Asp\textsuperscript{45} Is a Mg\textsuperscript{2+} Ligand in the ArsA ATPase*

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The ATPase activity of ArsA, the catalytic subunit of the plasmid-encoded, ATP-dependent extrusion pump for arsenicals and antimonials in *Escherichia coli*, is allosterically activated by arsenite or antimonite. Magnesium is essential for ATPase activity. To examine the role of Asp\textsuperscript{45}, mutants were constructed in which Asp\textsuperscript{45} was changed to Glu, Asn, or Ala. Cells expressing these mutated *arsA* genes lost arsenite resistance to varying degrees. Purified D45A and D45N enzymes were inactive. The purified D45E enzyme exhibited approximately 5% of the wild type activity with about a 5-fold decrease in affinity for Mg\textsuperscript{2+}. Intrinsic tryptophan fluorescence was used to probe Mg\textsuperscript{2+} binding. ArsA containing only Trp\textsuperscript{159} exhibited fluorescence enhancement upon the addition of MgATP, which was absent in D45N and D45A. As another measure of conformation, limited trypsin digestion was used to estimate the surface accessibility of residues in ArsA. ATP and Sb(III) synergistically protected wild type ArsA from trypsin digestion. Subsequent addition of Mg\textsuperscript{2+} increased trypsin sensitivity. D45N and D45A remained protected by ATP and Sb(III) but lost the Mg\textsuperscript{2+} effect. D45E exhibited an intermediate Mg\textsuperscript{2+} response. These results indicate that Asp\textsuperscript{45} is a Mg\textsuperscript{2+}-responsive residue, consistent with its function as a Mg\textsuperscript{2+} ligand.

The *ars* operon of conjugal R-factor R773 encodes an arsenite extrusion system that confers arsenite or antimonite resistance to *Escherichia coli* by extruding arsenicals and antimonials out of the cell, thus lowering their intracellular concentration (1). This efflux pump is composed of two types of subunits, ArsA and ArsB. The 63-kDa ArsA ATPase is the catalytic subunit. The 45-kDa ArsB subunit is an integral membrane protein that acts as the membrane anchor for ArsA and the oxyanion-translocating sector of the pump (2). When overexpressed, ArsA is found in the cytosol and purified as a soluble protein (3).

From its primary sequence, ArsA is composed of N-terminal (A1) and C-terminal (A2) halves that are homologous to each other, most likely the result of an ancestral gene duplication and fusion (4). Each half contains a consensus sequence for the phosphate binding loop (P-loop) of an ATP-binding site (5). Previous studies showed that both ATP-binding sites are required for catalysis and resistance (6, 7). The results of intergenic complementation and intragenic suppression studies have suggested a model in which catalysis occurs at the interface of the A1 and A2 ATP-binding sites (8). Recently, a highly conserved DTAP consensus sequence has been identified in ArsA homologues. These homologues are found in members of every kingdom from bacteria to humans (9). From the results of intrinsic tryptophan fluorescence, we have suggested this domain may act as a signal transduction domain that relays the communication between ATP-binding sites and the allosteric As(III)/Sb(III)-binding site.

From the sequence alignment of ArsA homologues with other enzymes such as nitrogenase iron protein (NifH) (10), RecA (11), and GTP-binding proteins, including Ras p21 (12), there is a highly conserved Asp-Pro (Fig. 1), which, in G-proteins, forms part of an effector loop. From the crystal structure of Ras p21 with GTP bound, the conserved Asp residue was shown to form a portion of the Mg\textsuperscript{2+}-binding site (12). In GTPases a magnesium ion brings together diverse components of the GTP-binding core, facilitating the information flow between domains. Elucidation of the Mg\textsuperscript{2+} coordination is therefore important for deciphering the mechanism of nucleotide-binding proteins.

Sequence alignment of ArsA with GTPases and other ATPases suggests that Asp\textsuperscript{45}-Pro\textsuperscript{46} are the corresponding residues in ArsA. In this study Asp\textsuperscript{45} was changed by mutagenesis to several other residues. The properties of the resulting mutant strains and the purified ArsAs support this postulate. First, an acidic residue is required at position 45 for ArsA ATPase activity. The wild type enzyme exhibited 20-fold more ATPase activity than the D45E derivative, with 5-fold higher affinity for Mg\textsuperscript{2+}. The D45A and D45N derivatives were inactive. Second, using intrinsic tryptophan fluorescence (13) and accessibility to trypsin to detect conformational changes upon ligand binding (3), Asp\textsuperscript{45} was shown to be required for Mg\textsuperscript{2+}-dependent responses.

**MATERIALS AND METHODS**

**Medium and Growth Conditions—** *E. coli* strains and plasmids used in this study are described in Table I. Cells were grown in Luria-Bertani medium (14) at 37 °C. Ampicillin (125 μg/ml) and tetracycline (12.5 μg/ml) were added as required. Sodium arsenite was added at the indicated concentrations.

**DNA Manipulations—** The conditions for plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation have all been described (15). Restriction enzymes and nucleic acid-modifying enzymes were obtained from Life Technologies, Inc. The Wizard\textsuperscript{TM plus} minipreps DNA purification system and the Wizard\textsuperscript{TM} DNA clean-up system (Promega) were used to prepare plasmid DNA for restriction enzyme digestion and to recover DNA fragments from low melting agarose gels, respectively.

**Oligonucleotide-directed Mutagenesis—** Mutations in the *arsA* gene were introduced by site-directed mutagenesis using the Altered Sites\textsuperscript{TM in vitro} mutagenesis system (Promega) with plasmid pTZ3H6 containing the *arsA* and *arsB* genes (9) in *E. coli* ES1301 mutS. In this plasmid the *arsA* gene was previously mutated to contain only a single tryptophan codon (Trp\textsuperscript{159}), and the sequence for six histidine codons was added at the 3′ end. This plasmid was used as the template to produce *arsA* mutants with D45E, D45A, or D45N substitutions. The mutagenic oligonucleotides used and the respective changes (underlined) obtained were as follows: D45A, 5′-TGAGGCCGGGCGGCTACTGAC-3′; D45E,
The soluble ArsA proteins were purified as described (9). ATPase activity was measured using an NADH-coupled assay (16) with 5 mM ATP.

Fluorescence Measurements—Fluorescence measurements were performed using an SLM-8000C spectrophotofluorometer with a built-in magnetic stirrer. The bandwidths for emission and excitation monochromators were 4 nm. Tryptophan fluorescence was monitored with an SLM-8000C spectrofluorometer with a built-in magnetic stirrer. The excitation wavelength of 295 nm and an emission wavelength of 337 nm. The fluorescence of the buffer (50 mM MOPS-KOH, pH 7.5) alone was subtracted from protein spectra. ArsA (1.25 μM), ATP (5 mM), and 2.5 mM MgCl₂ unless otherwise noted.

Limited Trypsin Digestion of ArsA—Limited trypsin digestion was performed at room temperature and terminated at the indicated times by the addition of a 3-fold excess of soybean trypsin inhibitor to the reaction mixture as described previously (3). The reaction mixtures were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by staining with Coomassie Blue, and the ATPase activity of each protein was determined using a Cy5-AUTOREAD sequencing kit with an ALFexpress system (Amersham Pharmacia Biotech). Plasmid DNA for sequencing was prepared with a Mini kit (QIAGEN).

Fluorescence Measurements—Fluorescence measurements were performed using an SLM-8000C spectrophotofluorometer with a built-in magnetic stirrer. The bandwidths for emission and excitation monochromators were 4 nm. Tryptophan fluorescence was monitored.

Limited Trypsin Digestion of ArsA—Limited trypsin digestion was performed at room temperature and terminated at the indicated times by the addition of a 3-fold excess of soybean trypsin inhibitor to the reaction mixture as described previously (3). The reaction mixtures were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (17).

RESULTS

Effect of D45X Substitutions on Resistance to Arsenite—E. coli cells harboring plasmid pTZ3H6 exhibited resistance to 4 mM sodium arsenite (Fig. 2). The arsA gene in this plasmid encodes a modified ArsA (W159H6) in which there is only a single tryptophan residue at position 159 and six histidine residues fused to the C terminus. Resistance conferred by this plasmid was the same as a plasmid bearing a wild type arsA gene (9), so this modified arsA gene was used as the parent for all D45X mutants. Mutational replacement of Asp45 with glutamic acid resulted in a reduction in arsenite resistance. Substitution of Asp45 with asparagine resulted in a further decrease in resistance, and cells expressing a D45A substitution lost arsenite resistance.

ATPase Activity of D45X ArsAs—The W159H6 ArsA and its D45X derivatives were purified with Ni²⁺ affinity chromatography to greater than 95% homogeneity, as judged by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Blue, and the ATPase activity of each protein was determined. In the presence of 5 mM ATP, 2.5 mM MgCl₂, and 0.1 mM potassium antimonal tartrate, D45E ArsA exhibited approximately 5% of wild type ATPase activity (data not shown). In contrast, no activity was observed with either the D45A or D45N enzymes.

ATPase activity was measured at varying concentrations of MgCl₂. With the parental enzyme, the concentration of MgCl₂ required for half-maximal ATPase activity was 0.8 mM (Fig. 3). The activity of the D45E enzyme was too low to obtain an accurate fit of the data, but it required at least 5-fold more divalent cation for maximal activity than the wild type. This suggests that the introduction of a glutamic acid residue at position 45 lowered the affinity for Mg²⁺ consistent with a role of Asp45 in coordination of the divalent cation.

Effect of Asp45 Substitutions on Intrinsic Fluorescence of Trp159—In the parental ArsA, Trp159 has a maximum emission at 337 nm in the native enzyme and at 353 nm in the denatured protein when excited at 295 nm (9). Each of the D45X proteins had the same emission maximum as the parental (Fig. 4A),
suggesting that the substitutions of Asp\(^{45}\) with Glu, Asn, or Ala per se have little effect on the overall protein conformation.

In the wild type ArsA the fluorescence of Trp\(^{159}\) has been shown to respond to MgATP binding (13). To produce an effect, both the divalent cation and ATP must be present (Fig. 4B).

Compared with the parental enzyme, fluorescence of the D45E ArsA exhibited a smaller response to MgATP addition. With both parental and D45E enzymes the fluorescent enhancement produced by MgATP was reversed by the addition of disodium EDTA. In contrast, neither the D45A nor the D45N enzyme responded to the addition of Mg\(^{2+}\).

Effect of D45X Substitutions on Accessibility to Trypsin—Limited trypsin digestion has been used to study the surface accessibility of arginine and lysine residues in the 63-kDa ArsA (3). The initial cleavage by trypsin produces a fragment of approximately 50 kDa with subsequent cleavage to smaller polypeptides. The rate of cleavage was decreased synergistically by the binding of both ATP and Sb(III), suggesting that the enzyme undergoes a conformational change when both substrate and allosteric effector sites are filled (13). However, those results were obtained in the absence of Mg\(^{2+}\). The addition of Mg\(^{2+}\) proved to be antagonistic to the protection conferred by ATP and Sb(III), increasing the rate of trypsin cleavage (Fig. 5). This suggests that the binding of Mg\(^{2+}\) to the enzyme in which the substrate and allosteric sites are already filled produces a conformational change that makes a tryptic site more accessible.

ATP and antimonite synergistically protected each D45X protein to the same extent as the parental ArsA, indicating that substitutions of Asp\(^{45}\) do not grossly affect the filling of the allosteric and substrate sites (Fig. 5). However, Mg\(^{2+}\) caused no effect on the tryptic pattern in the D45A or D45N proteins and a partial response with the D45E ArsA. The antagonism by Mg\(^{2+}\) on the parental and D45E ArsAs was reversed by EDTA, indicating that the effect of Mg\(^{2+}\) requires an acidic residue at position 45.

DISCUSSION

Coordination of Mg\(^{2+}\) in ATP- and GTP-binding proteins frequently involves aspartate residues (18–21). ArsA, the cat-
alytic subunit of the bacterial arsenite pump, hydrolyzes ATP to provide energy for arsenite efflux from cells (22). Mg\(^{2+}\) has been shown to be required for ArsA ATPase activity (3). The results described in this study indicate that the ArsA residue Asp45 is one ligand to the divalent cation.

First, sequence alignment of ArsA homologues demonstrates a conserved pair of residues that corresponds to Asp45-Pro46 in the R773 ArsA (Fig. 1). This DP sequence is conserved in other GTPases and ATPases, for example Asp33-Pro34 in Ras p21 (12), Asp\(^{33}\)-Pro\(^{34}\) in RecA (11), and Asp\(^{39}\)-Pro\(^{40}\) in the NifH iron protein subunit of nitrogenase (23) (Fig. 1). From the crystal structure of Ras p21 it was shown that in the GTP binding state Asp33 is a Mg\(^{2+}\) ligand. Similarly, from the crystal structure of NifH, Asp39 is in close proximity to the Mg\(^{2+}\) in the ATP-binding site. Based on the sequence comparisons, it is reasonable to hypothesize that Asp45 in ArsA may be involved in the coordination of the Mg\(^{2+}\) complexed with ATP.

Second, substitution of Asp45 in ArsA with Asn, Ala, or Glu

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**FIG. 4. Effect of D45X substitutions on intrinsic tryptophan fluorescence.** A, emission spectra of Trp\(^{159}\) in parental and D45X ArsAs. —, wild type; ---, D45E; --, D45N; ..., D45A. B, effect of D45X mutations on the response of Trp\(^{159}\) fluorescence to the addition of MgATP and EDTA. At indicated times, 5 mM ATP, 2.5 mM MgCl\(_2\), or 5 mM EDTA was added to 1.25 \(\mu\)M ArsA. Curve 1, wild type; curve 2, D45E; curve 3, D45N; curve 4, D45A.

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**FIG. 5. Effect of D45X substitutions on trypsin sensitivity.** Trypsin digestion was performed at room temperature with the indicated additions: 5.0 mM ATP, 0.5 mM potassium antimonyl tartrate (Sb(III)), 2.5 mM MgCl\(_2\), or 5.0 mM EDTA. ArsA proteins (16 mg/ml, final concentration) were mixed with trypsin (0.16 mg/ml, final concentration). At the indicated times, samples were removed, and the reactions were terminated by the addition of a 2-fold excess of soybean trypsin inhibitor. The tryptic products were analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels stained with Coomassie Blue. WT, wild type.
resulted in reduced arsenite resistance for cells harboring the respective plasmid, indicating that an aspartate residue at position 45 promotes ArsA catalysis. Although an aspartate residue is not absolutely required, because the D45E and D45N mutants retained partial resistance, the wild type enzyme is approximately 20-fold more active than the purified D45E ArsA and has about a 5-fold higher affinity for Mg\(^{2+}\) (Fig. 3).

Third, Mg\(^{2+}\) produces conformational changes in ArsA that can be detected from the effect of the cation on the intrinsic fluorescence of Trp\(^{159}\) (Fig. 4) or on the surface accessibility of the protein to trypsin (Fig. 5). As shown previously, the fluorescence of Trp\(^{159}\) responds specifically to Mg\(^{2+}\) and ATP (13).

With the D45E ArsA, the addition of Mg\(^{2+}\) and ATP induced an intermediate fluorescence enhancement that was reversed by EDTA. However, the fluorescence of the D45A or D45N proteins did not respond to MgATP. Moreover, the reversal of protection from trypsin digestion that Mg\(^{2+}\) afforded the wild type enzyme was absent in the D45N and D45A proteins and reduced in the D45E. These results strongly point to a role for Asp\(^{45}\) in binding Mg\(^{2+}\).

Several lines of evidence indicate that the substitutions did not affect the overall conformation of the proteins or their ability to bind the substrate ATP or the allosteric activator Sb(III). First, the emission spectra of all of the proteins were superimposable, indicating that the environment of Trp\(^{159}\) was the same in each (Fig. 4A). Second, the tryptic patterns of all the proteins were similar in the absence of ligands (data not shown) or in the presence of the nucleotide and the allosteric effector (Fig. 5), indicating that the surface of the proteins had reasonably equal accessibility to large molecules such as trypsin. The protection from trypsin afforded by the combination of ATP and Sb(III) (but in the absence of Mg\(^{2+}\)) shows that the binding of a substrate or an allosteric effector was not substantially altered by the replacement of Asp\(^{45}\) with another acidic residue (glutamate), a neutral residue (asparagine), or a smaller residue (alanine).

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