JB Review

J. Biochem. **123**, 16-23 (1998)

Metalloid Resistance Mechanisms in Prokaryotes

Chun Xu,* Tongqing Zhou,* Masayuki Kuroda,† and Barry P. Rosen*,[1]

Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, MI 48201, USA; and †Department of Microbiology, Kochi Medical School, Kohasu, Okoh-cho, Nankoku, Kochi 783

Received for publication, October 6, 1997

Resistance to antibiotics and other chemotherapeutic agents is becoming a wide spread health issue. The biochemical mechanisms of resistance vary, but active efflux of the toxic agents is one of the most common. Bacterial resistances to metals provide good model systems for transport-related resistances. One of the best understood metal resistance systems is the product of the *ars* operon, which provides resistance to arsenicals and antimonials. As a reflection of the ubiquity of arsenic in the environment, *ars* operons are found in all species of bacteria, carried in chromosomes, plasmids, and transposons. This review focuses on the biochemistry of the proteins of the *ars* operon of *R*-factor R773. The system is novel in several respects. First, it is regulated at the transcriptional and allosteric levels, and regulation is effected through cysteine thiol interaction with As(III) or Sb(III). Thus soft metal-thiol chemistry provides a high affinity digital switch to turn the regulated protein on with rapidity. The transport system that provides resistance, on the other hand, uses oxyanions of arsenic or antimony as substrates. This nonmetal chemistry allows for low affinity interactions of the membrane transporter with substrate, conductive with translocation and release of substrate on the outside of the cell membrane. Second, the transporter is uniquely capable of coupling to either electrochemical energy as a secondary carrier protein or the chemical energy of ATP when binding of a catalytic subunit converts it into an anion-translocating ATPase.

Key words: arsenic, antimony, metalloregulation, resistance, transport.

1. Introduction

Resistance has become a serious threat to the use of drugs for both antibacterial and anticancer chemotherapy (*1*). A variety of mechanisms produce resistance, but one of the most frequent is the development of transport systems that extrude drugs and toxic metals from the cell, reducing the intracellular concentration to subtoxic levels (*2*). Arsenicals and antimonials have long been used medicinally and were among the first chemotherapeutic agents used to treat infectious diseases. Paul Ehrlich introduced organic arsenical into clinical use for the treatment of syphilis and trypanosomal diseases. For his development of these drugs Ehrlich was awarded the Nobel Prize in 1908. His most famous arsenical drug was his *silver bullet*, Salvarsan. However, cells rapidly became resistant to Salvarsan, leading Ehrlich to postulate that first, arsenicals must be taken up by cells via a cell surface arsenical receptor in order to be effective, and second, that resistance could ensue from the loss of ability to take up the arsenical drug. Over the last fifteen years our laboratory has been investigating the mechanisms of resistance to arsenic and antimony (*3, 4*). We have shown that both prokaryotes and eukaryotes have extrusion systems for the salts of the metalloids As(III) and Sb(III), and that extrusion correlates with resistance (*5*).

Although resistance is becoming a major clinical problem, bacterial metal resistances were more common in the pre-antibiotic era than were resistances to antibiotics (*6*), perhaps because heavy metals are common in minerals, soil, and water and enter readily into the food chain (*7, 8*). The metal and metalloids salts to which bacterial resistances have been reported include arsenate, arsenite, antimony, lead, cadmium, zinc, bismuth, and mercury (*3, 9, 10*). The determinants for these resistances have been found on transmissible plasmids (*11, 12*), transposons (*13*), and chromosomes (*14*). One such is the *R*-factor R773, a large conjugative plasmid that carries a number of resistance determinants for antibiotics and metals (*11*). We cloned the arsenical resistance operon (*ars*) from R773 and have shown that it has five genes, *arsRDABC*. The first two, *arsR* and *arsD*, encode regulatory proteins (*15, 16*). The *arsA* and *arsB* genes encode the actual resistance, a pump that extrudes salts of trivalent arsenic or antimony (*17*). This pump exhibits no evolutionarily relationship to other families of transport ATPases. The last gene of the operon, *arsC*, encodes a reductase that catalyzes the conversion of As(V) to As(III), expanding the range of resistance to include pentavalent arsenicals (*18, 19*). In this review we will describe in detail the mechanism of the arsenical extrusion system and its regulation.

2. Metalloregulation by arsenic and antimony

A. The ArsR and ArsD repressors. Resistance to the metalloids arsenic and antimony in both Gram-positive and Gram-negative bacteria is associated with five-gene or

[1] To whom correspondence should be addressed. Phone: +1-313-577-1512, Fax: +1-313-577-2765, E-mail: brosen@med.wayne.edu

© 1998 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

three-gene *ars* operons (*11, 20, 21*) (Fig. 1). The first gene, *arsR*, in all operons encodes a *trans*-acting repressor protein (*12, 22, 23*). The ArsR protein of the Gram-negative plasmid R773 is the first identified member of a novel family of small metalloregulatory proteins (*22, 24, 25*). These include subgroups of proteins that respond to As(III)/Sb(III) [ArsR], Cd(II)/Zn(II) [CadC] (*26*), or Zn(II) [SmtB] (*27*) (Fig. 2). As a metal-repressive repressor protein that negatively regulates the transcription of a detoxification and/or metal efflux operon, each member of the ArsR family should have at least three domains, a metal binding domain, a DNA-binding domain, and a dimerization domain. A putative helix-turn-helix DNA-binding motif from residues 38 to 54 has been identified in ArsR protein (*25*). The ArsR sequence $ELC_{32}VC_{34}DLC_{37}$ has been proposed to form a portion of the metal binding domain, in which residues Cys32, Cys34, and Cys37 are ligands for As(III) and Sb(III) (*28*). Interestingly, while Cys37 is capable of interacting with As(III), it is not required for induction and is not conserved in all ArsRs. Using genetic and biochemistry assays, it was found that in the ArsR protein residues 1-8 and 90-117 are not required for ArsR dimerization, and a core sequence of about 80 residues has all of the information necessary for dimerization, repression and metal recognition (*29*).

How is the information from the sensing of environmental metals transduced into transcription of pump genes? We hypothesize that formation of the As-S bonds distorts the first putative helix of the DNA binding domain such that it can no longer interact with the operator site (Fig. 3). Preliminary computer modeling of the metal and DNA binding domains of ArsR suggests that for Cys37 to be able to be the third As(III) ligand, the first part of the DNA binding domain must go from α helix to β sheet. Thus the initial event in derepression would be As(III) binding to the thiolates of Cys32 and Cys34. The distortion produced by this binding may force the Cys37 thiolate into proximity with As(III), allowing Cys37 to be a third ligand to the arsenic without being involved in induction.

In five-gene *ars* operon the product of the second gene, *arsD*, that is missing from the three-gene arsenic resistance system, is also a *trans*-acting repressor (*16*).

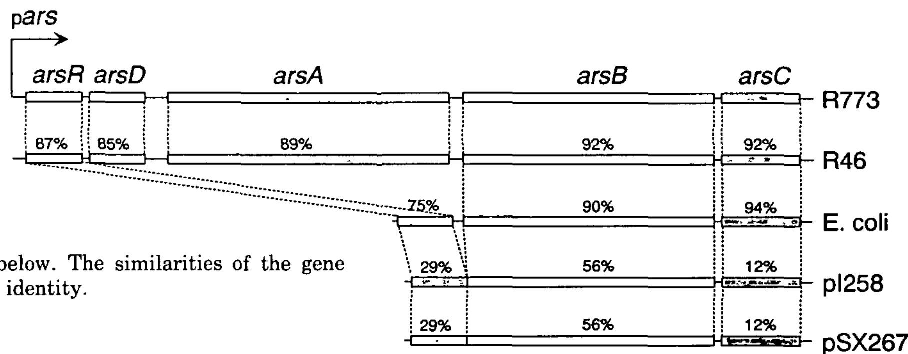

Fig. 1. **Bacterial *ars* operons.** In the top line the five genes of the *ars* operon of plasmid R773 are shown with the direction of transcription indicated by the arrow, starting with the promoter, p_{ars}. Genes are indicated by boxes, with the intergenic spaces as single lines. The genes of homologous *ars* operons of plasmid R46 (*62*), the *E. coli* chromosomal operon (*14*), and staphylococcal plasmids pI258 (*12*), and pSX267 (*23*) are aligned below. The similarities of the gene products to the R773 proteins are given as % identity.

```
ArsR R773                                  MLQLTPLQLFKNLSDETRLGIVL
ArsR E. coli                               MSFLLPIQLFKILADETRLGIVL
ArsR R46                                   MPEIASLQLFKILSDETRLGIVL
ArsR pI258                                 MSYKELSTILKVLSDPSRLEILD
ArsR pSX267                                MSYKELSTILKVLSDPSRLEILD
ArsR M. jannaschii                        MEKYEKAAEIFKAFGDPTRLMILK
CadC pI258         MKKKDTCEIFCYDEEKVNRIQGDLQTVDISGVSQILKAIADENRAKITY
CadC B. firmus     MNKKDTCEIFCYDEEKVNRIQGDLKTIDIVSVAQMLKAIADENRAKITY
CadX               MSYENTCDVICVHEDKVNNALSFLEDDKSKKLLNILEKICDEKKLKIIL
SmtB            MTKPVLQDGETVVCQGTHAAIASELQAIAPEVAQSLAEFFAVLADPNRLRLLS

                   Metal binding box  Helix Turn Helix
ArsR R773          LLREMGELCVCDLCMALDQSQPKISRHLAMLRESGILLDRKQGKWVHY
ArsR E. coli       LLSELGELCVCDLCTALDQSQPKISRHLALLRESGLLLDRKQGKWVHY
ArsR R46           LLREMGELCVCDLCTALEQSQPKTSRHLAMLRESGLLLDRKQGKWVHY
ArsR pI258         LL-SCGELCACDLLEHFQFSQPTLSHHMKSLVDNELVTTRKNGNKHMY
ArsR pSX267        LL-SCGELCACDLLEHFQFSQPTLSHHMKSLVDNELVTTRKNGNKHMY
ArsR M. jannaschii LLAENGSMCVCKIIDELKKPQPTISHHLNILKKAGIVKARKEGTWNFY
CadC pI258         ALCQDEELCVCDIANILGVTIANASHHLRTLYKQGVVNFRKEGKLALY
CadC B. firmus     ALCQDEESCVCDIANIIGITAANASHHLRTLHKQGIVRYRKEGKLAFY
CadX               SLIKEDELCVCDISLILKMSVASTSHHLRLLYKNEVLDFYKDGKMAYY
SmtB               LLARS-ELCVGDLAQAIGVSESAVSHQLRSLRNLRLVSYRKQGRHVYY

                       **
ArsR R773          RLSPHIPSWAAQIIEQAWLSQQDDVQVIARKLASVNCSGSSKAVCI 117
ArsR E. coli       RLSPHIPAWAAKIIDEAWRCEQEKVQAIVRNLARQNCSGDSKNICS 117
ArsR R46           RLSPHIPSWAALVIEQAWLSQQDDVQAIARKLASANCSGSGKAVCI 117
ArsR pI258         QLNHEFLDYINQNLDIINTSDQGCACKNMKSGEC             104
ArsR pSX267        QLNHEFLDYINQNLDIINTSDQRCACKNMKSGEC             104
ArsR M. jannaschii YIVDDRVKEIIKLVDEL                               89
CadC pI258         SLGDEHIRQIMMIALAHKKEVKVNV                      122
CadC B. firmus     SLDDEHIRQIMMIVLEHKKEVNVNV                      122
CadX               FIKDDEIREFFSKNHEGF                             115
SmtB               QLQDHHIVALYQNHLDHLQECR                         122
```

Fig. 2. **The ArsR family of metalloregulatory proteins.** Highly conserved regions are shaded. The shaded box shows the putative metal binding box, with a vicinal cysteine pair in all but the SmtB protein. Shown are the ArsR repressors of plasmids R773, the *E. coli* chromosomal operon, plasmid R46, pI258, pSX267, and *M. jannaschii*, the CadC repressors of plasmid pI258, chromosomally encoded CadC from *B. firmus*, the staphyloccocal CadX protein and the SmtB protein of *Synechococcus* PCC 7942. The locations of the helix-turn-helix DNA binding motif and the histidine pair of SmtB are indicted.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

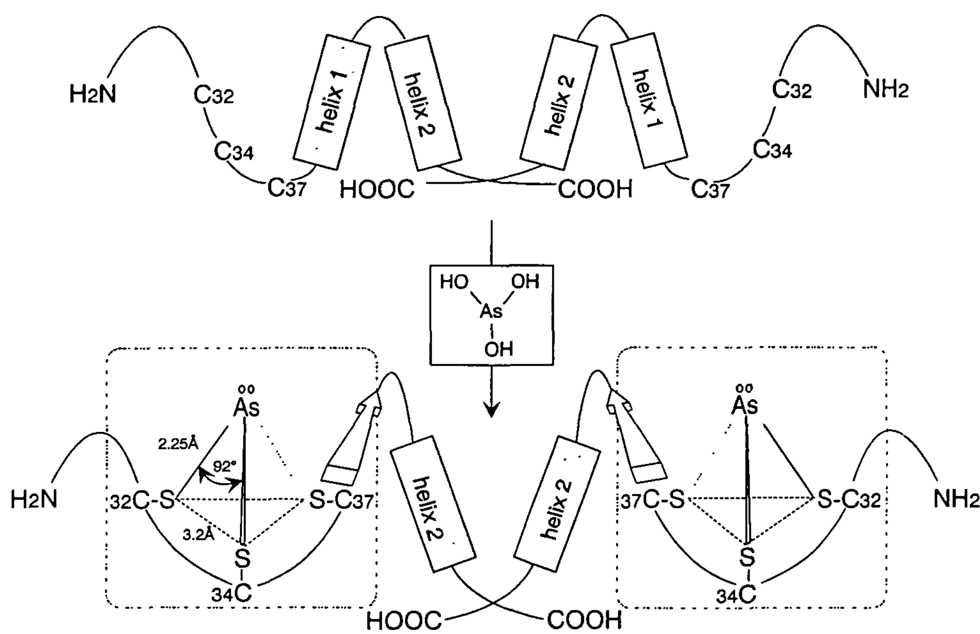

Fig. 3. **Model for metalloregulation by the ArsR repressor.** The ArsR homodimer binds to the operator/promoter region through a helix-turn-helix domain in each monomer, repressing transcription of the *ars* operon. Inducers, including arsenite, antimonite, and phenylarsine oxide, bind to Cys32, Cys34, and Cys37 through soft metal-thiol bonds, producing a conformational change in the DNA binding domain that results in dissociation of the repressor from the operator/promoter.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

Although both ArsR and ArsD are 13 kDa homodimers, they share no sequence similarity. From DNase I footprinting analysis, it was found that ArsD binds to the same site on the *ars* promoter element as the ArsR protein but with two orders of magnitude lower affinity (*30*). It was also found that ArsD is released from the *ars* DNA promoter by phenylarsine oxide, sodium arsenite, and potassium antimonyl tartrate, the same inducers to which ArsR responds, but *in vivo* repression by ArsR can be fully relieved with 10 μM sodium arsenite, while ArsD repression requires approximately 100 μM sodium arsenite for induction (*30*). These results suggest that ArsR has higher affinity for inducer than ArsD. Therefore a low level of environmental metalloid would cause dissociation of ArsR, resulting in transcription of the operon, increasing the amount of ArsD. As the intracellular concentration of ArsD exceeded the K_d for *ars* DNA, it would fill the *ars* operator site. Since its affinity for inducer is less than that of ArsR, the relatively low level of inducer present in the cell would not prevent its binding. On the other hand, exposure to high levels of environmental metalloid would cause dissociation of ArsD, effecting further expression of the *ars* genes and increased synthesis of the Ars extrusion pump. Synthesis of high levels of the pump proteins is itself toxic (*31*), so that there must be a balance between detoxification of the metalloid and expression of the pump genes. Thus action of the two repressors forms a homeostatic regulatory circuit that maintains the level of *ars* expression within a narrow range, with ArsR controlling basal level of expression, and ArsD controlling maximal expression.

B. Other members of the ArsR family: the CadC and SmtB repressors. While ArsR was the first identified member of a new family of metalloregulatory proteins, other metal-responsive repressors have since been identified. The Cd(II)/Zn(II) resistance (*cad*) operon from the staphylococcal plasmid pI258 has two genes, *cadA* and *cadC* (*32*). The *cadC* gene encodes CadC, the transcriptional regulator of the *cad* operon (*26*) and a member of the ArsR family. Silver and coworkers have mapped the operator site for CadC binding to the *cad* promoter (*33*) and have shown that there are two retarded species in gel mobility shift assays. They showed that CadC dissociated in the presence of high concentrations of Cd(II), Bi(III), and Pb(II) but not Zn(II). Since zinc is an inducer *in vivo*, the lack of a zinc effect *in vitro* is unexplained. From the deduced amino acid sequence, although CadC contains the metal binding motif having a consensus sequence of ELCVCDL, it does not respond to As(III)/Sb(III) (*33*).

SmtB is another member of the ArsR family. SmtB is a *trans*-acting repressor encoded by a divergently transcribed gene *smtB* of a metallothionein locus, *smt*, from the cyanobacterium *Synechococcus*. SmtB is required for Zn(II)-repressive expression of the metallothionein gene *smtA* (*27*). Recombinant SmtB has been expressed in *Escherichia coli* and purified (*34*). Based on gel mobility shift assays, methylation interference and molecular size calculations it was proposed that recombinant SmtB binds to the *smt* DNA promoter in multimeric fashion. At low concentrations of SmtB a single complex formed, and with higher concentrations of recombinant SmtB multiple complexes were detected in gel mobility shift assays (*34*). It was also shown that SmtB is capable of directly interacting with Zn(II), and complex formation was inhibited in the presence of ionic forms of various metals including zinc, copper, cadmium, cobalt, nickel, and chromium (*34*). Although SmtB is a member of the ArsR family of metalloregulatory proteins, it contains only one of the conserved cysteines within the proposed metal binding domain of ArsR family members. A different metal binding motif involving a vicinal histidine pair His105/His106 in the C-terminal region of the protein that is absent in ArsR and CadC has been suggested (*35*).

3. The Ars pump

Plasmid-mediated arsenical and antimonial resistance in *E. coli* cells is conferred by the operation of an ATP-driven efflux system that pumps the toxic compounds out of the cells (*2*). This pump is composed of two types of subunits

(Fig. 4A), the products of *arsA* and *arsB* genes in the R773 *ars* operon (*17*).

A. ArsA, the catalytic subunit of the Ars pump. From its predicted amino acid sequence, the 63 kDa ArsA protein has two homologous halves, the N-terminal A1 half and the C-terminal A2 half (*36*). Each contains the signature sequence of an ATP-binding site (*37*). Although functionally a peripheral membrane protein, when overexpressed ArsA can be purified from the cytosol of *E. coli* cells (*38*). Soluble ArsA exhibits As(III)/Sb(III)-stimulated ATPase activity.

1) Mechanism of ATP hydrolysis: Considering the presence of duplicated consensus sequences for nucleotide binding sites in A1 and A2, it was reasonable to ask whether both sites bind ATP; whether they interact with each other; and what the roles each plays in catalysis. These questions have been investigated by a variety of genetic, biochemical and biophysical approaches.

From the increase in fluorescence of the ATP analogue 2′,3′-*O*-(2,4,6-trinitrophenylcyclohexadienylidene)adenosine-5′-triphosphate (TNP-ATP), it was shown that both consensus nucleotide binding sites were capable of binding a nucleotide. Substitutions of residues in either site by mutation of codons in the correspond DNA led to inactivation of ATPase activity and loss of arsenite resistance (*39, 40*). The purified proteins similarly lost the ability to bind TNP-ATP at the substituted site but could still bind at the nonaffected site (*41*). Thus loss of binding at either site inactivated the enzyme, demonstrating that both are required for catalysis. Another assay for ATP binding involved photoactivation of the nucleotide with ultraviolet light, resulting in adduct formation between the adenine ring of ATP and ArsA. From analysis of peptides UV photolabeled with α-[^{32}P]ATP, a small peptide in the linker region connecting the A1 and A2 halves of the protein must be in contact with the adenine ring (*42*). It appeared that the nucleotide bound in the A1 site formed the photoadduct with α-[^{32}P]ATP. Thus it is possible that the A1 and A2 sites are not identical. However, since photoadduct formation requires the presence of a specific type of amino acid residue in the vicinity of the photoactivated adenine, the lack of interaction at the A2 site may simply reflect the lack of an appropriate residue nearby.

Genetic analyses have indicated that the A1 and A2 ATP binding sites functionally interact with each other. Two *arsA* genes, one with a mutation in the A1 site and the second with a mutation in the A2 site, complemented each other when expressed from compatible plasmids (*43*). Individually neither conferred arsenite resistance; when the two mutant genes were co-expressed in the same cells, those cells were resistant to arsenite. This suggests that two inactive ArsAs, one with a defective A1 ATP binding site and the other with a defective A2 site, can form an active ATPase complex. Biochemical reconstitution experiments confirmed this idea (*44*). Individually expressed peptides corresponding to the A1 and A2 halves were purified. By themselves they had no catalytic activity. If simply mixed together, they still had no activity. However, if denatured and refolded together, the resulting complex had ATPase activity. The results of recent studies utilizing intragenic suppression lend further support to the model of interacting A1 and A2 ATP-binding sites (*45*). A G15C substitution in the A1 ATP binding site resulted in a defective ArsA and reduced arsenite resistance. This mutant *arsA* gene was chemically mutagenized, and arsenite resistant strains selected. One mutant had both the original G15C substitution in the A1 site and a second A344V alteration just adjacent to A2 ATP binding site.

Several lines of evidence suggest that ArsA is a functional homodimer, including crosslinking experiments and light scattering (*46*). Since the results from both intergenic and intragenic complementation support the concept of interacting A1 and A2 ATP binding sites, we have hypothesized that there are two active catalytic sites in an ArsA dimer, each formed by a pair of interacting A1 and A2 ATP binding sites. This concept is similar to the mechanism of the H^+-translocating F_1-ATPase, in which residues from the α and β subunits form a catalytic site (*47*). It is not known whether the A1 and A2 ATP binding sites are from facing ArsA monomers or the same monomer. An experiment to test intersubunit interaction involved mixing of purified inactive and active ArsAs. The assumption was

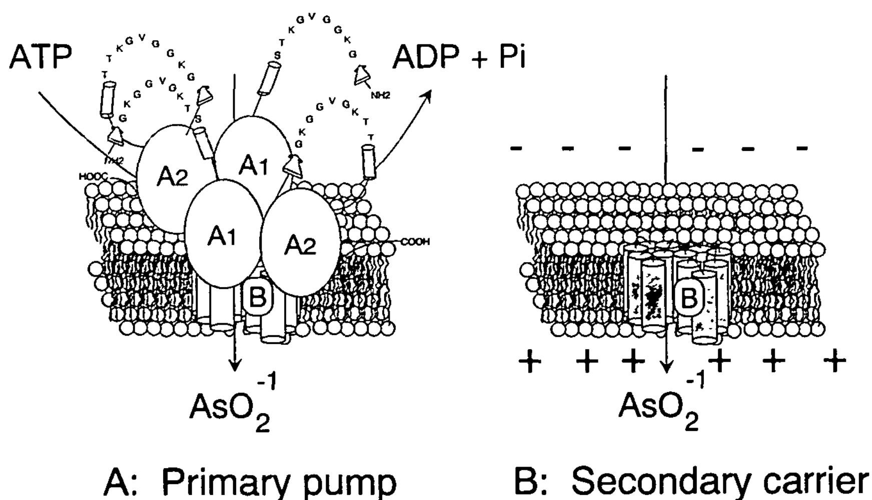

Fig. 4. **Dual energy coupling mechanisms of the Ars oxyanion-translocating system.** A: The complex of the ArsA and ArsB proteins forms an oxyanion-translocating ATPase that catalyzes extrusion oxyanions of As(III) or Sb(III). ArsA has two homologous halves, A1 (N-terminal) and A2 (C-terminal). Two subunits of the ArsA protein are indicated to reflect its structure in solution. ArsA is the catalytic subunit, exhibiting oxyanion-stimulated ATPase activity. The primary sequence of the two phosphate loops of the nucleotide binding domains in the A1 and A2 halves are shown. 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 this obligatory ATP-coupled pump. B: The ArsB protein also is capable of functioning in a second mode, as a $\Delta\psi$-driven carrier protein. When the ArsA protein is absent, the ArsB protein translocates arsenical and antimonial oxyanions, with energy derived from the proton pumping respiratory chain or F_0F_1 ATPase. When the ArsA protein is bound to the ArsB protein, the complex is an anion-translocating ATPase that is unable to utilize $\Delta\psi$.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

that formation of a dimer between active and inactive subunits would decrease the overall activity of the mixture, that is, the inactive subunit would "poison" the active ones. This was not observed (J. Li and B.P. Rosen, unpublished). Although a negative result, it is consistent with intrasubunit interaction of A1 and A2 sites within each monomer (Fig. 5). Finally, it remains to be determined whether both sites in the ArsA monomer are catalytic or one of them is regulatory.

2) Allosteric regulation: ArsA ATPase activity is activated by the oxyanions arsenite or antimonite (*38*). However, there is no obvious oxyanion binding motifs in the ArsA. What is the mechanism of allosteric activation? Cysteine modifying reagents such as methyl methanethiosulfonate and *N*-ethylmaleimide have been shown to inhibit ATPase activity, indicating the involvement of cysteines in catalysis (*48, 49*). ArsA has four cysteines, Cys26, Cys113, Cys172, and Cys422 (*49*). To investigate the role of cysteine residues in the regulation of ATPase activity, each of the four cysteines was mutated to a serine residue. The C26S ArsA has the same properties as wild type. Cells expressing the other three mutant enzymes lost their resistance to arsenite and antimonite. The purified C113S, C172S, and C422S enzymes have the same K_ms for ATP as wild type. However, the magnitude of stimulation and the affinity for arsenite and antimonite were reduced to different extent. These results suggest that Cys113, Cys172, and Cys422 are each involved in the metallostimulation of the ArsA ATPase. As soft metals As(III) and Sb(III) can inhibit the activity of enzymes *via* binding to two or three thiol groups in proteins. Usually this requires vicinal thiols, but in ArsA the thiols are spatially distant from each other in the primary sequence. For soft metal-thiol bond formation, ArsA must be folded so that Cys113, Cys172, and Cys422 are in close proximity in the tertiary structure. From the known structure of As(III)- or Sb(III)-thiol compounds, it can be predicted that the cysteine thiols should be 3-4 Å apart. To determine the distance between the cysteines residues, wild type ArsA and mutant ArsAs with cysteine to serine substitutions were treated with the bifunctional alkylating agent dibromobimane, which reacts with thiol pairs within 3-6 Å to form a fluorescent adduct. ArsAs with single cysteine mutation formed fluorescent adducts, as would be expected since those proteins retained two cysteine thiols. However, ArsAs with two of the three essential cysteines substituted did not form fluorescent adducts. These results demonstrate that Cys113, Cys172, and Cys422 are within in 6 Å in the native enzyme, consistent with their forming a novel trigonal three-coordinate As(III) or Sb(III) structure (*50*) (Fig. 5). While this resembles the As(III)-thiol structure in ArsR (Fig. 3), the two are clearly evolutionarily unrelated. Thus As-thiol structures involved in metalloregulation have evolved independently more than once.

3) The allosteric transition: the pathway of information transduction: How does filling of the allosteric site with As(III) or Sb(III) activate catalysis? There must be communication from the allosteric domain to the ATP binding domain(s). Recently, a 12-residue consensus sequence (DTAPTGHTIRLL) has been identified among ArsA homologues from eubacteria, archebacteria, fungi, plants, and animals (Fig. 6). The high degree of conservation implies that this DTAP motif has a conserved function. Intrinsic tryptophan fluorescence has been proven of value in the study of ArsA ATPase (*51*). To determinate the conformational change of the DTAP domain during the ATP

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

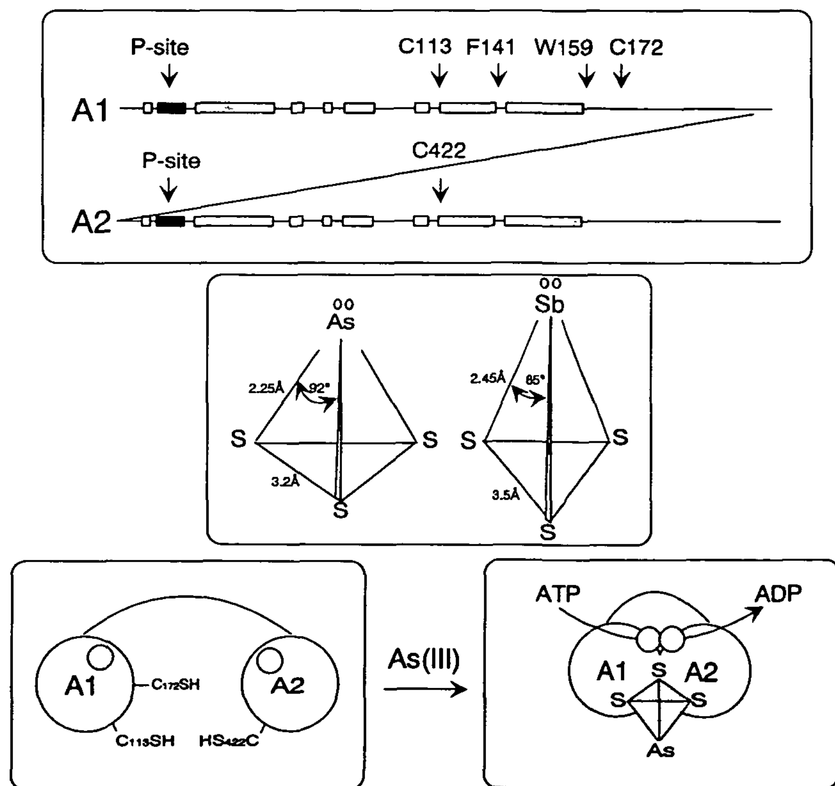

Fig. 5. **Metalloregulation of the ArsA ATPase.** Top: The 583 amino acid residue ArsA protein consists of two homologous halves, N-terminal A1 and C-terminal A2. The aligned boxes in the two halves indicate the regions of greatest sequence similarity. Each half has a consensus sequence for the binding site of phosphoryl groups of ATP (P-site). The location of the three cysteine residues in the allosteric binding site are indicated, as are the location of the residues used for spectroscopic probes of catalysis, Trp159 and F141. Middle: The structure of the soft metal-thiol complex in ArsA is postulated from the bond angles and distances found by crystallographic analysis of small molecules containing As-S or Sb-S bonds. This trigonal pyramidal structure contains three sulfur thiolates liganded to As(III) or Sb(III), with the metal at the apex. In ArsA the three sulfurs are the thiolates of Cys113, Cys172, and Cys422. Bottom: ArsA ATPase activity is allosterically regulated by binding of the As(III) or Sb(III) to the allosteric site. This is hypothesized to bring the A1 and A2 into contact to make a catalytic site at the interface between the two ATP binding sites.

```
136 FDHIIFDTAPTGHTIRLLQLPGAW  ArsA1
440 KRFVVMDTAPTGHTLLLLDATGAY  ArsA2
144 FSVVVFDTAPTGHTLRLLSLPDTL  Chlorobium vibrioforme
160 FDTVIFDTAPTGHTLRFLQLQNTL  Saccharomyces cerevisiae
152 FDVVIFDTAPTGHTLRFLGMPEVM  Methanococcus jannaschii
231 FTRIVFDTAPTGHTLRLLSLPDFL  Arabidopsis thaliana
152 FDVVVFDTAPTGHTLRLLQFPTLL  Caenorhabditis elegans
125 YDALVLDTAPTGETLRLLNFPTIV  Homo sapiens
125 YDVLIIDSAPTGTALRLLSLPEVG  Synechocystis sp.
225 FDVCLIDTAPTLGVGLAAALFAAD  incC, plasmid RK2
```

Fig. 6. **DTAP consensus sequence in ArsA homologs.** Highly conserved residues are shaded. Conserved residues are shaded. Shown are sequences from the A1 and A2 halves of ArsA, and from homologs from *C. vibrioforme, S. cerevisiae, M. jannaschii, Arabidopsis, C. elegans, H. sapiens, Synechocystis* sp., and plasmid RK2.

hydrolysis, two single tryptophan-containing ArsA protein were constructed and expressed, one with a tryptophan residue located at position 141, the N-terminal side of the DTAP domain and the second with a tryptophan residue located at position 159, the C-terminal side of the domain (*52*). From the results of fluorescence spectroscopy Trp141 is in a hydrophobic environment, while Trp159 is located in a relatively hydrophilic environment. During ATP hydrolysis the C-terminal end of the DTAP domain moves into a less polar region. As product is formed the N-terminal end enters a more hydrophilic environment. These results suggest that this conserved domain experiences a "rotational" or "flipping" movement during the catalytic cycle.

A distant homologue of ArsA is NifH, the iron protein subunit of the nitrogenase complex (*53*). NifH is a 32 kDa protein with a consensus ATP binding site. It functions as a 64 kDa homodimer with ATPase activity. Thus NifH is equivalent to an ArsA A1 or A2 domain, and the homodimer is equivalent to a whole ArsA. NifH passes electrons to the molybdenum-iron protein of nitrogenase, using the energy from ATP hydrolysis to lower the reducing potential of the [4Fe-4S] cluster in NifH. The cysteines that anchor the [4Fe-4S] cluster are equivalent to Cys117 and Cys422 in ArsA. It is instructive to consider the relationship of the ATP binding domain and [4Fe-4S] cluster in NifH. It has been proposed that in NifH the sequence between Asp125 and Cys132 is a MgATP signal transduction pathway, which spans the shortest distance between the MgATP binding site and the [4Fe-4S] cluster. Binding of MgATP results in a movement of this peptide chain and causes changes in environment of the [4Fe-4S] cluster (*54*). Similar sequences (commonly designated as the "Switch II" region) also exist in a class of GTPase signal transduction proteins (*55*); in those proteins nucleotide-binding and hydrolysis-induced conformational changes appear to provide kinetic triggers for other functions of the proteins. Strikingly, our preliminary results also showed that Asp142, located at the position corresponding to Asp125 in NifH, is important to the function of ArsA ATPase, possibly one ligand to the ATP bound Mg^{2+} (T. Zhou and B. P. Rosen, unpublished result). Preliminary modeling of ArsA on the crystal structure of NifH (*53*) leads us to hypothesize that the DTAP domain of ArsA and its homologues is a transduction domain that may be involved in the transmission of the energy of ATP hydrolysis to other functions of the Ars pump, such as transport of arsenite through the ArsB subunit or transmission of information from an allosteric to a catalytic domain.

B. The ArsA-ArsB complex is an obligatory ATP-coupled efflux pump for arsenicals and antimonials. As described above, ArsA is the catalytic subunit of the Ars pump and exhibits As(III)/Sb(III) stimulated ATPase activity. ArsB is a 45 kDa inner membrane protein which serves as a membrane anchor for the ArsA protein and functions as an anion-conducting pathway. To function as a primary pump, ArsA and ArsB form a tight membrane bound complex that can only be dissociated in the presence of high concentrations of urea or KCl (*56*). We have developed an *in vitro* assay for the quantitative determination of ArsA bound to ArsB-containing membrane vesicles (*17*). ArsA binds to the vesicles in saturable manner and the binding was stimulated in the presence of the substrate of the pump, arsenite or antimonite. As mentioned above, ArsA functions as a homodimer. Thus, ArsA-ArsB complex is expected to contain the ArsA homodimer (Fig. 4A), although this has not been directly demonstrated.

From *in vivo* and *in vitro* studies, we have demonstrated that the ArsA-ArsB complex is an obligatorily ATP-coupled primary pump (*17, 57*). In cells expressing both the *arsA* and *arsB* genes chemical energy was shown to be both necessary and sufficient for extrusion of arsenicals, while electrochemical energy was neither (*58, 59*). A *unc* strain of *E. coli* lacking the H^+-translocating ATPase that catalyzes the equilibrium between ATP and the electrochemical proton gradient was used. When starved for endogenous energy reserves, this strain will produce only chemical energy when given glucose in the presence of an inhibitor of respiration such as cyanide. On the other hand, it will generate only electrochemical energy when given a respiratory substrate such as succinate. This allows the establishment of intracellular conditions where either chemical energy or electrochemical energy is available for transport (*60*). In such experiments arsenical extrusion was conclusively demonstrated to be linked to chemical energy. While there was a temporal relationship of extrusion with ATP levels, the identity of the compound used as the energy source could not be determined from *in vivo* experiments. However, direct coupling of ATP to $^{73}AsO_2^-$ transport was demonstrated *in vitro* using everted (inside-out) membrane vesicles. Everted membrane vesicles have an orientation opposite to that in intact cells, so, while cells extrude arsenite, vesicles accumulate it. The vesicles were made from *E. coli* cells defective in the *unc* genes, so that coupling of the ArsA-ArsB complex to ATP hydrolysis was direct. Thus the R773 ArsA-ArsB complex is clearly an obligatorily ATP-coupled pump. ATP could not be replaced with other nucleoside triphosphates nor by nonhydrolyzable ATP analog, ATPγS. ATP-dependent transport by the ArsA-ArsB complex was insensitive to uncouplers. Transport in vesicles prepared from *unc* strains could not be driven by oxidation of lactate or NADH, demonstrating that electrochemical energy was neither necessary nor sufficient for the Ars pump. As with other transport ATPases, arsenite transport has an absolute requirement for divalent cation specifically Mg^{2+} or Mn^{2+}. Neither vanadate, an inhibitors of P-type ATPases, nor azide, an inhibitor of F-type ATPases, inhibited arsenite accumulation.

C. Dual modes of energy coupling of ArsB. While the ArsA-ArsB complex is clearly an ATP-coupled pump, several observations suggest that the situation is more

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

complicated. First, the secondary structure of the 429 residue ArsB protein, with twelve membrane spanning α-helices (*31*), is more similar to that of secondary carrier proteins than a pump subunit. Second, almost a dozen *ars* operons have been sequenced to date. Some have the same five gene *arsRDABC* operon structure, while others have only *arsRBC* (*23, 36, 61*). Third, expression of the R773 *arsB* gene in the absence of the *arsA* gene confers an intermediate level of arsenite resistance in *E. coli*, showing that it functions in the absence of an *arsA* gene (*60*). These quite surprising observations raise the question of how a transport ATPase could function without a catalytic subunit.

We therefore considered the possibility that ArsB could function as a secondary arsenite carrier in the absence of the ATPase subunit. The mode of energy coupling of ArsB-catalyzed efflux was determined in the presence and absence of an ArsA subunit (*62*). A primary pump would show dependency on chemical energy, while a secondary porter would require electrochemical energy. When *arsA* and *arsB* were co-expressed, the system required chemical energy, consistent with our previous observations (*60*). However, when the cells expressed only *arsB*, transport required electrochemical energy and became uncoupler sensitive. The results strongly indicate that, by itself, ArsB is a secondary carrier coupled to the proton motive force (Fig. 4B).

Again, the *in vivo* experiments were confirmed by uptake experiments in everted membrane vesicles (*62*). In contrast to vesicles containing the ArsA-ArsB pump, which used ATP for arsenite transport, ArsB-containing everted membrane vesicles accumulated $^{73}AsO_2^-$ dependent on formation of an electrochemical gradient, positive interior. With only ArsB no transport was observed with ATP. Accumulation was uncoupler sensitive but insensitive to weak bases that would dissipate only the chemical gradient of protons. These results suggest that ArsB catalyzes arsenite transport coupled to the membrane potential, perhaps as an electrophoretic uniporter.

Most likely the substrate is an oxyanionic form of As(III) or Sb(III). Soft metal chemistry is involved in the regulatory functions of the operon, with ArsR, ArsD, and ArsA each containing critical cysteine residues that interact with the metalloid. In contrast, ArsB has no critical cysteine residues. It has a single cysteine residue that was shown by site-directed mutagenesis experiments not to be essential for ArsB function. This illustrates the point that the system can use different types of chemistries for different functions. Soft metal-thiol interactions are very strong, approaching the strength of covalent bonds. This provides an excellent way to sense arsenic and to turn on the genes transcriptionally or the ArsA ATPase allosterically. However, for a transport system to be effective, it must be able to bind substrate on one side of the membrane and release it on the other; low affinity oxyanion binding is much appropriate for this type of reaction.

Why should there be two modes of energy coupling? If ArsB alone is sufficient for providing the resistance to arsenite and antimonite through a secondary transport mechanism, why is this not sufficient? Expression of *arsB* by itself confers a level of resistance that is lower than the *arsA* and *arsB* genes together (*60*), which probably reflects the fact that thermodynamically secondary carriers are less efficient mechanisms for creating concentration gradients than are primary pumps (*2*). It is reasonable to speculate that an *arsRBC* operon would have evolved first. Later capture of an ancestral *arsA* gene would have then allowed evolution of the more efficient pump. We have proposed that other multisubunit transport ATPases may have evolved similarly, with the catalytic and translocation sectors evolving independently as soluble ATPases and secondary transporters (*63*).

REFERENCES

1. Borst, P. and Ouellette, M. (1995) New mechanisms of drug resistance in parasitic protozoa. *Annu. Rev. Microbiol.* **49**, 427-460
2. Dey, S. and Rosen, B.P. (1995) Mechanisms of drug transport in prokaryotes and eukaryotes in *Drug Transport in Antimicrobial and Anticancer Chemotherapy* (Georgopapadakou, N.H., ed.) pp. 103-132, Dekker, New York
3. Rosen, B.P. (1996) Bacterial resistance to heavy metals. *J. Biol. Inorg. Chem.* **1**, 273-277
4. Mobley, H.L.T. and Rosen, B.P. (1982) Energetics of plasmid-mediated arsenate resistance in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **79**, 6119-6122
5. Dey, S. and Rosen, B.P. (1996) Heavy metal transporters in *Handbook of Biological Physics, Vol. II, Transport Processes in Membrane*, pp. 1-19, Elsevier, Amsterdam
6. Hughes, V.M. and Datta, N. (1983) Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature* **302**, 725-726
7. Foye, W.O. (1977) Antimicrobial activities of mineral elements in *Microorganisms and Minerals* (Weinberg, E.D., ed.) Microbiology series, Vol. 3, p. 387, Marcel Dekker, New York
8. Knowles, F.C. and Benson, A.A. (1983) The biochemistry of arsenic. *Trends Biochem. Sci.* **8**, 178-180
9. Novick, R.P. and Roth, C. (1968) Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. *J. Bacteriol.* **95**, 1335-1342
10. Silver, S. (1996) Bacterial resistances to toxic metal ions. *Gene* **179**, 9-19
11. Kaur, P. and Rosen, B.P. (1992) Plasmid-encoded resistance to arsenic and antimony. *Plasmid* **27**, 29-40
12. Ji, G. and Silver, S. (1992) Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid pI258. *J. Bacteriol.* **174**, 3684-3694
13. Summers, A.O. (1992) Untwist and shout: a heavy metal-responsive transcriptional regulator. *J. Bacteriol.* **174**, 3097-3101
14. Carlin, A., Shi, W., Dey, S., and Rosen, B.P. (1995) The *ars* operon of *Escherichia coli* confers arsenical and antimonical resistance. *J. Bacteriol.* **177**, 981-986
15. San Francisco, M.J.D., Hope, C.L., Owolabi, J.B., Tisa, L.S., and Rosen, B.P. (1990) Identification of the metalloregulatory element of the plasmid-encoded arsenical resistance operon. *Nucleic Acids Res.* **18**, 619-624
16. Wu, J.H. and Rosen, B.P. (1993) The *arsD* gene encodes a second *trans*-acting regulatory protein of the plasmid-encoded arsenical resistance operon. *Mol. Microbiol.* **8**, 615-623
17. Dey, S., Dou, D., and Rosen, B.P. (1994) ATP-dependent transport in everted membrane vesicles of *Escherichia coli*. *J. Biol. Chem.* **269**, 25442-25446
18. Oden, K.L., Gladysheva, T.B., and Rosen, B.P. (1994) Arsenate reduction mediated by the plasmid-encoded ArsC protein is coupled to glutathione. *Mol. Microbiol.* **12**, 301-306
19. Gladysheva, T.B., Oden, K.L., and Rosen, B.P. (1994) Properties of the arsenate reductase of plasmid R77. *Biochemistry* **33**, 7287-7293
20. Silver, S., Budd, K., Leahy, K.M., Shaw, W.V., Hammond, D., Novick, R.P., Willsky, G.R., Malamy, M.H., and Rosenberg, H. (1981) Inducible plasmid-determined resistance to arsenate, arsenite, and antimony (III) in *Escherichia coli* and *Staphylococcus aureus*. *J. Bacteriol.* **146**, 983-996

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

21. Gotz, F., Zabielski, J., Philipson, L., and Lindberg, M. (1983) DNA homology between the arsenate resistance plasmid pSX267 from *Staphylococcus xylosus* and the penicillinase plasmid pI258 from *Staphylococcus aureus*. *Plasmid* **9**, 126-137
22. Wu, J. and Rosen, B.P. (1991) The ArsR protein is a *trans*-acting regulatory protein. *Mol. Microbiol.* **5**, 1331-1336
23. Rosenstein, R., Peschel, A., Wieland, B., and Gotz, F. (1992) Expression and regulation of the antimonite, arsenite, and arsenate resistance operon of *Staphylococcus xylosus* plasmid pSX267. *J. Bacteriol.* **174**, 3676-3683
24. Wu, J. and Rosen, B.P. (1993) Metalloregulated expression of the *ars* operon. *J. Biol. Chem.* **268**, 52-58
25. Shi, W.P., Wu, J.H., and Rosen, B.P. (1994) Identification of a putative metal binding site in a new family of metalloregulatory proteins. *J. Biol. Chem.* **269**, 19826-19829
26. Yoon, K.P. and Silver, S. (1991) A second gene in the *Staphylococcus aureus cadA* cadmium resistance determinant of plasmid pI258. *J. Bacteriol.* **173**, 7636-7642
27. Morby, A.P., Turner, J.S., Huckle, J.W., and Robinson, N.J. (1993) SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene *smtA*: identification of a Zn inhibited DNA-protein complex. *Nucleic Acids Res.* **21**, 921-925
28. Shi, W.P., Dong, J., Scott, R.A., and Rosen, B.P. (1996) The role of arsenic-thiol interaction in metalloregulation of the *ars* operon. *J. Biol. Chem.* **271**, 9291-9297
29. Xu, C. and Rosen, B.P. (1997) Dimerization is essential for DNA binding and repression by the ArsR metalloregulatory protein of *Escherichia coli*. *J. Biol. Chem.* **272**, 15734-15738
30. Chen, Y. and Rosen, B.P. (1997) Metalloregulatory properties of the ArsD repressor. *J. Biol. Chem.* **272**, 14257-14262
31. Wu, J.H., Tisa, L.S., and Rosen, B.P. (1992) Membrane topology of the ArsB protein, the membrane subunit of an anion-translocating ATPase. *J. Biol. Chem.* **267**, 12570-12576
32. Nucifora, G., Chu, L., Misra, T.K., and Silver, S. (1989) Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium efflux ATPase. *Proc. Natl. Acad. Sci. USA* **86**, 3544-3548
33. Endo, G. and Silver, S. (1995) CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. *J. Bacteriol.* **177**, 4437-4441
34. Erbe, J.L., Taylor, K.B., and Hall, L.M. (1995) Metalloregulation of the cyanobacterial *smt* locus: identification of SmtB binding sites and direct interaction with metals. *Nucleic Acids Res.* **23**, 2472-2478
35. Turner, J.S., Glands, P.D., Samson, A.C., and Robinson, N.J. (1996) Zn^{2+}-sensing by the cyanobacterial metallothionein repressor SmtB: different motifs mediate metal-induced protein-DNA dissociation. *Nucleic Acids Res.* **24**, 3714-3721
36. Chen, C.M., Misra, T.K., Silver, S., and Rosen, B.P. (1986) Nucleotide sequence of the structural genes for an anion pump. The plasmid-encoded arsenical resistance operon. *J. Biol. Chem.* **261**, 15030-15038
37. Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982) Distantly related sequences in the α- and β-subunits of the ATP synthase, myosin kinase and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**, 945-951
38. Rosen, B.P., Weigel, U., Karkaria, C., and Gangola, P. (1988) Molecular characterization of an anion pump. The arsA gene product is an arsenite(antimonate)-stimulated ATPase. *J. Biol. Chem.* **263**, 3067-3070
39. Karkaria, C.E., Chen, C.M., and Rosen, B.P. (1990) Mutagenesis of a nucleotide-binding site of an anion-translocating ATPase. *J. Biol. Chem.* **265**, 7832-7836
40. Kaur, P. and Rosen, B.P. (1992) Mutagenesis of the C-terminal nucleotide-binding site of an anion-translocating ATPase. *J. Biol. Chem.* **267**, 19272-19277
41. Karkaria, C.E. and Rosen, B.P. (1991) Trinitrophenyl-ATP binding to the ArsA protein: the catalytic subunit of an anion pump. *Arch. Biochem. Biophys.* **288**, 107-111
42. Kaur, P. and Rosen, B.P. (1994) Identification of the site of [α-^{32}P]ATP adduct formation in the ArsA protein. *Biochemistry* **33**, 6456-6461
43. Kaur, P. and Rosen, B.P. (1993) Complementation between nucleotide binding domains in an anion-translocating ATPase. *J. Bacteriol.* **175**, 351-357
44. Kaur, P. and Rosen, B.P. (1994) In vitro assembly of an anion-stimulated ATPase from peptide fragments. *J. Biol. Chem.* **269**, 9698-9704
45. Li, J., Liu, S., and Rosen, B.P. (1996) Interaction of ATP binding sites in the ArsA ATPase, the catalytic subunit of the Ars pump. *J. Biol. Chem.* **271**, 25247-25252
46. Hsu, C.M., Kaur, P., Karkaria, C.E., Steiner, R.F., and Rosen, B.P. (1991) Substrate-induced dimerization of the ArsA protein, the catalytic component of an anion-translocating ATPase. *J. Biol. Chem.* **266**, 2327-2332
47. Abrahams, J.P., Leslie, A.G.W., Lutter, R., and Walker, J.E. (1994) Structure at 2.8Å resolution of F_1-ATPase from bovine mitochondria. *Nature* **370**, 621-628
48. Ksenzenko, M.Y., Kessel, D.H., and Rosen, B.P. (1993) Reaction of the ArsA adenosinetriphosphatase with 2-(4′-maleimidoanilino)naphthalene-6-sulfonic acid. *Biochemistry* **32**, 13362-13368
49. Bhattacharjee, H., Li, J., Ksenzenko, M.Y., and Rosen, B.P. (1995) Role of cysteinyl residues in metalloactivation of the oxyanion-translocating ArsA ATPase. *J. Biol. Chem.* **270**, 11245-11250
50. Bhattacharjee, H. and Rosen, B.P. (1996) Spatial proximity of Cys113, Cys172, and Cys422 in the metalloactivation domain of the ArsA ATPase. *J. Biol. Chem.* **271**, 24465-24470
51. Zhou, T., Liu, S., and Rosen, B.P. (1995) Interaction of substrate and effector binding sites in the ArsA ATPase. *Biochemistry* **34**, 13622-13626
52. Zhou, T. and Rosen, B.P. (1997) Tryptophan fluorescence reports nucleotide-induced conformational changes in a domain of the ArsA ATPase. *J. Biol. Chem.* **272**, 19731-19737
53. Georgiadis, M.M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J.J., and Rees, D.C. (1992) Crystallographic structure of the nitrogenase iron protein from *Azotobacter vinelandii*. *Science* **257**, 1653-1658
54. Ryle, M.J. and Seefeldt, L.C. (1996) Elucidation of a MgATP signal pathway in the nitrogenase iron protein: Formation of a conformation resembling the MgATP-bound state by protein engineering. *Biochemistry* **35**, 4766-4775
55. Kjeldgaard, M., Nyborg, J., and Clark, B.F.C. (1996) The GTP binding motif: variations on a theme. *FASEB J.* **10**, 1347-1368
56. Tisa, L.S. and Rosen, B.P. (1990) Molecular characterization of an anion pump: the ArsB protein is the membrane anchor for the ArsA protein. *J. Biol. Chem.* **265**, 190-194
57. Dey, S., Dou, D., Tisa, L.S., and Rosen, B.P. (1994) Interaction between the catalytic and the membrane components of an anion-translocating ATPase. *Arch. Biochem. Biophys.* **311**, 418-424
58. Mobley, H.L.T. and Rosen, B.P. (1982) Energetics of plasmid-mediated arsenate resistance in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **79**, 6119-6122
59. Rosen, B.P. and Borbolla, M.G. (1984) A plasmid-encoded arsenite pump produces arsenite resistance in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **124**, 760-765
60. Dey, S. and Rosen, B.P. (1995) Dual mode of energy coupling by the oxyanion-translocating ArsB protein. *J. Bacteriol.* **177**, 385-389
61. Bruhn, D.F., Li, J., Silver, S., Roberto, F., and Rosen, B.P. (1996) Arsenic resistance operon of IncN plasmid R46. *FEMS Microbiol. Lett.* **139**, 149-153
62. Kuroda, M., Dey, S., Sanders, O.I., and Rosen, B.P. (1997) Alternate energy coupling of ArsB, the membrane subunit of the Ars anion-translocating ATPase. *J. Biol. Chem.* **272**, 326-331
63. Rosen, B. P., Dey, S., Dou, D., Ji, G., Kaur, P., Ksenzenko, M. Y., Silver, S., and Wu, J. (1993) Evolution of an ion-translocating ATPase. *Ann. N.Y. Acad. Sci.* **671**, 257-272

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012