

Original Research Report

Structure-Function Analysis of the ArsA ATPase: Contribution of Histidine Residues¹

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The ArsA ATPase is the catalytic subunit of the ArsAB oxyanion pump in *Escherichia coli* that is responsible for extruding arsenite or antimonite from inside the cell, thereby conferring resistance. Either antimonite or arsenite stimulates ArsA ATPase activity. In this study, the role of histidine residues in ArsA activity was investigated. Treatment of ArsA with diethyl pyrocarbonate (DEPC) resulted in complete loss of catalytic activity. The inactivation could be reversed upon subsequent incubation with hydroxylamine, suggesting specific modification of histidine residues. ATP and oxyanions afforded significant protection against DEPC inactivation, indicating that the histidines are located at the active site. ArsA has 13 histidine residues located at position 138, 148, 219, 327, 359, 368, 388, 397, 453, 465, 477, 520, and 558. Each histidine was individually altered to alanine by site-directed mutagenesis. Cells expressing the altered ArsA proteins were resistant to both arsenite and antimonite. The results indicate that no single histidine residue plays a direct role in catalysis, and the inhibition by DEPC may be caused by steric hindrance from the carbethoxy group.

KEY WORDS: ArsA; ATPase; arsenite resistance; histidine; diethylpyrocarbonate; site-directed mutagenesis.

Plasmid R773 confers resistance to arsenicals and antimonials in *Escherichia coli* (Hedges and Baumberg, 1973) by extrusion of arsenite from the cells (Silver *et al.*, 1981) coupled to ATP (Moblely and Rosen, 1982). The *ars* operon of plasmid R773 consists of five genes, *arsRDABC* (Chen *et al.*, 1986; San Francisco *et al.*, 1990; Wu and Rosen, 1993). The *arsA* and *arsB* genes encode the pump, where the ArsA and ArsB are the subunits of an arsenite-translocating ATPase located in the inner membrane of *E. coli* (Tisa and Rosen, 1990). ArsA is the catalytic subunit that hydrolyzes ATP in the presence of either arsenite or antimonite (Hsu and Rosen, 1989), and the released energy is coupled to extrusion of the

oxyanions out of the cell through ArsB, an integral membrane protein (Dey *et al.*, 1994). In the absence of ArsB, ArsA accumulates as a cytosolic protein, facilitating over-expression and purification as a soluble ATPase (Rosen *et al.*, 1988).

The 583-residue ArsA polypeptide is composed of N-terminal (A1) and C-terminal (A2) halves with significant amount of internal homology, suggesting that the present *arsA* gene arose by gene duplication and fusion of its primordial ancestor (Chen *et al.*, 1986). Each half has a nucleotide binding domain (NBD) or the Walker A motif (GKGGVGKT) (Walker *et al.*, 1982) that interacts with the phosphate moiety of ATP. Both NBDs have been shown to be important for catalytic activity and resistance (Karkaria *et al.*, 1990; Kaur and Rosen, 1992). In the Walker B region of the A1 and A2 halves is a conserved aspartate residue that corresponds to Asp45 in the A1 half. By site-directed mutagenesis, Asp45 was shown to be a ligand to Mg²⁺ in the nucleotide-binding site (Zhou and Rosen, 1999). The A1 and A2 halves are held together by a 25-residue linker peptide (Li and Rosen, 2000). The molecular mechanism of ArsA activation has been well-studied (Rosen *et al.*, 1999). In the absence

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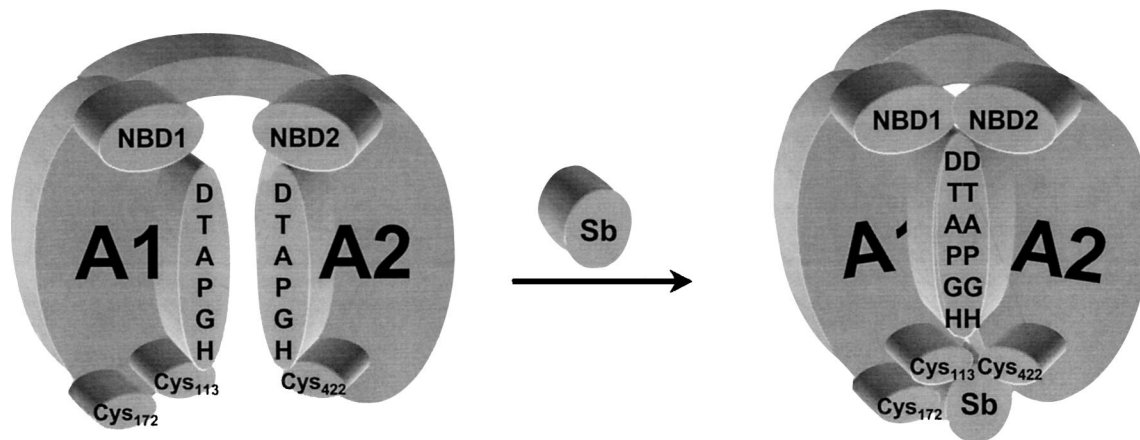


Fig. 1. Model of *ArsA* catalysis. In the absence of metalloid activator, the A1 and A2 halves of *ArsA* are only loosely associated, if at all. In this state the enzyme exhibits only a low, basal rate of ATP hydrolysis. Binding of metalloid, either Sb(III) or As(III), at the allosteric site, which contains Cys113, Cys172, and Cys422, produces a conformational change that results in an interface being formed between A1 and A2. The DTAP domains are involved in transmission of the metalloid status of the allosteric site to the nucleotide binding domains, promoting catalysis.

of the allosteric activator arsenite or antimonite, *ArsA* has a basal level of ATPase activity. The allosteric activators coordinate with Cys113 and Cys172 in the A1 half and Cys422 in the A2 half, producing a conformational change that brings the A1 and A2 NBDs into contact with each other, thus accelerating catalysis (Bhattacharjee *et al.*, 1995; Bhattacharjee and Rosen, 1996) (Fig. 1). The linker insures that the two halves of the protein are always in proximity of each other and thus facilitates the interaction of the two halves (Li and Rosen, 2000).

The single allosteric site and the two catalytic sites are connected by signal transduction or DTAP domains that consist of the sequences D₁₄₂TAPTGH₁₄₈ and D₄₄₇TAPTGH₄₅₃, respectively in the A1 and A2 halves (Zhou and Rosen, 1997). Conformational changes in the A1 DTAP domain have been visualized by monitoring intrinsic tryptophan fluorescence of mutants containing single tryptophans at either the N- or C-terminal ends of the domain (Zhou *et al.*, 1995; Zhou and Rosen, 1997). The catalytic cycle of the ATPase reaction has been elucidated from transient kinetic analysis of the fluorescence changes (Walmsley *et al.*, 1999).

The role of other residues in catalysis is the topic of current study. Histidine residues are frequently involved in general acid–base catalysis because the pK_a of the imidazolium nitrogen is near physiological pH (Jia *et al.*, 2000; Quirk *et al.*, 1998). In addition, histidine residues can stabilize reaction intermediates electrostatically and are also found in metal-ion binding sites (Drohat *et al.*, 1999; Lesburg *et al.*, 1997; Vinarov and Nowak, 1999). For these reasons the possible catalytic role of one or more of the thirteen-histidine residues of *ArsA* was investigated

using a combination of chemical modification and site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media

E. coli strains and plasmids used in this study are described in Table I. Cells were grown at 37°C in LB medium (Sambrook *et al.*, 1989). Ampicillin (125 µg/mL) and tetracycline (10 µg/mL) were added as required. Sodium arsenite, potassium antimonyl tartrate, or isopropyl β-D-thiogalactopyranoside were added at the indicated concentrations. All chemicals were obtained from commercial sources.

Oligonucleotide-Directed Mutagenesis

Mutations in the sequence of the *arsA* gene were introduced by site-directed mutagenesis using the Altered Sites® II *in vitro* Mutagenesis System (Promega). Plasmid pABH6 containing the *arsA* and *arsB* genes was used as the template to introduce the desired substitutions. The oligonucleotides used for mutagenesis of the *arsA* gene are shown in Table II.

DNA Manipulation and Sequence Analysis

Plasmid DNA was purified using QIAprep Spin Miniprep Kit (QIAGEN). DNA restriction endonuclease analysis, ligation and transformation were performed as

Table I. Strains and Plasmids

Strain/Plasmid	Genotype/Description	Reference
<i>E. coli</i> strains		
JM109	<i>endA1, recA1, gyrA96, thi, hsdR17</i> (r_k^- , m_k^+), <i>relA1, supE44, λ^-, $\Delta(lac-proAB)$, [F', <i>traD36, proA⁺B⁺</i>, <i>lacI^fZΔM15]</i></i>	(Sambrook <i>et al.</i> , 1989)
ES1301 <i>murS</i>	<i>lacZ53, mutS201::Tn5, thyA36, rha-5, metB1, deoC, IN(rrnD-rrnE)</i>	Promega
Plasmids		
pALTER [®] -1	Cloning and mutagenesis vector, Tc ^r	Promega
pALTER-AB (<i>arsAB</i>)	3.2-kilobase pair <i>HindIII-KpnI</i> fragment containing <i>arsA</i> and <i>arsB</i> genes cloned into the multiple cloning site of pALTER [®] -1 vector, <i>arsAB</i> , Tc ^r	(Bhattacharjee <i>et al.</i> , 1995)
pABH6 (<i>arsA</i> _{[His]₆B)}	pALTER-AB with six histidine codons added to 3'-end of <i>arsA</i> , Tc ^r	Zhou and Rosen, unpublished
pH138A (<i>arsA</i> _{H138A} -[His] ₆ B)	Site-directed mutagenesis of codon 138 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH148A (<i>arsA</i> _{H148A} -[His] ₆ B)	Site-directed mutagenesis of codon 148 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH219A (<i>arsA</i> _{H219A} -[His] ₆ B)	Site-directed mutagenesis of codon 219 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH327A (<i>arsA</i> _{H327A} -[His] ₆ B)	Site-directed mutagenesis of codon 327 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH359A (<i>arsA</i> _{H359A} -[His] ₆ B)	Site-directed mutagenesis of codon 359 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH368A (<i>arsA</i> _{H368A} -[His] ₆ B)	Site-directed mutagenesis of codon 368 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH388A (<i>arsA</i> _{H388A} -[His] ₆ B)	Site-directed mutagenesis of codon 388 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH397A (<i>arsA</i> _{H397A} -[His] ₆ B)	Site-directed mutagenesis of codon 397 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH453A (<i>arsA</i> _{H453A} -[His] ₆ B)	Site-directed mutagenesis of codon 453 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH465A (<i>arsA</i> _{H465A} -[His] ₆ B)	Site-directed mutagenesis of codon 465 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH477A (<i>arsA</i> _{H477A} -[His] ₆ B)	Site-directed mutagenesis of codon 477 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH520A (<i>arsA</i> _{H520A} -[His] ₆ B)	Site-directed mutagenesis of codon 520 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study
pH558A (<i>arsA</i> _{H558A} -[His] ₆ B)	Site-directed mutagenesis of codon 558 to Ala codon in <i>arsA</i> gene of pABH6, Tc ^r , Amp ^r	This study

described (Chung *et al.*, 1989; Sambrook *et al.*, 1989). All mutations were confirmed by sequencing using an ALFexpress system (Amersham Pharmacia Biotech) and Cy5-labeled primers. Plasmid DNA for sequencing was isolated with a QIAGEN Plasmid Mini Kit.

Purification of [His]₆-Tagged ArsA ATPases

Altered ArsA proteins were purified from cultures of *E. coli* strain JM109 harboring the indicated plasmids. Cells were grown at 37°C in LB medium to an A₆₀₀ of 0.6–0.8, at which point 0.1 mM isopropyl β -D-thiogalactopyranoside was added to induce ArsA expression. The

cells were grown for another 3 h before being harvested by centrifugation. The cells were suspended in buffer A (50 mM MOPS, pH 7.5, 20 mM imidazole, 500 mM sodium chloride, 20% glycerol, and 10 mM β -mercaptoethanol) and lysed by a single passage through a French pressure cell at 20,000 p.s.i. Diisopropyl fluorophosphate was added at 2.5 μ L/g of wet cells immediately following lysis. The lysate was centrifuged at 10,000 \times g for 30 min at 4°C. The supernatant solution containing the [His]₆-tagged protein was loaded onto Ni-NTA spin column (QIAGEN) preequilibrated with buffer A. Purification was done following the manufacturer's (QIAGEN) protocol except that buffer A was used as the wash buffer and buffer B (50 mM MOPS, pH 7.5, 250 mM imidazole,

Table II. Oligonucleotides Used for the Mutagenesis of R773 *E. coli* *arsA* Gene

Substitution	Primers (5' → 3')
H138A	ATC AAA AAT GAT <u>AGC</u> GTC AAA CCG CGT
H148A	CAG AAG GCG AAT GGT <u>GGC</u> ACC CGT CGG CGC GGT
H219A	GGC AAG TTC CAG <u>AGC</u> AGT CCG GGC GAC
H327A	CAT AAT CAG GCC <u>AGC</u> TTC ATT ACG GGC
H359A	AGA TGT TGT CAG <u>AGC</u> GAC ATC AAA TCC
H368A	GGT CAT GCT GAG <u>AGC</u> CGC CGC AGG ATC
H388A	TTC CGT TTC CTC <u>GGC</u> AGG ATC GAT CCT
H397A	TGT TTC AAG AAC <u>AGC</u> CTG ACG ATA GCG
H453A	CAG CAG CAA CAG CGT <u>GGC</u> TCC GGT CGG AGC CGT
H465A	CGC AAT TTC GCG <u>GGC</u> GTA CGC GCC TGT
H477A	CGG TGT GGT GAA <u>AGC</u> GCC TTT TTC TCC
H520A	CCA GCC CCA GGG <u>GGC</u> AAT GCC TGC ACG
H558A	GAC ACG GCT GGC <u>CGC</u> CTG GCG TTT AAC

Note. The mutated nucleotides are underlined.

500 mM sodium chloride, 20% glycerol, and 10 mM β -mercaptoethanol) was used as the elution buffer. The concentration of ArsA in purified preparations was determined from the absorption at 280 nm using a molar extinction coefficient of 33,480 (Rosen *et al.*, 1988). ATPase activity was assayed using an NADH-coupled assay method (Hsu and Rosen, 1989; Vogel and Steinhart, 1976).

Reaction of ArsA With DEPC

DEPC was purchased from Sigma. Prior to use, DEPC stock (6.9 M) was diluted in absolute ethanol to working concentrations and stored on ice. The concentration of DEPC was determined spectrophotometrically by reacting an aliquot with 20 mM imidazole buffer (pH 7.0) and measuring the increase in absorbance at 240 nm ($\epsilon_{240} = 3,200 \text{ M}^{-1} \text{ cm}^{-1}$) (Miles, 1977). Before reaction of ArsA with DEPC, dithiothreitol and imidazole were removed from the protein using a spin column (Penefsky, 1977) equilibrated with buffer C (50 mM MOPS-KOH buffer, pH 7.5, containing 0.25 mM EDTA). Modification of ArsA was performed in a reaction mixture containing 10 μM ArsA in buffer C and different concentration of DEPC (10–100 μM) at room temperature. For activity measurements, portions of the reaction mixture were removed at intervals, the reaction quenched by the addition of 20 mM dithiothreitol and 20 mM imidazole, and the residual activity measured by coupled assay method. An equivalent amount of ethanol instead of DEPC had no effect on ArsA activity.

Reactivation With Hydroxylamine

ArsA (10 μM) was incubated in the absence or presence of 0.1 mM DEPC in buffer C at room temperature.

After 10 min the reaction was quenched with 20 mM imidazole. Hydroxylamine (pH adjusted to 7.5) was then immediately added to the reaction mix to a final concentration of 500 mM. The reaction was incubated at room temperature for 5 h. Aliquots were withdrawn every hour and assayed for enzymatic activity.

RESULTS

Inactivation of ArsA by DEPC

Incubation of wild type ArsA with increasing concentrations of DEPC resulted in a time dependent loss of ATPase activity (Fig. 2). The inactivation followed pseudo-first-order rate kinetics at all of the DEPC concentrations examined. A second order rate constant of $6.3 \text{ mM}^{-1} \text{ min}^{-1}$ was determined by replotting the pseudo-first-order rate constants (k_{obs}) as a function of DEPC concentration (data not shown) (Eyzaguirre, 1987). When the log of the rate of inactivation (k_{obs}) was plotted against the log of the inhibitor concentration, a straight line with a slope of 1.05 was obtained (Fig. 2, inset). A slope of 1 indicates that one molecule of DEPC inactivates one molecule of ArsA (Eyzaguirre, 1987).

Although DEPC is considered to be a histidine specific reagent, it can still react with the nucleophilic side chains of cysteine, lysine, or tyrosine (Lundblad, 1995; Miles, 1977). Conversely, specific modification of histidine residues by DEPC can be reversed upon treatment with hydroxylamine. ArsA was incubated with a 10-fold molar excess of DEPC until the enzymatic activity declined to <5% of its initial value. Addition of 0.5 M hydroxylamine to the modified enzyme restored >70% of its original activity. This result suggests that the cysteines

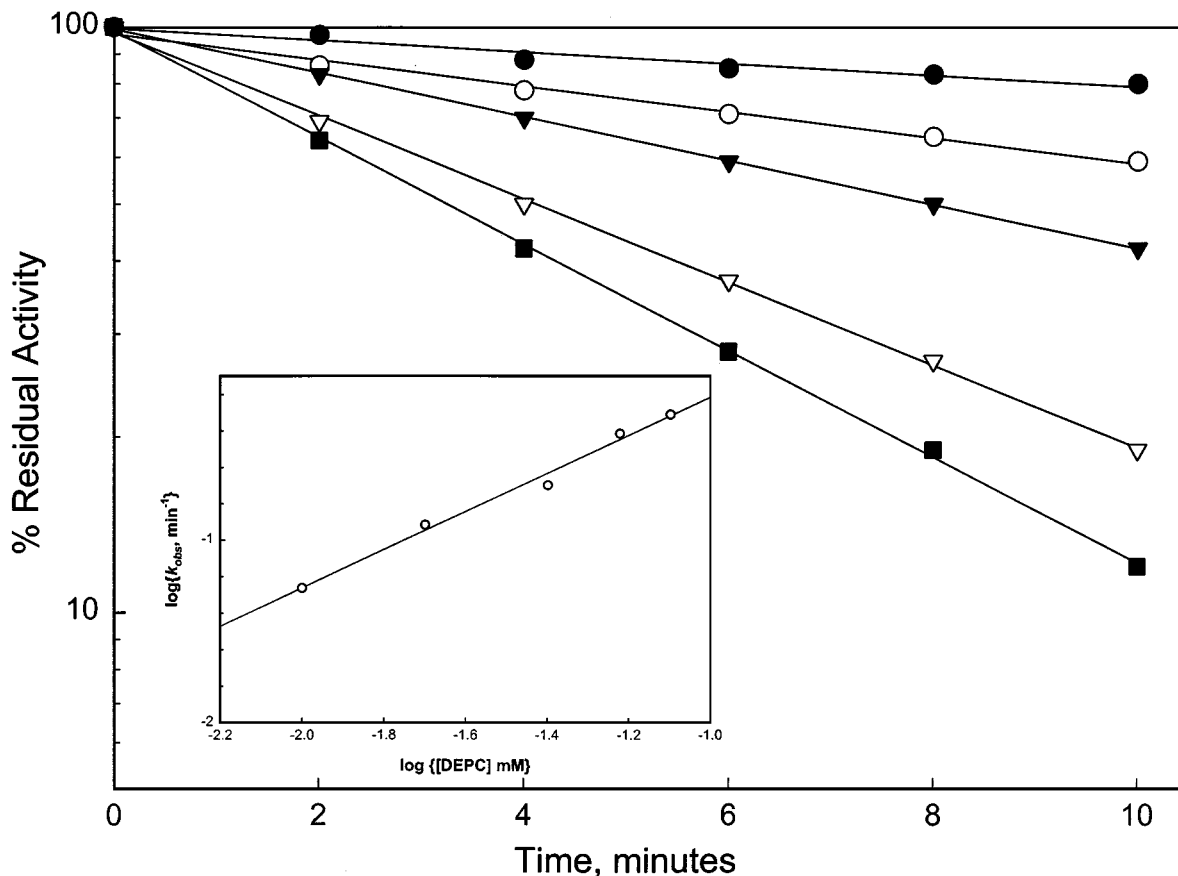


Fig. 2. Kinetics of inactivation of ArsA by DEPC. Purified ArsA (10 μM) was incubated with (●) 10 μM ; (○) 20 μM ; (▼) 40 μM ; (▽) 60 μM ; and (■) 80 μM of DEPC in buffer C at room temperature. Antimonite-stimulated ATPase activity was measured at the indicated times as described in the "Experimental Procedures." The log of percent activity remaining was plotted against time of inactivation. The pseudo-first order rate constants of inactivation (k_{obs}) were calculated from the slope of each of the lines. *Inset:* log of the pseudo-first order rate constants of inactivation (k_{obs}) versus log of the millimolar concentration of DEPC.

or primary amino groups of the protein were unaltered by DEPC, since modification of those residues results in derivatives that are stable to hydroxylamine. In addition, reversibility of DEPC modification negated the possibility of enzyme denaturation by irreversible alteration of ArsA conformation or to the formation of dicarboxyhistidyl derivatives. Furthermore, no change in the absorbance spectrum in the 275–280 nm region was noted, suggesting that the tyrosine residues did not react with the reagent.

Correlation Between Enzyme Activity and Histidine Residues Modified by DEPC

The difference spectrum of DEPC-treated ArsA versus unmodified ArsA revealed only a single peak at 240 nm that is characteristic of an *N*-carboxyimidazole derivative (data not shown). The relationship between activity and the number of histidine residues modified was deter-

mined (Fig. 3). Wild type ArsA was completely inactivated following modification of two of the histidine residues by DEPC. Modification of a single histidine resulted in greater than 70% loss of enzymatic activity. The second histidine reacted more slowly, correlating with loss of the residual activity.

Protection Against Inactivation by DEPC

To determine whether the loss of activity upon DEPC treatment is due to the modification of histidines located at or near the active site of ArsA, the enzyme was preincubated with either ATP and/or antimonite before being subjected to chemical modification (Fig. 4). Shielding the active site histidine by the substrate or activator should decrease the rate of inactivation by the modifier if it enters the active site. When the protein was preincubated with either ATP or Sb(III), the $t_{1/2}$ of inactivation by

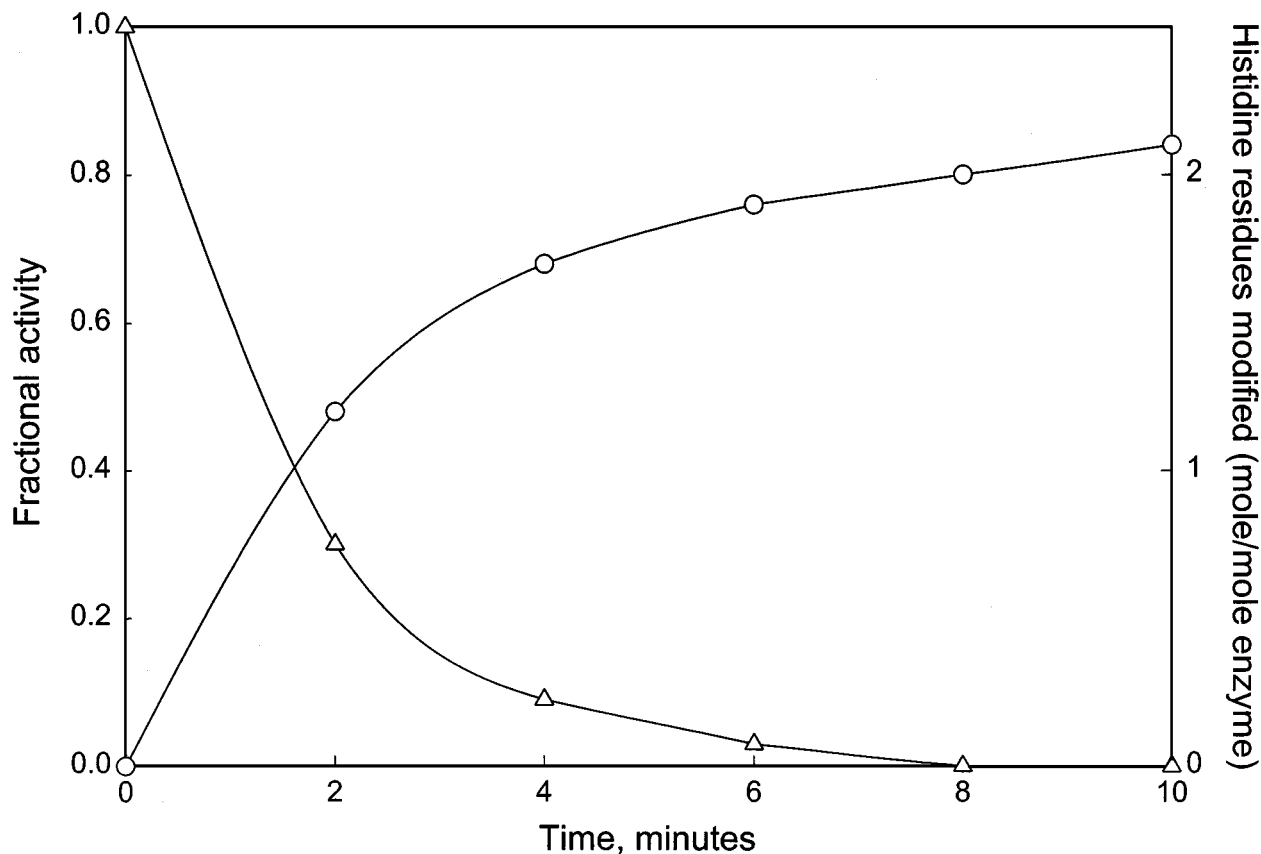


Fig. 3. Correlation of DEPC modification of histidine residues and inactivation of ATPase activity. A 10-fold molar excess of DEPC was added to purified ArsA (10 μ M), and the absorbance at 240 nm was monitored every 2 min for 10 min. The number of modified residues were determined using $\epsilon = 3,200 \text{ M}^{-1} \text{ cm}^{-1}$ (Miles, 1977). ArsA ATPase activity measurements were done under identical conditions. (Δ) ATPase activity; (\circ) number of histidines modified.

DEPC alone was 0.8 min, whereas the half time of inactivation reaction increased to 1.5 min and 2.5 min when preincubated with either antimonite or ATP, respectively. Synergistic protection was observed when both ATP and antimonite were added together, with $t_{1/2}$ increasing to 5.1 min.

Site-Directed Mutagenesis of ArsA

Each of the thirteen histidine residues in ArsA were individually changed to alanine residues, producing ArsA derivatives H138A, H148A, H219A, H327A, H359A, H368A, H388A, H397A, H453A, H465A, H477A, H520A, and H558A. Cells bearing the mutated *arsA* genes and wild type *arsB* genes were characterized phenotypically for arsenite resistance (Fig. 5). Cells expressing the wild type *arsA* and *arsB* genes could grow in medium containing concentrations of sodium arsenite in excess of 8 mM. Cells without an *ars* operon were sensitive above

1 mM sodium arsenite. Except for *arsA*_{H368A}, each of the single alanine mutants was resistant to sodium arsenite to the same level as wild type. Cells bearing the *arsA*_{H368A} gene showed a phenotype intermediate between sensitive and fully resistant cells. Similar results were obtained when the mutants were analyzed for antimony resistance (data not shown).

Analysis of the Altered ArsA Proteins

The steady state level of production of the altered ArsA proteins was examined. With the exception of H368A ArsA, each of the altered ArsA proteins was produced in similar levels as the wild type ArsA (Fig. 6). H368A ArsA was expressed at a 4-fold lower amount than the wild type protein. Western blot analysis using an antiserum against wild type ArsA showed that there was no difference between the wild type and altered proteins in terms of mobility on SDS-PAGE; nor was any degradation

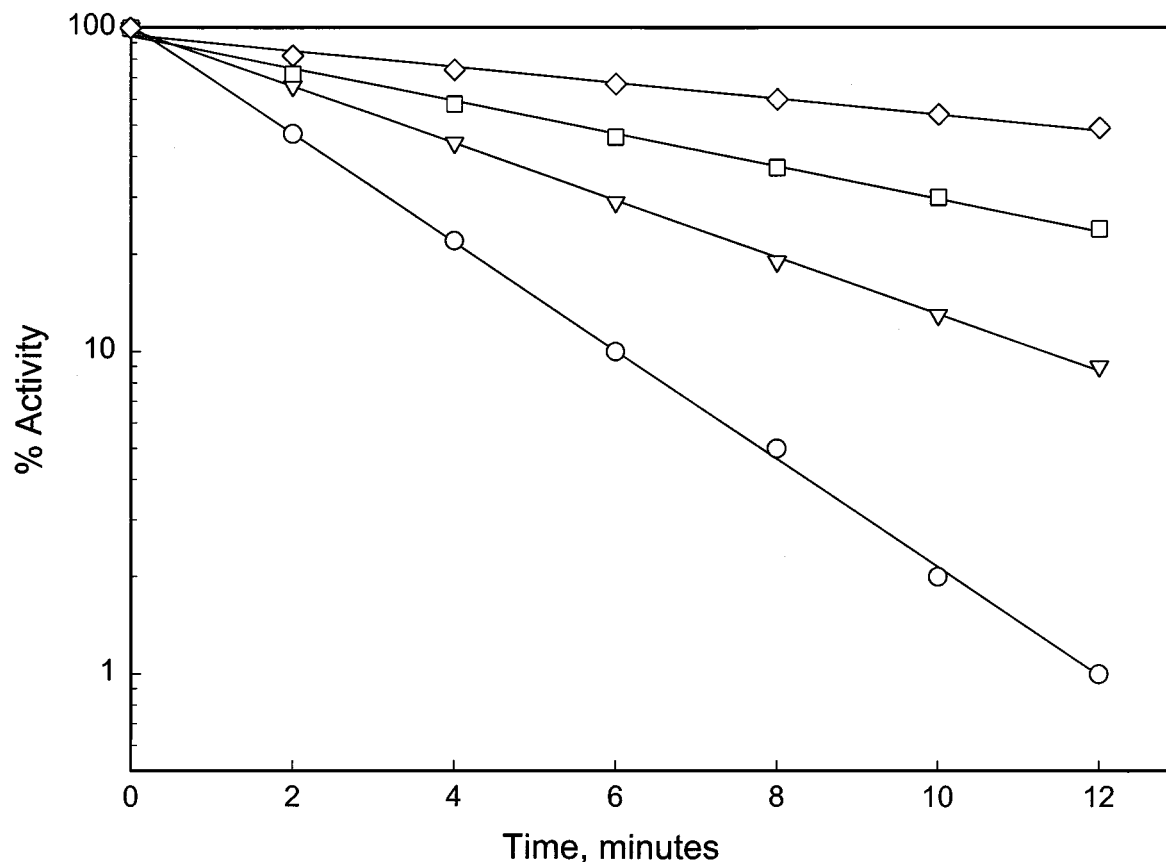


Fig. 4. Substrate protection of *ArsA* from DEPC inactivation. Purified *ArsA* (10 μ M) was incubated with ATP and/ or potassium antimonite tartrate for 20 min at room temperature. DEPC (80 μ M) was added, and the incubation continued. Antimonite-stimulated ATPase activity was measured at the indicated times. Additions were as follows: (○) none; (▽) 0.5 mM potassium antimonite tartrate; (□) 5 mM ATP; (◇) 0.5 mM potassium antimonite tartrate and 5 mM ATP.

of altered proteins observed (data not shown). Each altered protein was purified to >95% homogeneity as described in Experimental Procedures.

ATPase Activity of Altered *ArsA* Proteins

The purified proteins were analyzed for their ability to catalyze metalloid stimulated ATPase activity (Table III). Each of the alanine substituted altered *ArsAs* showed a basal ATPase activity similar to the wild type enzyme. Addition of antimonite further stimulated the rate of ATP hydrolysis. Nine of the altered *ArsA* proteins (H138A, H219A, H327A, H359A, H388A, H397A, H465A, H477A, and H520A) showed antimonite stimulated ATPase activity similar to the wild type *ArsA*. Altered *ArsA* with a substitution at residue 148, 368, or 453 exhibited approximately a 3-fold lowering of the metalloid stimulated ATPase activity. The H558A *ArsA*

Table III. V_{\max} of Oxyanion Stimulated ATPase Activity of *ArsA* Proteins

ArsA protein	V_{\max} (nanomoles of ATP hydrolyzed/min/mg of protein)		
	-Antimonite	+Antimonite	Antimonite-stimulated
WT	67	960	893
H138A	72	806	734
H148A	61	408	347
H219A	93	904	811
H327A	93	943	850
H359A	63	752	689
H368A	146	417	271
H388A	45	956	911
H397A	81	959	878
H453A	53	313	260
H465A	100	878	778
H477A	92	988	896
H520A	100	960	860
H558A	124	633	509

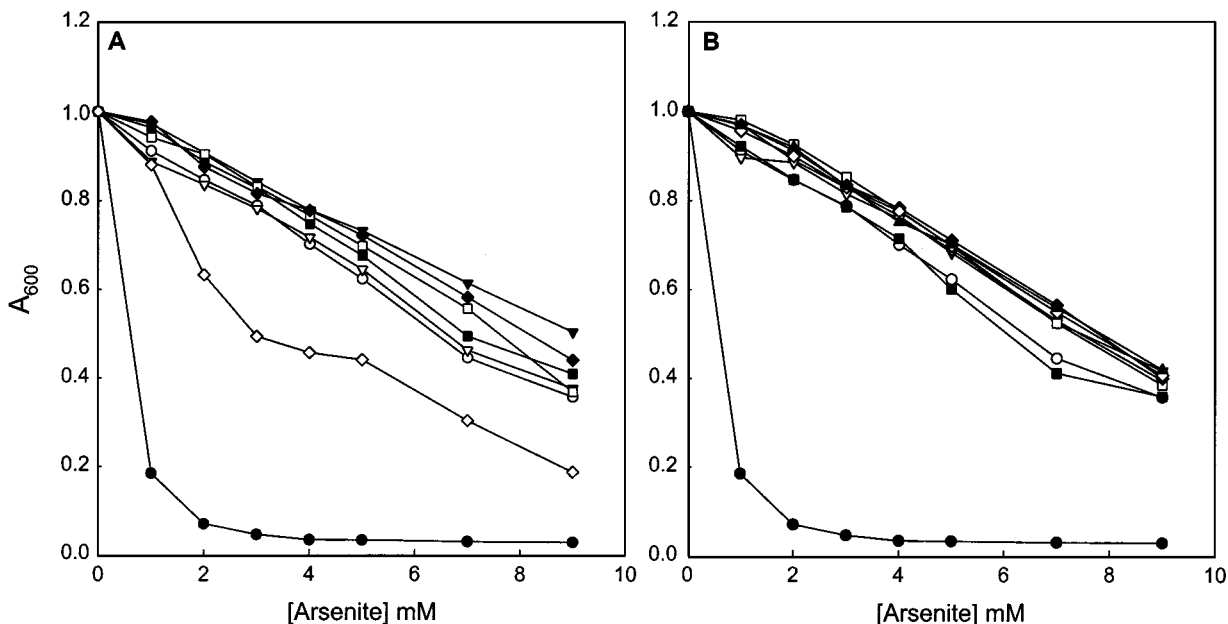


Fig. 5. Resistance to arsenite in cells expressing wild type and mutant *arsA* genes. Overnight cultures of *E. coli* strain JM109 bearing wild type and mutant *ars* plasmids were diluted 100-fold into fresh LB medium containing varying concentrations of sodium arsenite. Expression of the *ars* genes was induced with 0.1 mM isopropyl β -D-thiogalactopyranoside. After 5 h of growth at 37°C, the absorbance at 600 nm was measured in a spectrophotometer. **Panel A.** Plasmids: (○) pABH6 (*arsA*_{[His]6B}); (▼) pH138A (*arsA*_{H138A-[His]6B}); (▽) pH148A (*arsA*_{H148A-[His]6B}); (■) pH219A (*arsA*_{H219A-[His]6B}); (□) pH327A (*arsA*_{H327A-[His]6B}); (◆) pH359A (*arsA*_{H359A-[His]6B}); (◇) pH368A (*arsA*_{H368A-[His]6B}); (●) vector plasmid, pALTER[®]-1. **Panel B.** Plasmids: (○) pABH6 (*arsA*_{[His]6B}); (▼) pH388A (*arsA*_{H388A-[His]6B}); (▽) pH397A (*arsA*_{H397A-[His]6B}); (■) pH453A (*arsA*_{H453A-[His]6B}); (□) pH465A (*arsA*_{H465A-[His]6B}); (◆) pH477A (*arsA*_{H477A-[His]6B}); (◇) pH520A (*arsA*_{H520A-[His]6B}); (▲) pH558A (*arsA*_{H558A-[His]6B}); (●) vector plasmid, pALTER[®]-1.

exhibited a 50% lowering of the antimonite-stimulated rate compared to the wild type protein.

The altered ArsA proteins were treated with DEPC and assayed for ATPase activity. Assays were done after a 10 min incubation of the altered ArsAs with a 10-fold molar excess of DEPC. Each of the alanine-substituted proteins was as sensitive to DEPC as the wild type ArsA (data not shown).

DISCUSSION

Residues of functional importance for the ArsA ATPase include the cysteines of the allosteric site (Bhattacharjee *et al.*, 1995; Bhattacharjee and Rosen, 1996) and aspartates in the Mg²⁺ binding site (Zhou and Rosen, 1999). Other sequences that contribute to the activity of the enzyme include the glycine-rich flexible loops of the nucleotide binding domains (Karkaria *et al.*, 1990; Kaur and Rosen, 1992) and the DTAP sequences in the signal transduction domains (Zhou and Rosen, 1997). In an attempt to define other residues with catalytic or structural involvement, the contribution of each of the

thirteen-histidine residues of ArsA was examined using a combination of chemical modification and site-directed mutagenesis.

ArsA ATPase activity could be completely abolished following treatment with DEPC, a relatively specific reagent for modification of histidine groups of protein. The reaction between ArsA and DEPC was bimolecular and followed a pseudo-first-order kinetics. The inactivation could be reversed after treatment with hydroxylamine, signifying specific modification of histidine residues. The reversal of activity further suggested that DEPC did not modify either cysteines or primary amino groups. Additionally, the UV difference spectrum of DEPC-treated ArsA versus unmodified protein showed a single peak at 240 nm that is characteristic of protein bound *N*-carbethoxyhistidine. Furthermore, the absence of any absorbance change in the 275–280 nm region implied that the tyrosine groups of the protein were inert to the modifying reagent. The loss in activity of wild type ArsA could be correlated with the modification of only two histidines by DEPC. One of the histidine was fast reacting with ~70% loss in activity within 2 min of incubation with the reagent, followed by the modification of a

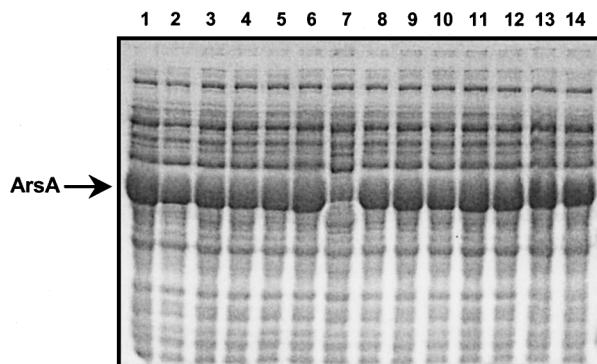


Fig. 6. Expression of altered *ArsA* proteins. Cultures of *E. coli* strain JM109 bearing the indicated plasmids expressing the wild type and mutant *arsA* genes were grown and induced as described in "Experimental Procedures." Samples were prepared by treating the lysate with SDS sample buffer for 10 min at 37°C, and analyzed by SDS-PAGE on 10% acrylamide gels. Plasmids: Lane 1, pABH6 (*arsA*_{[His]6B}); lane 2, pH138A (*arsA*_{H138A-[His]6B}); lane 3, pH148A (*arsA*_{H148A-[His]6B}); lane 4, pH219A (*arsA*_{H219A-[His]6B}); lane 5, pH327A (*arsA*_{H327A-[His]6B}); lane 6, pH359A (*arsA*_{H359A-[His]6B}); lane 7, pH368A (*arsA*_{H368A-[His]6B}); lane 8, pH388A (*arsA*_{H388A-[His]6B}); lane 9, pH397A (*arsA*_{H397A-[His]6B}); lane 10, pH453A (*arsA*_{H453A-[His]6B}); lane 11, H465A (*arsA*_{H465A-[His]6B}); lane 12, pH477A (*arsA*_{H477A-[His]6B}); lane 13, pH520A (*arsA*_{H520A-[His]6B}); lane 14, pH558A (*arsA*_{H558A-[His]6B}). The arrow indicates the position of migration of purified *ArsA* protein.

slow-reacting second histidine with complete loss of activity. Partial protection was afforded against DEPC inactivation by either the substrate ATP or the activator antimonite. Synergistic protection was observed when both ATP and antimonite were present together. All of these experiments suggested that modification of at least one of the histidines is responsible for the loss of catalytic activity.

Was the loss of activity due to a requirement for a specific histidine residue or due to the addition of a bulky modifying group? A molecular genetic approach was used to examine this question. There are thirteen histidine residues in *ArsA*, located at positions 138, 148, 219, 327, 359, 368, 388, 397, 453, 465, 477, 520, and 558. Each of these histidines was individually altered to alanine by site-directed mutagenesis. Cells expressing each of the altered *arsA* genes exhibited an oxyanion resistance phenotype similar to that of the wild type. The intermediate level of resistance in cells expressing H368A *ArsA* is likely because of a lower level of protein expression (Fig. 6). Each of the altered proteins were purified and assayed for the metalloid stimulated ATPase activity. Each enzyme exhibited a similar level of basal ATPase activity as the wild type *ArsA*. Addition of antimonite stimulated the ATPase activity significantly in each of the alanine substituted *ArsA*. Kinetic experiments suggested

that *ArsA* activity is lost primarily because of the modification of one histidine residue per molecule of *ArsA*. However, every alanine-substituted *ArsA* was as sensitive to DEPC as the wild type, suggesting that modification of several different histidine residues could lead to loss of activity.

It is clear from these results that none of the thirteen histidines is required for catalysis. The inactivation of *ArsA* ATPase activity by DEPC is most likely because of steric hindrance from addition of the bulky carboxy group. While none of the histidines is apparently catalytic, this does not mean that none serves a role in the mechanism of the enzyme. Several of the alanine-substituted enzymes had significantly reduced levels of activation by metalloid, including the H148A, H368A, and H453A proteins. It is of considerable interest that His148 and His453 are at the ends of the two signal transduction sequences. The possibility that these two histidine residues are involved in transmission of the signal of occupancy of the allosteric site to the catalytic sites is currently under investigation.

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