JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 911–913
0021-9193/96/$04.00+0

Vol. 178, No. 3

Soft Metal Thiol Chemistry Is Not Involved in the Transport of Arsenite by the Ars Pump

YANXIANG CHEN, SAIBAL DEY, AND BARRY P. ROSEN*

Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan 48201

Received 12 September 1995/Accepted 20 November 1995

The single cysteine in the ArsB protein subunit of the arsenite resistance pump was changed to serine and alanine residues. Resistance in cells expressing the two mutant *arsB* genes was the same as in the wild type, and the serine substitution had no effect on the arsenite transport properties. These results eliminate possible thiol chemistry in translocation. Thus, the pump uses soft metal chemistry for metalloactivation and nonmetal chemistry for oxyanion transport.

The ArsA and ArsB proteins encoded by the *ars* operon of R factor R773 are subunits of an ATP-coupled extrusion pump that produces resistance to arsenicals and antimonials in *Escherichia coli* (for recent reviews, see references 10 to 12). Arsenic and antimony are semimetals that can react with sulfur thiolates as soft metals. For example, in the complex of the product of the reaction of arsenite with dithiothreitol, the arsenite oxygens are displaced by the two sulfur thiolates and one hydroxyl oxygen of dithiothreitol (6). In this tricoordinate complex there are three heterocyclic rings, with an As-O bond of 1.83 Å (0.183 nm) and two As-S bonds of 2.24 and 2.25 Å (0.224 and 0.225 nm). The *ars* operon is transcriptionally regulated by the ArsR repressor, with arsenite and antimonite serving as inducers (16). Two cysteine residues, Cys-32 and Cys-34, have been identified as part of the inducer binding site, leading to the conclusion that release of the ArsR repressor from the operator site on the DNA results from a conformation change in the protein produced by coordination of As^{3+} or Sb^{3+} with the cysteine thiolates (15). Thus, transcriptional activation involves metal chemistry, not oxyanion binding. The ArsA subunit of the pump is an ATPase that is allosterically activated by coordination of As^{3+} or Sb^{3+} as soft metals to three cysteine thiolates, Cys-113, Cys-172, and Cys-422 (1). In the absence of the ArsA protein, the ArsB protein alone functions as a secondary carrier coupled to the proton motive force (9). However, the chemical nature of the transported species has not been determined. One possibility is that the oxyanions arsenite and antimonite are electrophoretically transported in response to the positive exterior membrane potential. Another possibility is that the transported species is a thiol conjugate of the soft metal. However, the results of in vitro transport studies in membrane vesicles eliminate that possibility (8). A third possibility is that As^{3+} or Sb^{3+} binds to cysteine thiolates in the ArsB protein and is transported as a soft metal (11). For that reason the role of the single cysteine, Cys-369, of the ArsB protein in transport and resistance was examined in this study.

Plasmids used in this study include the vector pBR322 (Ap^r and Tc^r) (2), pBC101 (*arsRDBC* Ap^r) (4), and plasmid pArsA3 (*arsA* Kn^r), which was derived from plasmid pArsA (*arsA*, Cm^r) (4) by insertion of the kanamycin resistance gene from plasmid pJBS633 (3) into the chloramphenicol resistance gene of pArsA. Plasmid pArsA was digested with *Nde*I, and the ends were made flush with the Klenow fragment of DNA polymerase I. The linearized plasmid was then digested with *Eco*RI, and the 6.2-kb fragment was recovered. Plasmid pJBS633 was digested with *Nco*I, the ends were made flush, the product was digested with *Eco*RI, and the 2.3-kb fragment was recovered. The two DNA fragments were ligated to produce plasmid pArsA3.

Using plasmid pBC101 as the template, the codon for Cys-369 of the *arsB* gene was changed to serine and alanine codons by site-directed mutagenesis by double-stranded DNA mutagenesis with two mutagenic primers (7) to introduce the codon changes in *arsB* and an additional primer to remove the unique *Sty*I site in the vector, allowing for enrichment and selection of mutants. The resulting plasmids were termed pBC102 ($arsB_{C369S}$) and pBC103 ($arsB_{C369A}$). The mutagenic primers (with the changes underlined) were GTCATTGG CAGCGATCTGGG and CAACGTCATTGGCGCCGATCT GGGGC for the serine and alanine changes, respectively, and AAACCAACCCCTGGCAGAACA for removal of the *Sty*I site. The identity of the mutations was confirmed by DNA sequencing. Double-stranded plasmid DNA was sequenced (14) with the Wizard Minipreps DNA purification system (Promega) and the Sequenase version 2.0 DNA sequencing kit (United States Biochemical). Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described elsewhere (5, 13). For assays of resistance to arsenite, cultures of *E. coli* AW10 (Δ*ars*::*cam*) (4) were grown overnight in Luria-Bertani (LB) medium (13) containing the appropriate antibiotics and diluted 100-fold into fresh medium without antibiotics and with various concentrations of sodium arsenite. The cultures were incubated at 37°C with shaking for 8 h, and the optical density at 590 nm was determined. For assays of the arsenite uptake in whole cells, cultures of *E. coli* AW10 containing various plasmids were grown for 8 h in LB medium at 37°C with shaking and diluted 100-fold into fresh LB medium containing 0.1 mM $NaAsO_2$. The cultures were incubated at 37°C with shaking overnight. Arsenite uptake assays were performed as described previously (9).

Cells bearing both plasmids pBC101 (*arsRDBC*) and pArsA (*arsA*) exhibited high-level resistance to arsenite (Fig. 1). When pBC101 bearing the wild-type *arsB* gene was replaced with either pBC102 or pBC103 bearing the mutant $arsB_{C369S}$ or $arsB_{C369A}$ gene, the level of arsenite resistant in the cells was

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, 540 E. Canfield Ave., Detroit, MI 48201. Phone: (313) 577-1512. Fax: (313) 577-2765. Electronic mail address: brosen@cms.cc.wayne.edu.

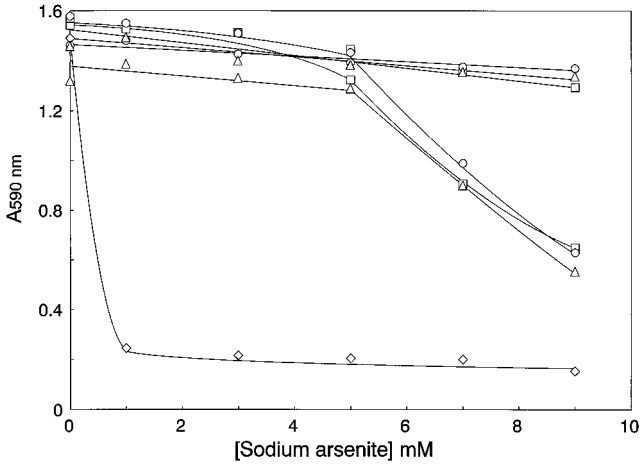

FIG. 1. Arsenite resistance in cells expressing the $arsB_{C369S}$ and $arsB_{C369A}$ genes. Arsenite resistance was assayed in cells of *E. coli* AW10 (Δ*ars*::*cam*) bearing the following plasmid(s): pBR322 (vector) and pArsA3 (*arsA*) (◆); pBC101 (*arsBC*) (□); pBC101 and pArsA3 (▤); pBC102 ($arsB_{C369S}C$) (○); pBC102 and pArsA3 (◍); pBC103 ($arsB_{C369A}C$) (△); or pBC103 and pArsA3 (▲).

unchanged. While expression of the R773 *arsA* and *arsB* genes confer high-level resistance, expression of the *arsB* gene in the absence of the *arsA* gene has been shown to confer an intermediate level of resistance to arsenite and antimonite (9). Cells with either pBC101, pBC102, or pBC103 alone exhibited the same intermediate level of arsenite resistance (Fig. 1). Thus, Cys-369 is not required for resistance.

The physiological response to arsenite results from the catalytic activity of the pump in extruding the toxic compound from the cell, and resistant cells exhibit reduced accumulation of arsenite compared with sensitive cells (10). The uptake of arsenite by intact cells expressing wild-type and mutant *arsB* genes was examined by using $^{73}AsO_2^-$ (Fig. 2). Cells with pBC101 and those with pBC102, both in the absence of the *arsA* gene, had equivalently reduced accumulation of $^{73}AsO_2^-$. When the *arsA* gene from plasmid pArsA was expressed in *trans* with either the wild-type or the mutant *arsB* gene, the level of accumulation decreased further than with *arsB* alone,

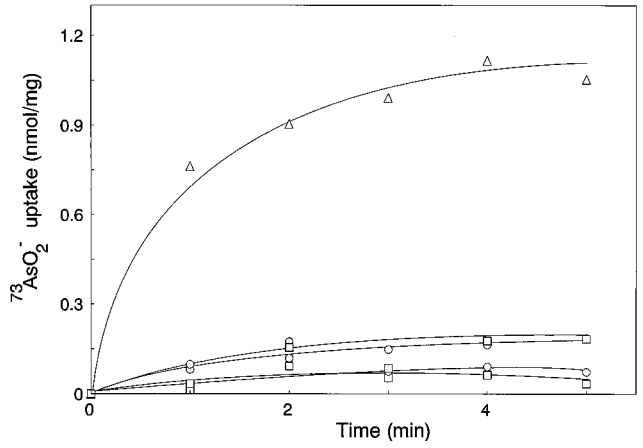

FIG. 2. ArsB-mediated arsenite exclusion. Accumulation of $^{73}AsO_2^-$ was measured in cells of *E. coli* AW10 bearing the following plasmid(s): pBR322 (vector) and pArsA3 (*arsA*) (▲); pBC101 (*arsBC*) (▤); pBC101 and pArsA3 (□); pBC102 ($arsB_{C369S}C$) (◍); or pBC102 and pArsA3 (○).

consistent with our previous observation that the ATP-coupled Ars pump, with both the ArsA and ArsB subunits, is a more efficient transport system than the ArsB secondary carrier (9). It is clear from these results that sulfur thiolate of the single cysteine residue in ArsB protein is not required for the arsenite transport activity of either the two-subunit pump or the single-subunit carrier.

In summary, the Ars anion transport system can be either an obligatory ATP-coupled primary pump or a secondary carrier coupled to the proton motive force, depending on the subunit composition of the transport complex (9). The ArsB protein can function as a secondary porter in the absence of the ArsA protein, and its topological structure is similar to those of many secondary porters (17). The ArsB protein contains a single cysteine residue, Cys-369, that has been predicted to be located in the 11th transmembrane membrane-spanning region. That residue was altered by site-directed mutagenesis to a serine or alanine residue. The $arsB_{C369S}$ and $arsB_{C369A}$ genes conferred resistance to the same levels of arsenite or antimonite as the wild-type protein, and the cells expressing the serine substitution exhibited the same arsenite transport properties as cells with the wild-type *arsB* gene. These results suggest that transport of arsenite by the ArsB protein does not involve metal thiol chemistry; electrophoretic anion transport is the most likely alternative. In contrast, the ArsR and ArsA proteins bind As^{3+} or Sb^{3+} as soft metals through interaction with cysteinyl residues in the transcriptional regulation by the ArsR repressor and activation of the ArsA ATPase (1, 12, 15). Consequently, the molecule of arsenite that interacts with the ArsA protein to allosterically activate is most likely not the molecule of arsenite translocated by the ArsB protein. We conclude that the Ars pump uses two distinct arsenic (or antimony) chemistries: soft metal binding for environmental recognition of arsenic and antimony compounds by the ArsR repressor and for metalloactivation of the ArsA ATPase subunit, and nonmetal chemistry for oxyanion transport catalyzed by the ArsB protein.

This work was supported by U.S. Public Health Service grant AI19793.

REFERENCES

1. **Bhattacharjee, H., J. Li, M. Y. Ksenzenko, and B. P. Rosen.** 1995. The role of cysteinyl residues in metalloactivation of an oxyanion-translocating ATPase. J. Biol. Chem. **270:**1–6.
2. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heyeneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:** 95–113.
3. **Broome-Smith, J. K., and B. G. Spratt.** 1986. A vector for the construction of translational fusions to TEM β-lactamase and the analysis of protein export signals and membrane protein topology. Gene **49:**341–349.
4. **Carlin, A., W. Shi, S. Dey, and B. P. Rosen.** 1995. The *ars* operon of *Escherichia coli* confers arsenical and antimonial resistance. J. Bacteriol. **177:** 981–986.
5. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
6. **Cruse, W. B. T., and M. N. G. James.** 1972. The crystal structure of the arsenite complex with dithiothreitol. Acta Crystallogr. Sect. B **28:**1325–1331.
7. **Deng, W. P., and J. A. Nickoloff.** 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. **200:**81–88.
8. **Dey, S., D. Dou, and B. P. Rosen.** 1994. ATP-dependent arsenite transport in everted membrane vesicles of *Escherichia coli*. J. Biol. Chem. **269:**25442–25446.
9. **Dey, S., and B. P. Rosen.** 1995. Dual mode of energy coupling by the oxyanion-translocating ArsB protein. J. Bacteriol. **177:**385–389.
10. **Dey, S., and B. P. Rosen.** 1995. Mechanisms of drug transport in prokaryotes and eukaryotes, p. 103–132. *In* N. H. Georgopapadakou (ed.), Drug transport in antimicrobial and anticancer chemotherapy. Marcel Dekker, Inc., New York.
11. **Dey, S., and B. P. Rosen.** Heavy metal transporters. *In* Handbook of biolog-

ical physics, vol. II. Transport processes in membrane, in press. Elsevier, New York.
12. **Rosen, B. P., H. Bhattacharjee, and W. P. Shi.** 1995. Mechanisms of metalloactivation of an anion-translocating ATPase. J. Bioenerg. Biomembr. **27:** 85–91.
13. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
14. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
15. **Shi, W., J. Wu, and B. P. Rosen.** 1994. Identification of a putative metal binding site in a new family of metalloregulatory proteins. J. Biol. Chem. **269:** 19826–19829.
16. **Wu, J., and B. P. Rosen.** 1993. Metalloregulated expression of the *ars* operon. J. Biol. Chem. **268:**52–58.
17. **Wu, J. H., L. S. Tisa, and B. P. Rosen.** 1992. Membrane topology of the ArsB protein, the membrane component of an anion-translocating ATPase. J. Biol. Chem. **267:**12570–12576.