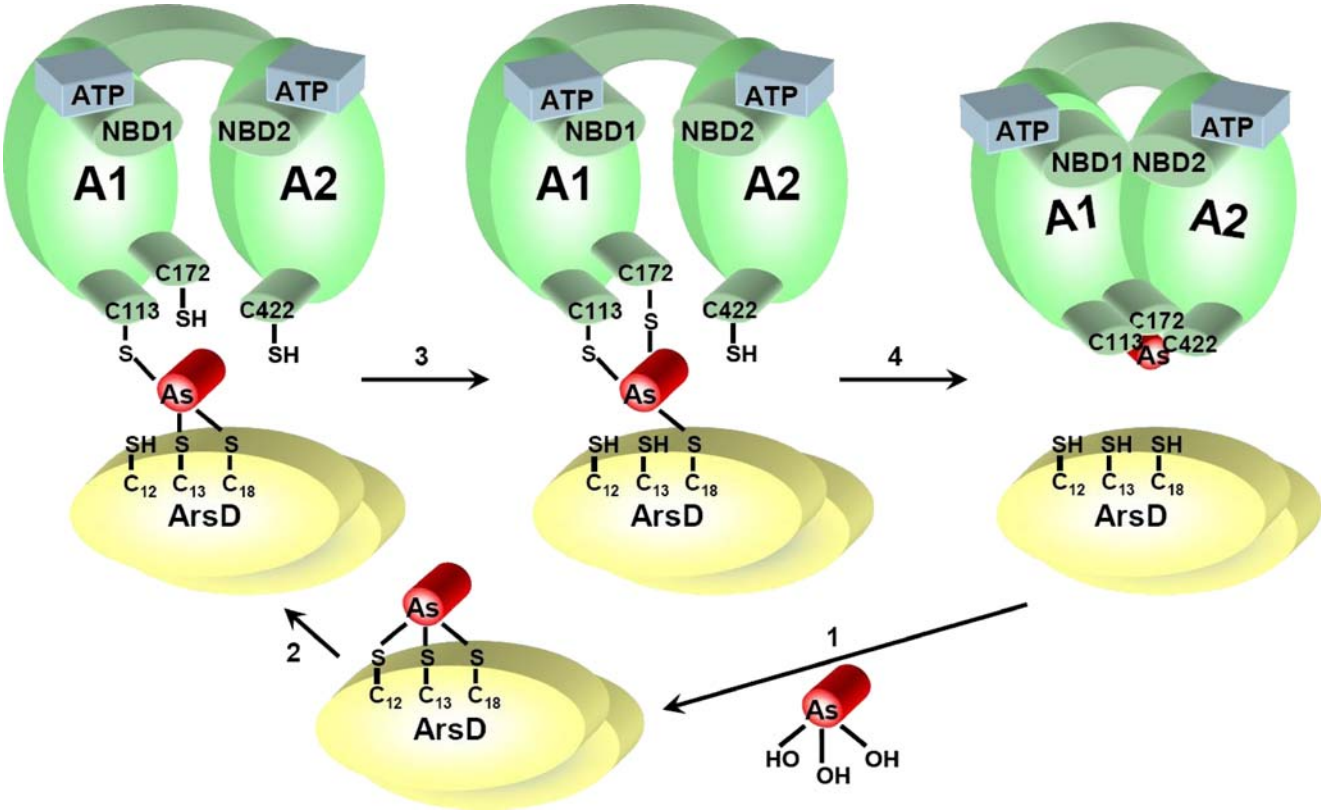

Fig. 2 Hypothetical reaction scheme for transfer of As(III) from ArsD to ArsA. *Step 1* ArsD binds As(III) by exchange of the three hydroxyls of $As(OH)_3$ for the thiols of MBS1 residues Cys12, Cys13 and Cys18. Although ArsD is a dimer, transfer from only one subunit is shown for simplicity. *Step 2* 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 order of ligand exchange in the transfer reaction is not known

to ArsA and in activation of ArsA ATPase activity. Cells expressing a mutated $arsD_{1–118,C112A/C113A}$ gene, which encodes an ArsD derivative having only MBS1, in trans with *arsAB* accumulated As(III) to nearly the same extent as those expressing wild type *arsDAB*. These cells also have a modest reduction in arsenite resistance compared to cells expressing wild type *arsDAB*. However, the cells expressing other ArsD mutants lacking MBS1 ($arsD_{C12/C13A}$ or $arsD_{C18A}$) accumulate slightly more As(III) than cells with only *arsAB* and were no more resistant to As (III) than cells with only *arsAB*. These in vivo results strongly support the hypothesis that MBS1 (Cys12, Cys13 and Cys18), but not MBS2 or MBS3, is required for metallochaperone activity.

The fact that the MBSs in ArsA and MBS1 in ArsD appear to be involved in interaction suggests that metalloid binding sites in both proteins are brought into close proximity, allowing transfer of metalloid directly from the binding site on ArsD to the binding site on ArsA. It is plausible that this interaction destabilizes the metalloid binding sites on ArsD, reducing its affinity, while stabilizing the metalloid binding sites on ArsA by occluding the metalloid within the complex, thus enabling transfer of metalloid to ArsA. In this manner, the thermodynamically unfavorable process of transferring the metalloid from a high to a low-affinity site could be overcome, although, as mentioned above, this might not be a concern if the transferred metalloid is rapidly extruded from the cell. While the details of metalloid transfer from ArsD to ArsA are unknown at this time, the mechanism of transfer of copper from the metallochaperone CCS to the superoxide dismutase SOD1 is instructive (Lamb et al. 2001; Torres et al. 2001). The cysteine residues of CCS project into the active site of SOD1. By doing so, Cu(I) bridges the donating cysteine residues of CCS and the receiving histidine residues, stabilizing the heterodimer and facilitating metal transfer. In effect, transfer of copper between the Atx1 and Ccc2p is ‘catalysed’ by the metallochaperone. We propose a similar mechanism of As(III) transfer from ArsD to ArsA in which there is a step-wise exchange of sulfur ligands from ArsD cysteines to ArsA cysteines (Fig. 2).

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