Journal of Bioenergetics and Biomembranes, Vol. 27, No. 1, 1995

Mechanisms of Metalloregulation of an Anion-Translocating ATPase

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Received December 1, 1994; accepted December 12, 1994

The *ars* (arsenical resistance) operon cloned from R-factor R773 has five genes that encode two repressor proteins, ArsR and ArsD, and three structural proteins, ArsA, ArsB, and ArsC. The ArsA and ArsB proteins form a membrane-bound pump that functions as an oxyanion-translocating ATPase. The substrates of the pump are the oxyanions arsenite or antimonite. The ArsC protein is an arsenate reductase that reduces arsenate to arsenite, which is subsequently pumped out of the cell. This review deals with the mechanism of transcriptional regulation by the ArsR repressor and allosteric regulation of the ArsA protein, the catalytic subunit of the pump. The chemical nature of the inducer plays an important role in regulation. In solution arsenite or antimonite exist as oxyanions and reacts with the cysteines in proteins. In both transcriptional regulation by the ArsR repressor and allosteric regulation of the ArsA ATPase, the ability of As(III) and Sb(III) to interact with the cysteines of the proteins, involves their action as effector.

KEY WORDS: Arsenic; antimony; regulation, ion pump; ion-translocating ATPase.

INTRODUCTION

In bacteria the expression of some genes that encode ion pump proteins is regulated by the substrate ions or environmental stress. For example, the P-type ATPases for ions of magnesium and cadmium are transcriptionally regulated by their substrates, while the Kdp ATPase for potassium uptake is regulated by the osmolarity of the medium (Corbisier, *et al.*, 1993; Maguire, 1992; Siebers and Altendorf, 1993). In contrast, expression of genes for some eukaryotic ATPases appears to be regulated developmentally or hormonally, and others are expressed constitutively, but regulation by substrates does not appear to be a major factor (Carafoli, 1994). However, the rate of hydrolysis of ATP by most ion-translocating ATPases is low in the absence of the transported ion, suggesting that regulation at the level of enzymatic activity may occur. The oxyanion-translocating ATPase responsible for bacterial resistance to arsenicals and antimonials is regulated at both the transcriptional and enzymatic levels. A discussion of those mechanisms forms the basis of this review.

Paul Ehrlich developed the first antimicrobial agent, Salvarsan, an arsenical drug, for treatment of infectious diseases such as syphilis (Ehrlich, 1960). Arsenical and antimonial drugs are still used clinically for the treatment of tropical diseases (Berman, 1988). Perhaps because of their past or present use, arsenical resistance is frequently found on clinically isolated resistance factors (Kaur and Rosen, 1992). A conjugative R-factor, R773, isolated from an arsenic-resistant strain of *Escherichia coli* from a patient with a urinary track infection (Elek and Higney, 1970), was shown to confer arsenical resistance (Hedges and Baumberg, 1973). R773 arsenical resistance was shown to be correlated with reduced accumulation of $^{74}AsO_4^{-3}$ in bacterial cells (Silver *et al.*, 1981). Resistance and efflux were both inducible by arsenite or antimonite (Silver *et al.*, 1981). Induced cells loaded

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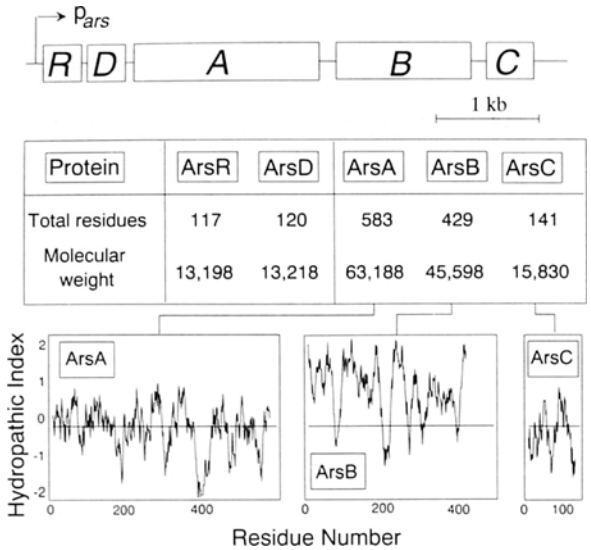

Fig. 1. Physical map of the *ars* operon. Top: the five genes of the operon are shown with the direction of transcription indicated by the arrow, starting with the promoter, p_{ars}. The genes are indicated by boxes proportional to their length in kilobase pairs (kb) of DNA. Middle: the five gene products are listed with the number of amino acid residues and molecular masses in daltons (Da). Bottom: the hydropathic profiles of the ArsA, ArsB, and ArsC proteins are shown.

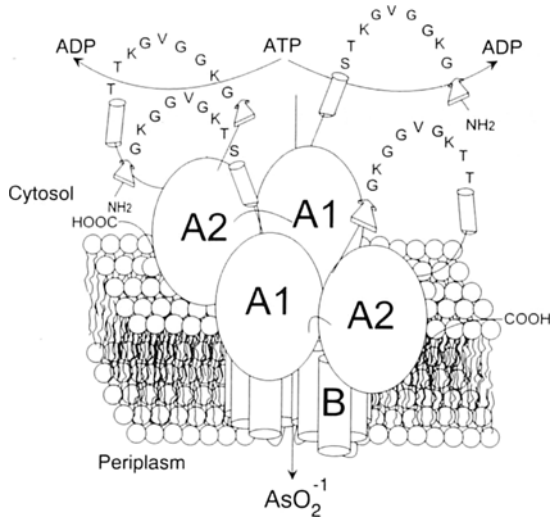

Fig. 2. The oxyanion pump. The complex of the ArsA and ArsB proteins forms an oxyanion-translocating ATPase that catalyzes extrusion oxyanions of arsenic and antimony in the III oxidation state from the cytosol (top) to the periplasm (bottom). The complex is shown with a dimer of the ArsA subunit, its active form in solution, and a single ArsB subunit, but the stoichiometry of the ArsA and ArsB proteins in the complex has not been determined. The ArsA protein, the catalytic subunit, exhibits Sb(III)- or As(III)-stimulated ATPase activity. The ArsB protein is an inner membrane protein in *E. coli* and serves both as the membrane anchor for the ArsA protein and as the anion-conducting subunit of the pump.

with $^{74}AsO_4^{-3}$ extruded the radioactivity in an energy-dependent manner (Mobley and Rosen, 1982; Rosen and Borbolla, 1984; Silver and Keach, 1982). The transported compound was originally thought to be arsenate, but a more recent examination of this question has demonstrated that the cells must reduce arsenate to arsenite prior to transport (Ji and Silver, 1992). *In vivo* extrusion in *E. coli* is coupled to chemical energy and not electrochemical, indicative of a primary arsenite pump. Membrane vesicles made from resistant cells exhibit ATP-driven accumulation of radioactive arsenite, while a membrane potential is neither necessary nor sufficient for uptake (Dey and Rosen, 1994). These *in vitro* experiments demonstrate that arsenite transport is catalyzed by an oxyanion-translocation ATPase.

From the nucleotide sequence and genetics of the *ars* (arsenical resistance) operon cloned from R-factor R773, five genes were identified (Mobley *et al.*, 1983; Chen *et al.*, 1986). The five genes encode two repressor proteins, ArsR and ArsD, and three proteins that produce resistance, the ArsA, ArsB, and ArsC proteins (Fig. 1). The ArsC protein is an arsenate reductase that reduces arsenate to arsenite, thus expanding the range of resistance from As(III)-containing compounds to As(V)-containing compounds. The ArsA and ArsB proteins form a membrane-bound pump that functions as an arsenite-translocating ATPase (Fig. 2). This review will focus on the mechanism of transcriptional regulation by the ArsR repressor and allosteric regulation of the catalytic subunit of the pump, the ArsA protein. The chemical nature of the inducer or activator is an important question. In solution arsenite or antimonite form oxyacids or oxyanions. It seems likely that the transported substrates of the pump are those oxyacids or oxyanions. However, as metalloids they have the potential to react as soft metals. As described below, in both transcriptional regulation by the ArsR repressor and allosteric activation of the ArsA ATPase, soft metal chemistry accounts for the ability of As(III) and Sb(III) to serve as effector.

METALLOREGULATED TRANSCRIPTION OF THE *ars* OPERON

Transcription of the *ars* operon is regulated by a substrate-responsive repressor, the ArsR protein. This

metalloregulatory protein plays both metal sensory and transcriptional regulatory roles in a switching mechanism, controlling the basal level of the *ars* operon expression (Wu and Rosen, 1991, 1993). The ArsR protein belongs to a novel metalloregulatory family in which the ArsR protein is the first identified member. Other known members include functionally equivalent ArsR proteins from the *E. coli* chromosome (Carlin *et al.*, in press) and staphylococcal plasmids (Ji and Silver, 1992; Rosenstein *et al.*, 1994), the CadC regulatory proteins of cadmium-zinc resistance operons (Yoon *et al.*, 1991), and the SmtB regulatory protein of the cyanbacterial cadmium-zinc metallothionein gene *smtA* (Morby *et al.*, 1993). Other *cadC*-like genes have been identified (Shi *et al.*, 1994); note that the CadC protein regulates the gene for a P-type ATPase. Although only the ArsR proteins have been studied in detail, it is believed that each member of the ArsR family is a metal-responsive repressor protein that negatively regulates the transcription of a detoxification and/or metal efflux operon in prokaryotes. To regulate transcription each must be a DNA-binding protein. A putative helix-turn-helix DNA-binding motif has been identified in the ArsR protein, and this region exists in other members of the ArsR family (Shi *et al.*, 1994). In each protein there is the highly conserved consensus sequence ELCVCDL located on the N-terminal side of the helix-turn-helix DNA-binding domain. This conserved region includes a vicinal cysteine pair in all but the SmtB protein. As described below, this cysteine pair is involved in binding of the metal ion inducers.

Although the cysteine pair is required for metal binding, it cannot be responsible for recognition, since the range of ions recognized varies. The ArsR proteins can be released from the *ars* operator region by antimonite (Sb(III)) and arsenite (As(III)) (Wu and Rosen, 1993; Rosenstein *et al.*, 1994), but Cd(II) is not an inducer (unpublished). The CadC protein recognizes Cd(II) and Zn(II) but not arsenite or antimonite (Yoon *et al.*, 1991). Transcription of the metallothionein gene *smtA* is increased by Cd(II), Zn(II), or Cu(II) *in vivo* (Huckle *et al.*, 1993), and Zn(II) dissociates the DNA-SmtB protein complex *in vitro* (Morby *et al.*, 1993). However, the SmtB protein does not appear to recognize arsenite or antimonite. Thus the ion recognition domains of these proteins must include sequences in addition to the cysteines required for ion binding. Interestingly, each of the Cd(II)/Zn(II) metalloregulatory members of the ArsR family has an N-terminal extension of 25–30 residues not present in the As(III)/Sb(III) regulatory proteins (Shi *et al.*, 1994). This N-terminal domain has 1–2 additional cysteines that could be involved in Cd(II)/Zn(II) recognition.

The ArsR protein of R773 exists as a dimer in the cytosol (unpublished). It specifically binds to the *ars* operator DNA, a region of imperfect dyad symmetry located just upstream of the promoter. From hydroxyl radical footprinting analysis, only two sites were protected within this operator region: 4 base pairs from nucleotide −61 to −58 and 4 base pairs from nucleotide −50 to −47 (Wu and Rosen, 1993). Formation of the ArsR-DNA complex can be prevented by arsenite and antimonite but not by arsenate *in vitro*, although the last can induce expression of *ars* operon *in vivo*. Arsenate induces *in vivo* because it is first reduced to arsenite by the ArsC protein (Oden *et al.*, 1994). The putative helix-turn-helix DNA-binding domain spans residues 36–52. When residue H50, located within the second helix, was altered to a tyrosinyl residue, the mutation resulted in constitutive expression of the *ars* operon, consistent with its involvement in DNA binding (Shi *et al.*, 1994). When a wild type *arsR* gene was co-expressed from a compatible plasmid, the constitutivity of the $arsR_{H50Y}$ mutant was restored to inducibility, confirming that the ArsR protein is a *trans*-acting factor.

To determine the nature of the inducer binding domain of the ArsR protein, the *arsR* was chemically mutagenized *in vitro* using hydroxylamine, and a novel positive selection was used to isolate mutants with decreased ability to respond to inducer (Shi *et al.*, 1994). In this selection the gene for a toxic protein was put under control of the *ars* promoter. This prevented isolation of ArsR proteins that would no longer bind to the operator, since constitutive mutants would express the gene for the toxic protein and die. Similarly, ArsR proteins that could respond to inducer would die. Only ArsR proteins that still bound to the operator but remained repressed in the presence of inducer would survive. Three mutants were isolated, and each had altered cysteines within the conserved $ELC_{32}VC_{34}DL$ region. Each of the three altered ArsR proteins (C32Y, C32F, and C34Y) retained the ability to bind specifically to the *ars* operator and to repress transcription of the operon. However, consistent with the phenotype of the mutants, inducers were less effective in dissociating the altered ArsR proteins from the promoter *in vitro*, and *in vivo* did not elicit an increase in *ars* operon expression. To eliminate the possibility that the mutant phenotype resulted from

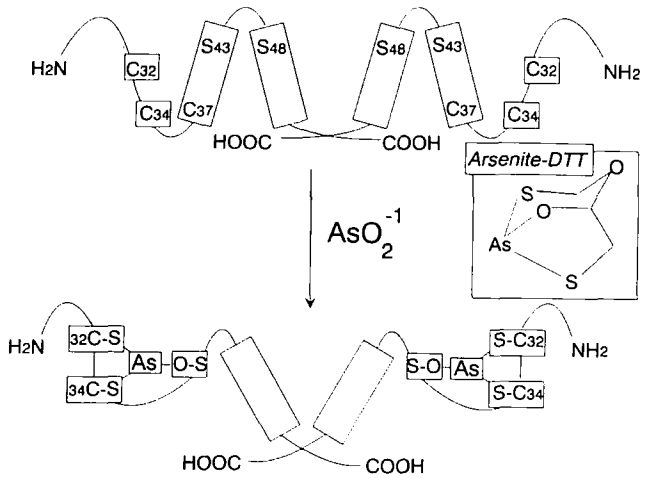

Fig. 3. Metalloregulation by the ArsR repressor. The ArsR protein is a homodimer that binds to the operator region through a helix-turn-helix domain in each monomer, repressing transcription of the *ars* operon. Either arsenite or antimonite are inducers that bind to the cysteine pair C32 and C34 as through covalent metal–sulfur bonds. Formation of a third bond with the oxygen of a serine residue in one of the DNA-binding helices disrupts the structure of the helix, abolishing DNA binding. In the inset is shown the structure of the arsenite–dithiothreitol complex, in which As(III) forms three covalent bonds with one oxygen and two sulfur atoms.

introduction of bulky phenylalanine or tyrosine groups, additional mutations were created using directed mutagenesis to introduce glycine residues in place of the cysteine residues. The C32G and C34G proteins exhibited the same phenotype as the original randomly created mutants (unpublished). We have thus proposed that the sequence ELCVCDL forms a metal binding box in members of the ArsR family (Shi *et al.*, 1994), with arsenic– or antimony–thiolate bonds to the two cysteinyl residues. In other metal regulatory proteins CXC or CXXC sequences have been implicated as potential metal-binding domains (O'Halloran, 1993).

Several questions remain. Are C32 and C34 sufficient for soft metal binding? How does sensing the various inducers such as Sb(III), As(III), Zn(II), Cu(II), and Cd(II) by the members of the ArsR family result in transcriptional activation? The answers to these questions may be related. Although *in vivo* expression of a reporter gene under control of the *ars* promoter was not inducible with high levels of either arsenite or antimonite, *in vitro* the C32Y and C34Y proteins gave partial responses to a 10-fold higher concentrations of antimonite required for maximal response of the wild type ArsR repressor. This result suggested that C32 and C34 are not solely responsible for binding of inducer. As(III) is complexed by three closely spaced cysteinyl residues in the rat glucocorticoid receptor (Chakraborti *et al.*, 1992), so participation of an additional cysteinyl residue in the ArsR protein was explored. The ArsR protein contains five cysteinyl residues, C32, C34, C37, C108, and C116. The latter two were shown to be unnecessary for ArsR function (Wu and Rosen, 1991). When C37 was altered to a serine residue, the C37S protein was indistinguishable from the wild type repressor in *in vitro* gel retardation assays (unpublished). Thus the third ligand to As(III) or Sb(III) does not appear to be a sulfur. Another possibility is a histidine residue. Cd(II) and Zn(II) binding motifs frequently include histidine residues (O'Halloran, 1993), and H50 is highly conserved in members of the ArsR family. As mentioned above, a H50Y alteration resulted in constitutive expression, but the protein was not examined for arsenite binding.

There are other possibilities. Information obtained from model compounds such as the complex formed by reaction of arsenite with dithiothreitol is instructive (Delnomdedieu *et al.*, 1993). Arsenite reacts as a soft metal, with elimination of the oxygens of arsenite and formation of covalent arsenic–sulfur bonds. The third ligand to As(III) is the oxygen of one of the hydroxyl groups of dithiothreitol, forming a pyramidal tricoordinate complex with three heterocyclic rings (Fig. 3). The As–O bond (1.83 Å) is shorter than the two As–S bonds (2.24 and 2.25 Å), indicating a stronger, more covalent character. Participation of serine or threonine hydroxyl groups in the ArsR protein must therefore be considered. Even though the hydroxyl protons are not physiologically dissociable, formation of a covalent arsenic– or antimony–oxygen bond should not require prior ionization. Residues S43 and S48, both highly conserved residues in members of the ArsR family, are located within the putative DNA binding domain of these proteins. The role of S43 or S48 in metal binding in the ArsR protein is currently being investigated. From knowledge of the participation of residues C32 and C34 and the location of the putative DNA binding domain as immediately adjacent, we propose a model for induction in which As(III) or Sb(III) form a tricoordinate species with the two cysteinyl thiols and the oxygen of S43 or S48 (Fig. 3). Binding of the metal to a residue within one of the helices of the DNA-binding domain would induce a conformational change in that domain, resulting in dissociation of the repressor from the *ars* operator, hence induction.

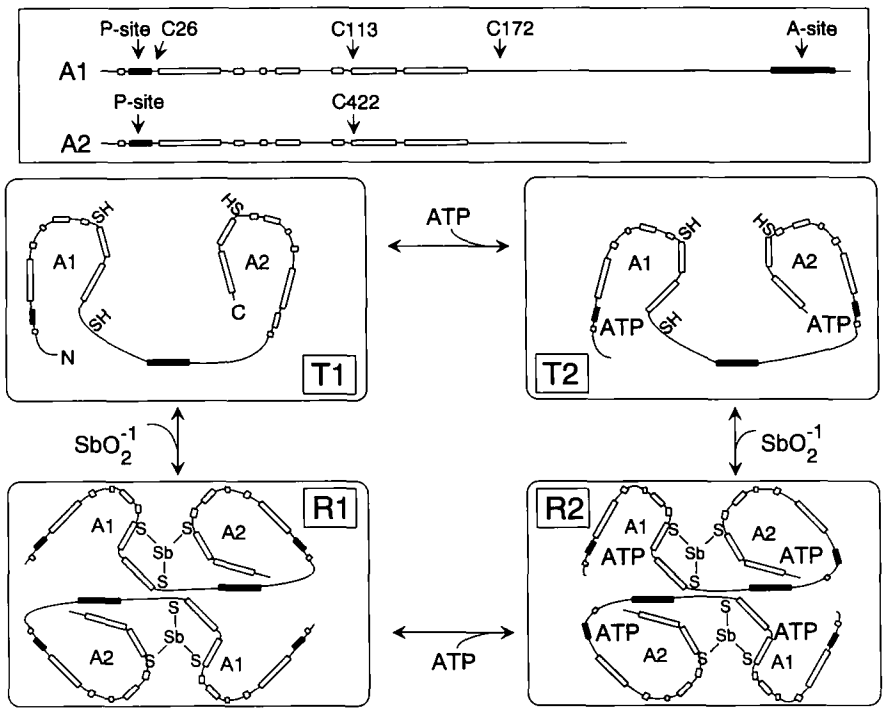

Fig. 4. Allosteric metalloactivation of the ArsA protein. Top: The 583 amino acid residue ArsA protein has two homologous halves, the N-terminal A1 half and the C-terminal A2 half, with the regions of greatest similarity boxed. The A1 and A2 portions of the ArsA protein both have ATP binding sites; the phosphate binding loops (P-sites) of each are identified. The location of the four cysteine residues in the primary sequence are indicated. Connecting the A1 and A2 halves is a short linker region. A residue within this region forms a photoadduct with the adenine ring of the ATP bound to the A1 nucleotide binding site, indicating that this sequence is in or near the adenine binding site (A-site). Bottom: The 63-kDa ArsA protein has independent binding sites for antimonite and ATP. The purified soluble protein exists primarily as a monomer in an inactive T1 form. ATP binding is associated with a unique conformational change to the T2 form, which has a low basal level of ATPase activity. The T1 monomer exists in equilibrium with dimer, which has the R1 conformation. Metalloids Sb(III) or As(III) bind preferentially to the R1 conformation, increasing by mass action the concentration of dimer. The fully active R2 conformation has both ATP and metalloid binding sites filled. As a membrane-bound subunit of the pump, the ArsA protein exists at all times as dimer. In the absence of substrate or effector both ArsA subunits exist in an assortment of each of the conformations, but the equilibrium favours primarily the inactive T1 form; binding of ATP and metalloid increases by mass action the amount of the enzyme in the active R2 conformation, promoting catalysis.

ALLOSTERIC METALLOREGULATION OF THE ArsA PROTEIN

The primary sequence of the ArsA protein did not reveal the presence of any conserved metal binding motifs. An indication that cysteines might be important for the activity of the ArsA protein came from the extreme sensitivity to sulfhydryl reagents such as *N*-ethylmaleimide and methyl methanethiosulfonate (unpublished). Unlike Hg(II), which can inactivate enzymes by reacting with a single cysteine thiol, inhibition of enzymes by arsenite usually requires binding to the thiol groups of spatially proximate cysteine residues (Torchinskii, 1981). This implies that for arsenite or antimonite to inhibit (or activate) an enzyme, the protein would be expected to have two or more cysteine residues in close proximity in the tertiary structure, if not in the primary sequence. In the ArsA protein there are only four cysteines, C26, C113, C172, and C422, all located in different regions of the primary sequence. For arsenite or antimonite to act as an allosteric modulator, two or

more of the cysteines should be close together in the folded protein to form As(III)– or Sb(III)–S bonds.

To investigate the role of cysteine residues in the allosteric activation of the ArsA protein, each of the four cysteine residues were altered to serine residues by site-directed mutagenesis of the *arsA* gene (Bhattacharjee *et al.*, submitted). Cells expressing the $arsA_{C26S}$ mutant exhibited no change in resistance to arsenite or antimonite, and the purified C26S protein had wild type ATPase activity. In contrast, cells expressing the $arsA_{C113S}$, $arsA_{C172S}$, and $arsA_{C422S}$ mutants were less resistant to arsenite or antimonite, and each of the three altered proteins exhibited reduced ATPase activity. It was shown by a variety of methods that replacement of each of the cysteines with serine residues did not grossly perturb the tertiary structure of the ArsA protein. However, the altered proteins differed in their activation of ATPase activity by antimonite or arsenite. The kinetic parameters of the C26S ArsA protein were essentially the same as that of wild type protein. Thus C26 does not appear to be involved in either activation or catalysis. When the kinetic parameters of the purified C113S, C172S, and C422S ArsA proteins were investigated, for each the K_m for ATP was found to be within an order of magnitude of the wild type enzyme. However, the concentration of oxyanion required for activation was substantially increased, most likely reflecting a decrease in affinity for oxyanion. Alteration of C422 appears to have more of an effect than alteration of C113 or C172. The C113S and C172S proteins exhibited a 20-fold increase in the concentration of antimonite required for half maximal activation, while the C422S required 200-fold more. These results would suggest that all three residues are involved in activation.

The results of limited trypsin digestion also supports the idea that the activator binding site of the C113S, C172S, and C422S proteins is modified. Surface accessibility to trypsin has been used to assess the structure of the ArsA protein (Hsu and Rosen, 1989). The effect of ATP and oxyanions on the rate of trypsin digestion has been shown to reflect the binding of substrate and effector and the interaction of the two types of binding sites in the ArsA protein. The presence of both antimonite and ATP has been shown to synergistically protect the 63-kDa wild type ArsA protein (Hsu and Rosen, 1989). The synergistic effect was observed with the C26S protein but not the C113S, C172S, or C422S proteins. Activation of purified soluble ArsA protein by arsenite or antimonite has been correlated with formation of a homodimer (Hsu *et al.*, 1991). It has been postulated that the monomer, with low basal activity, exists in equilibrium with dimer, the catalytically active conformation. A reasonable model is that Sb(III) or As(III) preferentially bind to the active conformation in coordination with the cysteinine thiolates 113, 172, and 422 (Fig. 4). What accounts for the greater activation by Sb(III) over As(III)? Although the chemical properties of the two elements are similar, the ionic radius of Sb(III) is 0.76 Å compared to 0.58 Å for As(III). Moreover, the length of an As–S bond is approximately 2.2–2.3 Å (Cruse and James, 1972), while an Sb–S bond is approximately 2.5–2.9 Å (Kavounis *et al.*, 1982). Most likely the local geometry in the activator binding site governs the preferential formation of Sb(III) cysteinine thiolate bonds, while formation of As(III)–S bonds results in a more strained conformation of the enzyme.

CONCLUSIONS AND SPECULATIONS

We have shown that environmental sensing of arsenic and antimony compounds by the ArsR repressor and activation of the ArsA ATPase involves their reaction as soft metals. What is the chemical nature of the species transported by the pump? In contrast to the ArsR and ArsA proteins, the membrane sector of the pump, the ArsB protein, has no critical cysteine residues. It has only a single cysteine residue, C369, and mutagenic alteration of this residue had no effect on resistance (unpublished). The topological structure of the ArsB protein is similar to many secondary transport proteins (Wu *et al.*, 1992), and, in the absence of the ArsA protein, it can function as a secondary porter (Dey and Rosen, in press). The most reasonable speculation is that the ArsB protein transports the nonmetallic oxyanions arsenite or antimonite and does not interact with arsenic or antimony as soft metals. Indeed, binding of soft metals to cysteine thiolates is so strong that it is not likely that they could be removed from the pump without considerable disruption of the structure of the complex. Thus it appears that the Ars pump uses two distinct arsenic (antimony) chemistries: soft metal binding to activate the catalytic subunit, which channels energy into the membrane subunit, which transports the oxyanion. Implicit in this scenario is that the arsenic bound as activator is not the arsenic transported. This separation of activation and transport domains may be common

in other transport ATPases. For example, the P-type ATPases for Cd(II) (Silver *et al.*, 1993) and Cu(II) (Bull *et al.*, 1993; Chelly *et al.*, 1993; Mercer *et al.*, 1993; Petrukhin *et al.*, 1993; Tanzi *et al.*, 1993; Vulpe *et al.*, 1993) contain cytosolic N-terminal domains with single or multiple cysteine pairs. These may function as activation domains, since transfer of the metals from the cysteine thiolates to a putative membrane site, with subsequent release on the other side of the membrane, seems energetically unfeasible. We would speculate that even among groups I and II metals recognition domains in ion-translocating ATPases will be separate from transport domains, and that this will be found to be a common feature of pumps.

ACKNOWLEDGMENT

This work was supported by United States Public Health Service Grant AI19793.

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