Steric Limitations in the Interaction of the ATP Binding Domains of the ArsA ATPase*

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Jiaxin Li and Barry P. Rosen‡

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

ArsA, the catalytic subunit of an anion-translocating ATPase, has two consensus nucleotide binding sites, one N-terminal and one C-terminal. A mutation producing a G15C substitution in the N-terminal domain resulted in substantial reductions in arsenite resistance, transport, and ATPase activity. A second site revertant (A344V) adjacent to the C-terminal nucleotide binding site was previously shown to restore arsenite resistance, suggesting the interaction of the nucleotide binding sites in ArsA (Li, J., Liu, S., and Rosen, B. P. (1996) J. Biol. Chem. 271, 25247–25252). In this study, it is shown that alteration of Ala-344 to bulkier residues, including Cys, Thr, Pro, Asp, Leu, Phe, Tyr, or Arg, also suppressed the G15C substitution. However, A344G or A344S substitutions only marginally suppressed the primary mutation. Alteration of Gly-15 to Ala, Cys, Asp, Tyr, or Arg each resulted in decreased arsenite resistance. The larger the residue volume of the substitution, the lower the resistance, with a G15R substitution producing the least resistance. Resistance in a strain expressing an arsA gene encoding the G15R substitution could be rescued by A344S, A344T, A344D, A344R, or A344V second site suppressors. The larger the residue is then the greater the suppression is. The in vitro ArsA ATPase activities from purified wild type, G15A, G15C, and G15R exhibit an inverse relationship between activity and residue volume. Purified G15A and G15C exhibited both an increase in the $K_m$ for ATP and a decrease in $V_{max}$. The results are consistent with a physical interaction of the two nucleotide binding domains and indicate that the geometry at the interface between the N- and C-terminal nucleotide binding sites places spatial constraints on allowable residues in that interface.

Abstractive text

Escherichia coli plasmids R773 (1) and R46 (2) carry arsenical resistance (ars) operons encoding a pump that produces arsenite and antimonite resistance by ATP-coupled extrusion (3). This ATP-coupled oxyanion transporter consists of two types of subunits. The 63-kDa ArsA subunit is the catalytic component, with As(III)/Sb(III)-stimulated ATPase activity, and is allosterically activated by binding of As(III) or Sb(III) to a triad of cysteine thiols (4, 5). The 45.5-kDa intrinsic membrane ArsB component has 12 transmembrane $\alpha$-helices (6)

and functions as both the membrane anchor for ArsA and the anion-translocating sector of the pump (7).

ArsA has two homologous halves, A1 and A2, most likely the result of an ancestral gene duplication and fusion (1). Each half has a consensus glycine-rich sequence similar to a Walker A motif or P-loop (8–10). The results of genetic complementation (12) and biochemical reconstitution (13) suggested that an A1 and A2 domain interacted to form a catalytic unit. The isolation of intragenic suppressors supported the concept that a single catalytic site was formed at the interface of the A1 and A2 ATP binding sites (14).

In this study, amino acid residues 15 and 344 of ArsA were substituted with a variety of other residues. In single mutants, the smaller the residue at position 15, the greater the phenotypic resistance, with a G15R substitution producing the least resistance. In contrast, the larger the residue at position 344, the greater the phenotypic suppression of arsA15G and arsA15SR mutations. Wild type, G15A, and G15C ArsAs were purified and characterized. There was an inverse relationship between ATPase activity and residue volume at position 15, consistent with the resistance phenotype of the mutants. Enzymes with larger residues at position 15 had an increased $K_m$ for ATP and a decrease in $V_{max}$. These results suggest that there are spatial constraints in allowable residues at the interface of the A1 and A2 nucleotide binding sites.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—E. coli strains and plasmids used in this study are described in Table I. Cells were grown in Luria-Bertani medium (15) at 37°C. Ampicillin (125 μg/ml) and tetracycline (10 μg/ml) were added as required. Sodium arsenite, potassium antimonyl tartrate, and isopropyl β-D-thiogalactopyranoside were added at the indicated concentrations.

DNA Manipulations—The conditions for plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation have all been described (16). Restriction enzymes and nucleic acid modifying enzymes were obtained from Life Technologies, Inc. Wizard™ Plus minipreps DNA purification system and Wizard™ DNA clean-up system (Promega). Plasmid pALTER-AB (4) containing the arsA and arsB genes was used as the template to obtain G15X mutants, and pG15C.1 (Table I) to obtain pG15C/A344X double mutants. The mutagenic oligonucleotides used were as follows: G15A, G15R, and G15Y, CCGCCTCCTTATACGCTGA/GCTTTAAGACAG; G15D, CGCCCTCCTTATACGCTGATTAAAGACAG; A344P, A344S, A344T, A344Y, ACAGCAATGCCAGCCAGCA/TACGCTGCTACGGT; CATCCTGCTTATACGCTGATTAAAGACAG; A344R and A344L, ACAGCAATGCCAGCCAGCA/TGGTACGCTGCTTATACGCTGATTAAAGACAG; A344G to A344C, GCAATGCCAGCAACAGA/GTTTACGCTGCTTATACGCTGATTAAAGACAG; A344F, ACACGCTGCTTATACGCTGATTAAAGACAG; A344E, TACGCTGCTTATACGCTGATTAAAGACAG; A344D, ACAGCAATGCCAGCCAGCA/TACGCTGCTTATACGCTGATTAAAGACAG; and A344C, GCAATGCCAGCAACAGA/GTTTACGCTGCTTATACGCTGATTAAAGACAG.

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, 540 E. Canfield Ave., Detroit, MI 48201. Tel.: 313-577-1512; Fax: 313-577-2765; E-mail: brosen@med.wayne.edu.
Oligonucleotides synthesized with a degeneracy have the bases present in the mixture indicated in parentheses. Substitutions that resulted in mutation are underlined. The identity of each mutation was confirmed by DNA sequencing of the entire mutated gene. Double-stranded plasmid DNA was prepared using the Qiagen DNA Purification System. Sequencing was performed using the Pharmacia Cy5 labeled automated sequencer kit (Pharmacia Biotech Inc.) and ALFexpress apparatus by the method of Sanger et al. (17). Other single or double mutants were constructed by molecular subcloning using a combination of two restriction endonucleases, either HindIII and SphI or SphI and KpnI (Table I). Inhibition by arsenite was tested on solid Luria-Bertani media. The minimal inhibitory concentration is the concentration at which no growth was detected. Cells were grown overnight and streaked to single colonies on agar plates containing varying concentrations of sodium arsenite. Growth was monitored after 24 h at 37 °C.

**purification and assay of the ArsA ATPase—**Proteins were purified from the cytosol of 2.5 liters of induced cultures as described previously (4, 14). Each ArsA was judged to be greater than 95% homogeneous by sodium dodecyl sulfate electrophoresis on 8% polyacrylamide gels (18). The concentrations of purified ArsAs were determined using a bicinchoninic acid protein assay (Pierce) or from the absorption at 280 nm using a molar extinction coefficient of 33,480 (19). ATPase activity was measured using a coupled assay (20), as described previously (21).

**results**

**arsenate resistance phenotype of G15X mutants—**To examine the effect of residue volume at position 15 on arsenite resistance, Gly-15 was altered to Ala, Asp, Cys, Tyr, and Arg. An inverse relationship was observed (Fig. 1). The larger the residue then the resistance was less, with the phenotype of cells expressing an arsA<sup>G15R</sup> mutation similar to cells with vector alone.

**suppression of G15C or G15R by A344X second site alterations—**By random mutagenesis, an A344V alteration was found to suppress the arsenite-sensitive phenotype of an arsA<sup>G15C</sup> mutant (14). The effect of single mutations at residue 344 on arsenite resistance was examined. Cells expressing six single arsA<sup>G15C</sup> genes (X = G, S, T, D, R, and V) each exhibited an arsenite-resistant phenotype, with only slight differences in resistances at higher concentrations of arsenite (Fig. 2). To examine the relationship of residues 15 and 344 in more detail, the codon for Ala-344 in the arsA<sup>G15C</sup> gene was changed to 11 different codons by site-directed mutagenesis with mixtures of oligonucleotides, producing ArsA derivatives G15C/A344X, where X = G, S, C, T, V, P, D, L, F, Y, or R. Cells harboring the mutated arsA genes and wild-type arsB gene were phenotypically characterized for arsenite resistance (Fig. 3). Cells expressing the wild-type arsB genes could tolerate up to 10 mM Na<sub>2</sub>AsO₃; cells containing only vector were unable to grow when the concentration of arsenite was more than 1 mM. Alteration of Ala-344 to bulkier residues, including Cys, Thr, Pro, Asp, Leu, Phe, Tyr, or Arg each suppressed the G15C substitution. Moreover, the larger the residue at position 344, the higher the concentration of arsenite required to inhibit growth. However, the smaller A344G did not suppress, and a substitution of serine, which has a volume near that of alanine, only marginally suppressed the primary mutation. Similarly, when the G15R substitution was combined with substitutions at residue 344, the larger the residue at position 344, the higher was the resistance of the double mutant (Fig. 4). While the A344R substitution suppressed both G15C or G15R, the secondary mutation was less effective than expected from a simple steric effect, suggesting a slight negative effect of a basic residue at position 344. Overall, these results suggest that the primary effect of the side chain at residue 344 is a steric one, rather than that of charge or polarity.

**effect of G15X and A344X substitutions on ArsA ATPase activity**—It was of interest to examine the effect of single and double mutations on the catalytic activity of the ArsA ATPase. None of the mutations reported here resulted in reduced synthesis of ArsA (data not shown). However, just as was reported by the A344V substitution (14), most A344X derivatives formed inclusion bodies, with very little enzyme found in the cytosol. Attempts to purify the residual soluble portions were unsuccessful; the altered proteins appeared to degrade to smaller polypeptides during chromatography. The formation of inclusion bodies and increased degradation may result from defective folding of the abnormal proteins. However, the G15A, G15C, and G15R singly substituted derivatives could be purified. Consistent with the phenotypic effect of arsA<sup>G15R</sup>, the G15R enzyme was catalytically inactive. When the catalytic parameters of G15C and G15R were compared with wild-type enzyme, their affinity for ATP was decreased from 0.05 to 0.7 mM, and the V<sub>max</sub> for G15A and G15C was decreased 60 and 90%, respectively.

**Discussion**

The glycine-rich P-loop is conserved in purine binding enzymes from prokaryotes to eukaryotes (8). Similar three-di-
mensional structures from RecA (22), myosin (23), the ras oncogene product p21 (24) and F1-ATPase (25) imply that the function of the P-loop is conserved in each enzyme, even though the primary sequence of the P-loop for each specific purine NTPase is variant, and each enzyme has different biological activity (26). Using cassette mutagenesis, Shen et al. (26) substituted each residue of the primary sequence of the yeast mitochondrial F1-ATPase β-subunit P-loop and found that there were constraints imposed by only four residues in the P-loop (G190XXXXGKT197). Furthermore, stereochemical constraints were observed in the P-loops of the E. coli ATP synthase β-subunit (27) and dinucleotide binding proteins (28). An inverse relationship was observed between the volume of β-subunit residue 174 of the E. coli ATP synthase and ATPase activity, suggesting that residue 174 was spatially near the ATP binding site (29), a prediction that was subsequently verified in the crystal structure (25).

ArsA, the catalytic subunit of the Ars pump, has two P-loops (1) and an allosteric effector binding site (4, 5). Previous studies demonstrated the necessity for Gly-15 (14), Gly-18, Gly-20, Thr-22 (30), and Lys-21 (31) in the A1 P-loop (G15KGGVGKT22). Substitutions of residues Gly-334 (14) and Lys-340 (31) in the A2 nucleotide binding domain (G334KGGVGKT341) were better tolerated than the equivalent substitutions in the A1 P-loop. From a combination of results from genetic complementation (12), biochemical reconstitution (13), and intragenic suppression (14), a model has been proposed in which the A1 and A2 nucleotide binding domains of ArsA must physically interact for catalysis to occur, requiring the two domains to be located near each other in the quaternary structure of the enzyme.

In the absence of structural information on ArsA, this hypothesis was tested genetically. An intragenic suppressor of a G15C substitution in the A1 P-loop was found to be in the codon for Ala-344 (14). In nucleotide binding sites, the P-loop is usually preceded by a β-sheet and followed by an α-helix (11). Ala-344 is at the beginning of a putative α-helix which follows the A2 P-loop. In this study, the effect of mutation of the codon for Ala-344 to the codons for eleven different residues was examined. The substitutions include residues representing each type of side group: nonpolar (Gly, Ala, Val, Pro, and Leu), and polar (Glu, Ser, Thr, Asp, and Arg).

Fig. 1. Effect of arsA<sub>G15X</sub> mutations on arsenite resistance. Overnight cultures of E. coli strain JM109 bearing plasmids with wild-type and mutant arsA genes and a wild-type arsB gene were diluted 100-fold into fresh Luria-Bertani medium containing varying concentrations of sodium arsenite. A<sub>600 nm</sub> was measured after 8 h of growth at 37°C and the values normalized to growth in the absence of arsenite. Cells had the following plasmids: ■, pALTER-AB (arsAB); ○, pG15A (arsA<sub>G15A</sub>B); ●, pG15C (arsA<sub>G15C</sub>B); ▲, pG15D (arsA<sub>G15D</sub>B); □, pG15Y (arsA<sub>G15Y</sub>B); ●, pG15R (arsA<sub>G15R</sub>B); and △, vector plasmid pALTER™1.

Fig. 2. Effect of arsA<sub>A344X</sub> mutations on arsenite resistance. Phenotypes of cells expressing single arsA<sub>A344X</sub> mutations were determined as described in the legend to Fig. 1. Cells had the following plasmids: □, pALTER-AB (arsAB); ○, pA344T (arsA<sub>A344T</sub>B); ■, pA344G (arsA<sub>A344G</sub>B); ▲, pA344V (arsA<sub>A344V</sub>B); ●, pA344S (arsA<sub>A344S</sub>B); □, pA344D (arsA<sub>A344D</sub>B); △, pA344R (arsA<sub>A344R</sub>B); and ●, vector plasmid pALTER™1.
polar (Ser, Thr, and Cys), aromatic (Phe and Tyr), negatively charged (Asp), and positively charged (Arg). The ability to suppress the phenotype of the primary mutation correlated only with size and not charge or polarity, with bulkier residues at position 344 providing better suppression of the change at position 15 (Figs. 3 and 4).

The effect of side group substitution at position 15 was examined. A series of single mutants were created by changing the codon for Gly15 in the A1 P-loop to codons for nonpolar (Ala), polar (Cys), aromatic (Tyr), negatively charged (Asp), and positively charged (Arg). Again, the effects correlated primarily with side chain volume and not charge or polarity (Fig. 1). In contrast to the effect of residue size at position 344, there was an inverse effect of residue 15 side group size on arsenite resistance. However, considering that the volumes of arginine (197 Å³) and tyrosine (191 Å³) are similar, an arginine substitution produced less resistance than anticipated. Nevertheless, the G15R phenotype could be effectively rescued by introduction of a larger residue at position 344 (Fig. 4). Thus the primary effect is one of size. These results demonstrate that the interaction of the two nucleotide binding domains can be spatially constrained by the steric effects of residue side chain volume, presumably at an interface between the two domains.

Many transport ATPases have multiple nucleotide binding domains, including ArsA (1), the F₁-ATPase (25) and ABC transporters such as the P-glycoprotein (32). The existence of multiple sites may provide control of catalysis. For example, multisite catalysis of F₁ results from positive cooperativity between nucleotide binding sites (25). P-glycoprotein has two drug binding sites and an allosteric site for modulators of drug transport (32). Moreover, conformational communication between a drug binding site and the nucleotide binding domains of the P-glycoprotein has also been reported (33). Information flow between metal or drug binding sites and catalytic sites in transport ATPases is both expected and necessary for function, both in P-glycoprotein and ArsA. We postulate that this transduction of information is most likely communicated by conformational changes in the enzymes that bring the nucleotide binding sites together, with catalysis accelerated at the interface of the two domains.
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