Interaction of ATP Binding Sites in the ArsA ATPase, the Catalytic Subunit of the Ars Pump*

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The ArsA ATPase is the catalytic subunit of the Ars pump that catalyzes arsenical extrusion in *Escherichia coli*, thus providing resistance. The active form of ArsA is a homodimer with four nucleotide binding sites, two from each monomer. The codons for Gly-15 in the N-terminal consensus nucleotide binding sequence and Gly-334 in the C-terminal sequence were individually mutated to cysteine codons. Cells expressing an arsA\(_{G15C/G334C}\) mutation retained arsenite resistance, while an arsA\(_{G15KGGVGKTSI25}\) mutation resulted in substantial reductions in arsenite resistance, transport, and ATPase activity. Selection for suppression of the G15C mutation that restored arsenite resistance yielded an A344V substitution. Ala-344 is located adjacent to the C-terminal nucleotide binding sequence. The second site mutation did not suppress the loss of resistance resulting from G18D, G20S, or T22I substitutions in the N-terminal nucleotide binding motif (Walker et al., 1991a, b). This allosteric control ensures that the pump does not hydrolyze ATP without coupled anion translocation.

The 63-kDa ArsA ATPase contains two consensus ATP-binding motifs (Walker et al., 1982) and is an A1 and A2 domain. The glycine-rich sequence of the consensus binding sites for the γ-phosphate of ATP (P-loop) of ArsA is G\(^1\)\(KGGVGKTSIS\)\(^{25}\) and G\(^{334}\)KGGVGKTTMA\(^{344}\), respectively, in the A1 and A2 halves (Chen et al., 1986). The results from genetic complementation (Kaur and Rosen, 1993) and biochemical reconstitution (Kaur and Rosen, 1994a) suggested that each catalytic unit in the active dimer consists of an A1 domain from one monomer and an A2 domain from the other monomer. To test this idea, we adopted second site suppressor analysis, which has been utilized effectively to study intergenic subunit-subunit (Omote et al., 1994) and intragenic domain-domain (Harris et al., 1991; Iwamoto et al., 1993) interactions. In this approach, if a second site mutation suppresses a defined first mutation, the two altered amino acid residues may be spatially proximate (although it should be emphasized that this is not the only way that intragenic complementation can occur). Based on this hypothesis, Iwamoto et al. (1993) mutagenized the codon for the first glycine residue in the P-loop of the β subunit of the F1 ATPase to a cysteine codon and then isolated revertants of the βG149C mutant.

In this study, we used site-directed mutagenesis to alter the first glycine residues in the A1 and A2 P-loops. Gly-149 of the β subunit of the F1 ATPase corresponds to Gly-15 and Gly-334 of the two P-loops in ArsA. These were altered individually or in combination to cysteine residues. The G334C mutant exhibited a wild type phenotype and was not studied further. However, both the G15C and G15C/G334C mutants exhibited moderate sensitivity to arsenite. An arsenite resistant revertant of the G15C mutation was found to have a single substitution of A344V. The G15C/A344V revertant had wild type levels of arsenite transport in vivo. The single A344V mutant also showed wild type properties. The A344V mutation suppressed a G15C mutation but not G18D, G20S, or T22I mutations, each of which has been shown to prevent binding of ATP to the A1 P-loop (Karkaria et al., 1990). These results suggest spatial proximity of Gly-15 and Ala-344 and support a model in which the two types of ATP binding sites interact to form a catalytic unit.
EXPERIMENTAL PROCEDURES

Materials—All restriction enzymes and nucleic acid modifying enzymes were obtained from Life Technologies, Inc. Oligonucleotides were synthesized in the Macromolecular Core Facility of Wayne State University School of Medicine. 73AsO₃²⁻ was purchased from Los Alamos Laboratories, Los Alamos, NM. All other chemicals were obtained from commercial sources.

Media and Growth Conditions—E. coli strains and plasmids used in this study are described in Table I. Cells were grown in either TEGA medium supplemented with 0.5% glycerol, 2.5 μg of thiamine per ml, 0.5% peptone, and 0.15% succinate or in LB medium (Sambrook et al., 1989) at 37 °C. Ampicillin (125 μg/ml), tetracycline (10 μg/ml), chloramphenicol (17 μg/ml), and kanamycin (10 μg/ml) were added as required. Sodium arsenite, potassium antimony tartrate, and isopropyl-1-thio-β-D-galactopyranoside (IPTG) were added at the indicated concentrations.

DNA Manipulations—The conditions for plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation have all been described (Sambrook et al., 1989).

Oligonucleotide-directed Mutagenesis—Mutations in the arsA gene were introduced by site-directed mutagenesis using the Altered Sites™ In Vitro Mutagenesis System (Promega). Plasmid pALTER-AB containing the arsA and arsB genes was used as the template (Bhattacharjee et al., 1995). The mutagenic oligonucleotides used and the respective changes (underlined) introduced were as follows: G15C, CGCCTCCTT-TACACTGTTAAAAACGACG; G334C, CCCCCGACCTTTATAACGACGCAAAACGACG; G15C/A344V, TACACGTAAAAAACAG; G334C, CCCACGCCAACCCTTTACACATCA-GACTTCTTTACACTGTTAAAAACGACG. The identity of the mutations was confirmed by DNA sequencing each mutant gene. Double-stranded plasmid DNA was prepared using the Qiagen DNA Purification System. Sequencing was performed using the Pharmacia Cy5-labeled autosum kit (Pharmacia Biotech Inc.) and ALFexpress apparatus by the method of Sanger et al. (1977). Selection of Revertants—Hydroxylamine was used to mutate plasmid DNA by a modification of the method of Humphreys et al. (1976). Five μg of DNA from plasmid pG15C were incubated with 0.4 M NH₄OH in 2 ml of a buffer consisting of 0.1 M KPO₄, pH 6.0, containing 1 mM dithiothreitol and 1 mM EDTA and precipitated with ethanol (Sambrook et al., 1989). Plasmid DNA was transformed into cells of E. coli strain JM109. Plasmid DNAs from single colonies showing resistance to 5 mM NaAsO₃ were sequenced, resulting in the isolation of pG15C/A344V (arsA_G15C/arsB_A344V). Other A1 P-loop/A344V double mutants (Table I) were constructed by molecular cloning of the A1 P-loop mutations into pMad45. ¹The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis.

Quantitation and Cellular Localization of Altered ArsA— Cultures of cells containing the appropriate plasmids were grown in LB medium at 37 °C to an A₆₀₀ nm = 0.8, followed by induction with 0.1 mM IPTG for 2.5 h. One ml of cells was pelleted and suspended in 0.5 ml of SDS sample buffer. After boiling for 5 min, the proteins from 2 μl of sample were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 5% polyacrylamide gels (Laemmli, 1970). Immunoblotting was performed utilizing a chemiluminescence assay (DuPont NEN) and exposed to x-ray film at room temperature as described earlier (Dey et al., 1994).

To determine the cellular localization of the expressed proteins, 100 μl of induced cells were pelleted by centrifugation and washed with 100 μl of 10 mM Tris-HCl, pH 7.5, containing 0.1 M KCl. The cells were suspended in 5 ml of a buffer consisting of 10 mM Tris-HCl, pH 7.5, containing 2 mM dithiothreitol, 0.5 mM diethiothreitol, and 20% (v/v) glycerol and lysed by a single passage through a French Press at 20,000 psi, followed by immediate addition of 2.5 μl of the serine protease inhibitor diisopropylfluorophosphate per g wet weight of cells. Insoluble protein bodies were pelleted at 10,000 × g for 10 min and suspended in the original volume of the same buffer. Cytosol was produced by removing membranes at 150,000 × g for 1 h. Samples (30 μl) were mixed with 10 μl of 4% concentrated SDS sample buffer and boiled for 5 min. One μl of each sample was analyzed by Western blotting.

²AsO₃²⁻ Transport in Cells—²⁷AsO₃²⁻ was prepared by reduction of ⁷³AsO₃²⁻ (Reay and Asher, 1977). ²⁷AsO₃²⁻ uptake by whole cells reflects the efflux activity of the Ars pump, where lack of uptake is equivalent to active extrusion (Rosen and Borbolla, 1984; Dey and Rosen, 1995b). Cells bearing plasmids were grown overnight at 37 °C in 5 ml of supplemented TEA medium containing 10 μg/ml tetracycline. The culture was diluted into 50 ml of prewarmed supplemented TEA medium. At an A₆₀₀ nm = 0.6, expression of the arsA and arsB genes was induced by addition of 0.1 mM IPTG for 1 h. The cells were harvested, washed, and suspended in 0.5 ml of TEA medium, all at room temperature. To initiate arsenite transport, 40 μl of cells (approximately 1 mg of protein) was diluted into 0.6 ml of TEA medium containing 20 mM glucose, 0.1 mM NaAsO₃, and 1.25 μCi of ⁷³AsO₃²⁻. Portions (0.1 ml) were withdrawn at intervals, filtered through nitrocellulose filters (0.45 μm pore diameter, Whatman), and washed with 5 ml of TEA medium. The filters were dried, and radioactivity was quantified by liquid scintillation counting.

Purification and Assay of the ArsA ATPase—Proteins were purified from the cytosol of 2.5 liters of induced cultures as described previously (Hsu and Rosen, 1989). Each ArsA was judged to be >95% homogeneous by Coomassie Blue staining of samples separated by SDS-PAGE. The concentrations of purified ArsAs were determined using the method of Lowry et al. (1951). ATPase activity was measured using a coupled assay (Vogel and Steinhardt, 1976), as described previously (Hsu and Rosen, 1989).
Fig. 1. Resistance to arsenite in cells expressing wild type, arsA\textsubscript{G15C} and arsA\textsubscript{G334C} 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. A\textsubscript{600 nm} was measured after 8 h of growth at 37°C. Cells had the following plasmids: ■, pA334C (arsA\textsubscript{A344V}); ▲, pALTER-AB (arsAB); ●, pG15C/G334C (arsA\textsubscript{G15C/G334C}); △, pG15C (arsA\textsubscript{G15C}); □, vector plasmid pALTERNM-1.

RESULTS

Isolation of a Primary Mutant—Mutations in the codons for residues in the A1 and A2 consensus nucleotide binding sequences, G\textsuperscript{15}GKVGGKTIS\textsuperscript{19} and G\textsuperscript{334}GKVGGKTTMA\textsuperscript{344}, respectively, have been previously shown to reduce or eliminate arsenite resistance (Karkaria \textit{et al.}, 1990; Kaur and Rosen, 1992). Substitutions in the A1 P-loop (G18D, G18R, G20S, T22I (Karkaria \textit{et al.}, 1990) and K21E (Kaur and Rosen, 1992)) or A2 P-loop (G337R, K340E, and K340N (Kaur and Rosen, 1992)) each abolished the ability of the Ars pump to extrude arsenite, and the purified altered proteins were each catalytically inactive. From those results it was concluded that Gly-18, Gly-20, and Gly-337 in the two P-loops are required for resistance, transport, and ATPase activity.

The roles of Gly-15 and Gly-334 have not been studied previously. The equivalent glycine residue in the P-loop of the β chain of the E. coli \textit{F}\textsubscript{1} ATPase was changed to a cysteine with loss of function (Iwamoto \textit{et al.}, 1993). Using site-directed mutagenesis, the codons for Gly-15 and Gly-334 were changed to cysteine codons separately and in combination, producing ArsA derivatives G15C, G334C, and G15C/G334C. Cells harboring the mutated \textit{arsA} genes and wild type \textit{arsB} gene were phenotypically characterized for arsenite resistance (Fig. 1). Cells expressing the wild type \textit{arsAB} genes could tolerate 5 mM Na\textsubscript{2}AsO\textsubscript{4}; cells containing only vector were unable to grow when the concentration of arsenite was more than 1 mM. Only a single isolate was identified as having a single point mutation in the \textit{arsA} gene; when the entire \textit{arsA} gene was sequenced the codon for Ala-344, GCT, was found to have been mutated to GTT, creating a valine substitution. Note that residue 344 immediately follows the highly conserved A2 P-loop sequence. The phenotype of cells expressing the \textit{arsA}\textsubscript{G15C/G334C} double mutation was identical to that of the wild type (Fig. 2A). To examine the effect of the A344V substitution in the absence of G15C, an \textit{arsA} gene containing just the A344V mutation was constructed. Cells expressing the \textit{arsA}\textsubscript{A344V} mutation alone exhibited a wild type phenotype.

To address the question of the allelic specificity of the A344V suppression, the effect of the A344V substitution on other A1 P-loop mutants was examined. Three other mutants in the A1 P-loop, G18D, G20S, and T22I, were combined with the A344V mutation. In none of the A344V substitution able to suppress the phenotypic effect of the P-loop mutations (Fig. 2B), indicating the suppression of A344V on the G15C allele was specific.

Expression and Cellular Location of Altered ArsAs—The steady state level of wild type and altered ArsAs in cells was estimated by Western blot analysis using antisera to wild type ArsA. Each altered protein was produced in approximately the same amount and migrated with the same mobility as wild type ArsA (Fig. 3A). The cellular location of the altered proteins was determined. Although ArsA is functionally a component of the membrane-bound pump, it is found in the cytosol when expressed in high amounts (Rosen \textit{et al.}, 1988). The G15C protein was similarly found predominantly in the cytosol (Fig. 3B). In contrast, the G15C/A344V and A344V proteins were found nearly exclusively as insoluble aggregates. Although the instability of G15C/A344V and A344V enzymes prevented biochemical characterization, from the \textit{in vivo} results, it is likely that they would have properties more similar to those of the wild type than to the G15C enzyme.

\textsuperscript{23}AsO\textsubscript{4}\textsuperscript{3-} Transport—Cells with an active Ars pump exhibit a low net accumulation of arsenite (Mobley and Rosen, 1982; Dey and Rosen, 1995a). In contrast, cells without the pump are unable to extrude arsenite and exhibit a much higher level of accumulation. Uptake of \textsuperscript{23}AsO\textsubscript{4}\textsuperscript{3-} was assayed in cells expressing the various mutant \textit{arsA} genes (Fig. 4). Cells without an \textit{ars} operon (vector only) accumulated arsenite, while cells expressing a wild type \textit{ars} operon had a 10-fold lower steady state level of arsenite accumulation. The steady state level of arsenite accumulation in cells expressing the \textit{arsA}\textsubscript{G15C} gene was intermediate, but indicates that the \textit{arsA}\textsubscript{G15C} gene product is partially functional \textit{in vivo}. Cells expressing the \textit{arsA}\textsubscript{A344V} or \textit{arsA}\textsubscript{G15C/A344V} genes exhibited the same or lower accumulation compared to wild type. These results correlate well with

![Graph showing resistance to arsenite](image.png)

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| Protein | \( K_m \) for ATP (\( 10^{-4} \text{M} \)) | \( V_{\text{max}} \) (\( \text{nmol ATP hydrolyzed/mg protein/min} \)) |
|---------|-----------------|-----------------|
| Wild type | 0.06 | 904 |
| G15C | 3.7 | 57 |

\( ^* \) Sb(III) was in the form of 0.1 mM potassium antimonyl tartrate.

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\(^*\) Assays performed with 5 mM ATP.
observed when one plasmid contained a wild type sequence and the other a wild type with plasmids carrying combinations of the point mutations in the two halves of the ArsA gene were used to produce enzymes that had the following plasmids. pCMC1010 (arsA), pALTER-AB (arsAB), pG15C (arsA), pALTER-AB (arsAB), pCMC1010 (arsA), and analyzed by SDS-PAGE on 8% acrylamide gels followed by immunoblot analysis using anti-ArsA serum. A, cells had the following plasmids: lane 1, 0.1 μg of purified ArsA; lane 2, vector plasmid pALTER™-1; lane 3, pALTER-B (arsB); lane 4, pALTER-AB (arsAB); lane 5, pG15C (arsA); lane 6, pG15C/A344V (arsA); lane 7, pA344V (arsA); lane 8, cytosol and insoluble aggregates from cells expressing the following plasmids: lane 1, 0.1 μg of purified ArsA; lanes 2 and 3, plasmid pALTER™-1; lanes 4 and 5, pG15C (arsA); lanes 6 and 7, pG15C/A344V (arsA); lanes 8 and 9, pA344V (arsA); lanes 3, 5, 7, and 9, insoluble aggregates.

DISCUSSION

In a previous study (Kaur and Rosen, 1993), the sequences for the A1 and A2 halves of ArsA were cloned into compatible plasmids. While neither alone was sufficient to confer resistance, their co-expression restored resistance. Genetic complementation was also observed between ArsA genes with point mutations in the two halves of the ArsA gene and in cells with plasmids carrying combinations of the ArsA1 or ArsA2 subclones and point mutations. Complementation was only observed when one plasmid contained a wild type ArsA sequence and the other a wild type ArsA sequence. In a subsequent study, subclones of the ArsA gene were used to produce the individual A1 and A2 polypeptides (Kaur and Rosen, 1994b). Neither purified polypeptide alone exhibited ATPase activity, nor did simple mixing of the polypeptides restore activity. However, when the two halves of the enzyme were denatured and renatured together, an active ATPase complex could be reconstituted. From the combination of genetic complementation and biochemical reconstitution, a model was proposed in which the ArsA dimer has two catalytic units, each composed of an A1 domain from one monomer and an A2 from the other monomer. However, none of those results provided direct evidence that the A1 and A2 nucleotide binding domains are located near each other in the quaternary structure of the enzyme.

The successful use of second site suppressors to investigate interacting domains in the F1F0-ATPase (Omote et al., 1994, Iwamoto et al., 1993; Harris et al., 1991) suggested that a similar approach might provide useful information on the interactions of the subunits of the Ars ATPase. Of particular relevance is the suppression of an E. coli βG149C mutation by βG172D, βS174F, βE192V, and βV198A (Iwamoto et al., 1993), each of which was subsequently shown to be located near the site of binding of the γ-phosphate of ATP (Abrahams et al., 1994). The suppression of a F1 βS174F mutation by an αR296S mutation (Omote et al., 1994) is of particular interest, demonstrating that residues at the interface of the α and β subunits must interact to form a catalytic unit, a premise supported by structural information gained from the solution of the crystal structure of the F1-ATPase (Abrahams et al., 1994).

Gly-149 of the β subunit of the F1-ATPase corresponds to Gly-15 and Gly-334 of the two P-loops in ArsA. In this study we similarly used site-directed mutagenesis to alter the Gly-15 and Gly-334, the first glycine residue in the A1 and A2 P-loops,
to cysteine residues. The G334C substitution appeared to be neutral, but the G15C substitution resulted in an enzyme that had lost >90% of its $V_{\text{max}}$ while retaining its allosteric regulation. The effect of the mutation in vivo was partial loss of arsenate resistance and transport. The partial phenotypes suggested that the G15C mutation would be a good candidate for suppressor analysis. After several rounds of screening arsenite-resistant clones, only a single intragenic suppressor in the A2 site of the other (Fig. 5). With information about the regions involved in this interface provided by the present study, more directed mutagenic studies will be designed to test this hypothesis.

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