Alternate Energy Coupling of ArsB, the Membrane Subunit of the Ars Anion-translocating ATPase*

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The arsenical resistance (ars) operon of the conjuga-
tive R-factor R773 confers resistance to arsenical and antimonial compounds in Escherichia coli, where resistance results from active extrusion of arsenite catalyzed by the products of the arsA and arsB genes. Previous in vivo studies on the energetics of arsenite extrusion showed that expression of both genes produced an ATP-coupled arsenite extrusion system that was independent of the electrochemical proton gradient. In contrast, in cells expressing only the arsB gene, arsenite extrusion was coupled to electrochemical energy and independent of ATP, suggesting that the Ars transport system exhibits a dual mode of energy coupling depending on the subunit composition. In vitro the ArsA-ArsB complex has been shown to catalyze ATP-coupled uptake of $^{73}$AsO$_2^-$ in everted membrane vesicles. However, transport catalyzed by ArsB alone has not previously been observed in vitro. In this study we demonstrate everted membrane vesicles prepared from cells expressing only arsB exhibit uptake of $^{73}$AsO$_2^-$ coupled to electrochemical energy.

Resistance to arsenical and antimonial compounds in bacterial cells is mediated by active extrusion of oxyanions of As(III) or Sb(III) from the cells (1–3). These efflux systems are encoded by ars operons. The ars operon of the conjugal R-factor R773 of Escherichia coli has been shown to encode an anion-translocating ATPase composed of two types of subunits, ArsA and ArsB. ArsA, the catalytic subunit, is a 63-kDa As(III)/Sb(III)-catalyzing ATPase composed of two types of subunits, ArsA and ArsB. ArsB, the membrane subunit, is a 63-kDa As(III)/Sb(III)-stimulated ATPase (4). ArsB is a 45-kDa inner membrane protein that catalyzes oxyanion translocation (5). The ArsA-ArsB complex has been shown both in vivo (6–8) and in vitro to be an obligatorily ATP-coupled primary pump (9).

However, recent results from several laboratories have shown that an arsB gene in the absence of an arsA gene is sufficient for resistance. While the ars operons of plasmids R773 (10) and R46 (11) have arsA genes, the homologous operons of the staphylococcal plasmids pI258 (12) and pSx267 (13) and the chromosomal ars operon of E. coli (14, 15) do not contain an arsA gene. Cells expressing the pI258 and E. coli chromosomal ars operons extrude arsenite (15, 16). There are at least two possible interpretations of these results. First, there could be a chromosomal arsA gene or homologue. Although this possibility cannot be ruled out, there are no data supporting it. Second, these Ars proteins could function independently of ArsA, perhaps as a secondary carrier protein. The chromosomally encoded ArsB is 79% identical and overall 90% similar to the R773 ArsB. It seems intuitively unlikely that such close homologues would catalyze different reactions. When the R773 arsB gene was expressed in the absence of arsA, it conferred arsenical resistance and active extrusion (8). These findings suggest that the ArsB protein alone can catalyze energy-dependent efflux in the absence of a catalytic subunit.

For these reasons the energetics of ArsB-catalyzed efflux was compared with that of the ArsAB pump (8). In cells expressing arsB alone, arsenite transport was coupled only to electrochemical energy, not chemical energy, suggesting that a chromosomally encoded ArsA protein is probably not involved. Interestingly, the transmembrane structure of the R773 ArsB is topologically identical to secondary membrane carriers, with 12 membrane spanning segments (5). From the aggregate of these results, we postulated that ArsB functions as a secondary arsenite transporter in the absence of an ArsA subunit, a novel dual mechanism of energy coupling of a transport system (8).

However, all of the data suggesting a role of ArsB in secondary anion translocation were from physiological studies. In this study, we provide the first direct in vitro evidence that ArsB catalyzes electrophoretic anion transport. We have constructed an arsB expression plasmid that has enabled us to measure arsenite transport activity in everted membrane vesicles. NADH respiration provided electrochemical energy for ArsB-mediated transport in vesicles. Transport was sensitive to the addition of uncouplers and depolarizing permeant anions, indicating that in these everted vesicles anion transport is coupled to an electrochemical proton gradient, positive interior.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction enzymes and nucleic acid modifying enzymes were purchased from Life Technologies, Inc. and Promega. Oligonucleotides were synthesized in the Macromolecular Core Facility of Wayne State University School of Medicine. Carrier-free $^{73}$AsO$_2^-$ was obtained from Los Alamos National Laboratories. All other chemicals were obtained from commercial sources.

Strains, Plasmids, and Media—E. coli strains and plasmids used in this study are listed in Table I. E. coli strains harboring indicated plasmids were grown in LB medium (17) at 37°C. Ampicillin (100 μg/ml), chloramphenicol (35 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml) was added as required. NaAsO$_2$ or isopropyl-1-thio-β-D-galactopyranoside (IPTG) was used at concentrations indicated.

DNA Manipulations—Plasmid DNA propagation, restriction enzyme treatment, ligation, and transformation were performed by minor mod-

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1 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; PIPES, 1,4-piperazinediethanesulfonic acid; Bicine, N,N-bis[2-hydroxyethyl]glycine.
ifications of published procedures (17). Plasmid DNA was isolated with Qiagen Plasmid kit (Qiagen) for sequence analysis by the method of Sanger et al. (18). Oligonucleotide-directed mutagenesis was performed using the Altered Sites™ II in vitro Mutagenesis System (Promega) according to the manufacturer’s directions. All mutations introduced were confirmed by sequencing using a Cy5-labeled sequencing kit and the ALFexpress system from Pharmacia Biotech Inc.

**Construction of an arsB Expression Plasmid—** Plasmid pcB101 was digested with EcoRI and KpnI, and a 2.9-kilobase fragment containing the entire gene was cloned into vector plasmid pALTER-1 (Promega), yielding plasmid pcB101. The BamHI site in the gene was not present in pcB101 and was added by Klenow fragment (T7, lac, and SP6 promoters, Tc). The resulting plasmid was used to clone the arsB gene into HindIII-digested pJHW101, and the cell suspension was incubated into LB medium containing kanamycin and grown overnight. Plasmid DNA was prepared and digested by EcoRI, and the product was transformed into JM109. Plasmid DNA was prepared and screened for loss of the EcoRI and Bgl II sites. The arsB fusion was confirmed by DNA sequencing. The resulting plasmid, pAO-BC, was used to clone the arsB gene fusion into plasmid pKM11. A Csp45I-HindIII fragment from plasmid pAO-BC into Csp45I-HindIII-digested pKM11, yielding plasmid pKMO-BC.

The ArSBC chimera was detected by immunoblotting of membrane protein separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Membrane protein was suspended in SDS sample buffer and heated at 80 °C for 10 min, following which the proteins were separated by SDS-PAGE on 15% polyacrylamide gels (21). Proteins were electrophoretically transferred to a nitrocellulose membrane (0.2 μm pore size, Schleicher & Schuell) overnight at 25 V and 4 °C. Antisera raised against purified ArsR was used to detect the ArSBC chimera. Immunoblotting was performed utilizing an enhanced chemiluminescence assay (DuPont NEN) and exposed to x-ray film at room temperature, as described previously (22).

**Other Methods—** Protein content was determined by a microdetermination of the procedure of Lowry et al. (23) using bovine serum albumin as a standard. ^73AsO_3^- was prepared by reduction of ^75AsO_4^2- (24).

**RESULTS**

**Construction of an arsB Expression Plasmid—** ATP-driven ^73AsO_3^- transport has been measured in membrane vesicles from cells expressing the arsA and arsB genes from the native
ars promoter. When the arsA gene was deleted from the R773 ars operon, the cells retained a low level resistance to arsenite (8), similar to that conferred by the chromosomal ars operon (15), which also lacks an arsA gene (14). However, no arsenite transport was observed in membrane vesicles from cells expressing arsB from the ars promoter regardless of the source of energy (data not shown). Previous attempts to express the R773 arsB gene at increased levels have been unsuccessful. The reasons for this are obscure, but possible explanations include instability of the polycistronic mRNA (25) and lethality of ArsB itself (26). In an attempt to increase expression, arsB was cloned behind the tac promoter of plasmid pKK223-3, producing plasmid pKMB1. This plasmid was transformed into three strains of E. coli in which the chromosomal ars operon had been disrupted: strains AW10, AW3110, and LE392ΔuncICΔars. However, only strain AW10 could be stably transformed, perhaps because, among the three strains, only AW10 contains a lacP promoter to control basal level of expression of arsB. Even in the absence of exogenously added IPTG, expression of arsB from pKMB1 produced low level arsenite resistance similar to that conferred by the chromosomal ars operon, and higher level resistance was observed when arsA was expressed in trans (Fig. 1). Although it is difficult to quantify the amount of ArsB due to the lack of a specific ArsB antisera, relative amounts of ArsB produced from the ars promoter could be compared with those from the tac promoter in pKMB1. In both plasmids, the arsB gene was fused in frame to the downstream arsC gene, and the chimeras were detected with antisera directed against ArsC (Fig. 2). The arsBC gene fusion produced arsenite resistance comparable to the wild type arsB gene (data not shown). In neither case could the ArsBC chimera be visualized by Coomassie staining of the gels. Even using very sensitive chemiluminescent methods, the ArsBC chimera produced from the ars promoter could not be detected (Fig. 2, lane 2). However, under control of the tac promoter, the ArsBC chimera could be detected immunologically (Fig. 2, lanes 3 and 4). ArsB was produced even in the absence of inducer, with a several fold increase following IPTG induction. As shown above, phenotype expression of arsenite resistance from the tac promoter likewise did not require induction (Fig. 1).

AsO₂⁻₃ Transport in Everted Membrane Vesicles—The results of the in vivo study of energetics of transport suggested that ArsB alone could catalyze arsenite extrusion coupled to electrochemical energy (8). To test this hypothesis, ⁷⁸AsO₂⁻₃ uptake was measured in everted membrane vesicles prepared from cells of E. coli strain AW10 bearing plasmid pKMB1. In these experiments, the cells were induced with IPTG to maximize expression of ArsB. The membrane vesicles exhibited time- and NADH-dependent ⁷³AsO₂⁻₃ accumulation (Fig. 3). Membrane vesicles prepared from cells harboring vector plasmid pKK223-3 showed no transport. Transport required NADH oxidation, as shown by the complete inhibition by KCN. The uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) completely reversed NADH-dependent ⁷³AsO₂⁻₃ uptake (Fig. 3), as did the combination of valinomycin plus nigericin (Table II). These results clearly show that arsenite transport catalyzed by ArsB alone is coupled to the electrochemical proton gradient established by NADH respiration. In contrast, little transport was observed with ATP (Table II).

Effect of Permeant Anions and Weak Base on ⁷³AsO₂⁻₃ Transport—The effect of permeant anions and a permeant weak base was examined (Table II). In these experiments, the vesicles were prepared in a sulfate-containing buffer. Under such conditions, the electrochemical gradient has been shown to be primarily in the form of a membrane potential, positive interior, with little or no pH gradient (27). NH₄⁺, which dissipates the remaining ΔpH, had a small effect on the initial rate of ⁷³AsO₂⁻₃ accumulation. NH₄Cl was more inhibitory, attributable to an effect of Cl⁻ as a permeant anion. The effect of SCN⁻ and ClO₄⁻ ions even more permselective than Cl⁻, considerably reduced transport activity.

Concentration Dependence for Arsenite—The concentration dependence for arsenite exhibited saturation kinetics, with an apparent Kₘ of 0.14 mM (Fig. 4). This is essentially identical with the apparent Kₘ of 0.1 mM for the ArsA-ArsB pump (9), suggesting that the mechanism of transport by ArsB is independent of the source of energy.

Effect of pH and Oxyanions on ⁷³AsO₂⁻₃ Transport—Transport activity was maximal at pH 7 and decreased between 7 and 9 (Fig. 5). The effects of oxyanions on ⁷³AsO₂⁻₃ transport via ArsB was examined. Among the oxyanions tested, the sodium or potassium salts of ⁷³AsO₂⁻₃, PO₄³⁻, NO₃⁻, NO₂⁻, SO₄²⁻, and SeO₃²⁻ had little effect on the arsenite transport (Table III), indicating that ArsB does not catalyze nonspecific anion movement.

In contrast, potassium antimonyl tartrate was found to stimulate ⁷³AsO₂⁻₃ transport 4- to 5-fold. The effect was specific for Sb(III) salts; sodium potassium tartrate had no effect on ⁷³AsO₂⁻₃ transport, and the same stimulation was observed with antimony trichloride, which would be expected to hydrate to an
antimonite oxyanion. This stimulatory effect of antimonite was further investigated. ArsB was required for Sb(III)-stimulated $^{73}$AsO$_2$$_2$ accumulation (Fig. 6). Transport required NADH oxidation in the presence and absence of antimonite, and in both cases FCCP inhibited. Thus, this effect appears to be a property of the ArsB-mediated transport system and not a nonspecific effect of Sb(III). The degree of stimulation required stoichiometric amounts of antimonite and arsenite (Fig. 7). Importantly, at each concentration of arsenite examined, the maximal stimulation occurred at an equimolar concentration of Sb(III) (Fig. 7).

The solution chemistry of arsenicals and antimonials is not well characterized. In solution, it might be expected that the arsenite oxyanion ($\text{AsO}_2$$_2$) would be hydrated to $\text{As(OH)}_2$O$^-$, which is in equilibrium with the protonated form As(OH)$_3$. However, other reasonable forms could be suggested, such as $\text{O(HO)-As-O-As(OH)}_2$ or, when arsenite and antimonite are added together, a mixed salt such as $\text{O(HO)-As-O-Sb(OH)}_2$ might be formed. If antimonial oxyanions were better substrates for the transport system than arsenicals, such mixed salts could explain the substantial stimulation of $^{73}$As(III) uptake by Sb(III). In both transcriptional regulation by ArsR and allosteric regulation by ArsA, Sb(III) is more effective than As(III). Thus, it would not be surprising if antimonials were a better substrate for ArsB than arsenicals. Al-
Alternatively, an allosteric effect of Sb(III) on the carrier itself cannot be ruled out. Sb(III) allosterically activates the ArsA ATPase, but it does by binding to a triad of cysteine thiolates (4), while there are no essential thiols in ArsB (28). Further arguing against an allosteric effect is the fact that maximal stimulation requires stoichiometric amounts of As(III) and Sb(III) at all concentrations of As(III). These results suggest that a mixed salt containing both As(III) and Sb(III) can be transported by the carrier.

**DISCUSSION**

From the results of previous in vivo and in vitro studies, we concluded that the arsA and arsB gene products of the R773 ars operon form a membrane-bound complex that functions as an obligatorily ATP-coupled arsenite pump. First, arsenical extrusion from cells of *E. coli* exhibited dependence on chemical energy; electrochemical energy was neither necessary nor sufficient (6, 7). Although the form of chemical energy could not be unambiguously identified from those physiological studies, there was a correlation between ATP levels and extrusion. Second, the transport system was shown to be a complex of two subunits, ArsA and ArsB (22), where ArsA is an As(III)/Sb(III)-stimulated ATPase. Third, everted membrane vesicles containing the ArsA-ArsB complex exhibited energy-dependent accumulation of $^{73}\text{AsO}_2^-$ (9). In vitro transport had an absolute requirement for ATP. Again, electrochemical energy was neither necessary nor sufficient. In these cells, the $\text{H}^+$-translocating F$_0$F$_1$ ATPase was deleted, so coupling of the ArsA-ArsB complex to ATP hydrolysis was direct.

However, as described above, several findings posed a question with respect to the energy coupling of the Ars system. Most intriguing was the observation that three of five ars operons lack an arsA gene. Only the operons from *E. coli* plasmids R773 (10) and R46 (11) have arsA genes. The two staphylococcal (12, 13) and the *E. coli* chromosomal (14) operons do not. Obviously it would be difficult to have an ATP-coupled pump without an ATPase subunit. One possibility is that the two types of extrusion systems have different biochemical mechanisms. The close similarity of the R773, R46, and *E. coli* chromosomal ArsB proteins (each exhibits over 90% similarity to the other two) would suggest that the proteins should have a common mechanism. Even the ArsBs from the staphylococcal plasmids, which are less than 60% similar to the proteins from the *E. coli* proteins, have essentially superimposable hydropathic profiles, suggesting similar membrane topology (29). Indeed, chimeras of the ArsB proteins from the Gram-positive and Gram-negative bacteria constructed by gene fusions of the arsB genes are functional (29). A reasonable deduction is that all ArsBs have the same biochemical mechanism.

Do ArsBs function as components of primary pumps, as the in vivo energetics studies indicate (6, 7)? Are they secondary carriers, as suggested by their transmembrane topology (5, 28)?
These possibilities are not mutually exclusive. Indeed, results of in vivo transport studies suggested that the ArsB protein mediates the electrochemical energy-dependent arsenite efflux in the absence of the ArsA protein while the ArsA-ArsB complex catalyzes chemical energy-dependent transport (8). The p258-encoded ArsB similarly had been proposed to utilize electrochemical energy (30). The results in this study clearly demonstrate that ArsB functions as a secondary arsenite transporter under conditions where there is only \( \Delta \psi \) (27), consistent with the carrier catalyzing electrophoretic anion movement coupled to the membrane potential. The fact that some \( \text{ars} \) operons have both \( \text{arsA} \) and \( \text{arsB} \) genes and thus encode pumps, while others have only the \( \text{arsB} \) gene and encode secondary systems suggests that the acquisition of a gene for a catalytic subunit might be a recent evolutionary event. Independence from electrochemical gradients would make the cell less susceptible to depolarizing environmental poisons. Therefore, the Ars system is a novel transport system that physiologically has two possible modes of energy coupling depending on its subunit composition (Fig. 8).

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