Tryptophan Fluorescence Reports Nucleotide-induced Conformational Changes in a Domain of the ArsA ATPase*

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The ars operon of plasmid R773 encodes an ATP-dependent extrusion pump for arsenite and antimonite in Escherichia coli. The ArsA ATPase is the catalytic subunit of the pump protein, with two nucleotide binding consensus sequences, one in the NH2-terminal half and one in the COOH-terminal half of the protein. A 12-residue consensus sequence (DTAPTGHTIRLL) has been identified in ArsA homologs from eubacteria, archaebacteria, fungi, plants, and animals. ArsA enzymes were constructed containing single tryptophan residues at either end of this conserved sequence. The emission spectrum of the fluorescence of the tryptophan on the COOH-terminal end (Trp-159) indicated a relatively hydrophilic environment for this residue. An increase in intrinsic tryptophan fluorescence and a blue shift of the maximum emission wavelength were observed upon addition of MgATP, indicating movement of Trp-159 into a less polar environment. No fluorescence response was observed with MgADP, with nonhydrolysable ATP analogs, or with MgATP by catalytically inactive enzymes. This suggests that the location Trp-159 is shifted only during hydrolysis of ATP. In contrast, the emission spectrum of Trp-141, located on the NH2-terminal side of the consensus sequence, indicated a relatively nonpolar environment. The maximum emission wavelength red shifted upon addition of MgADP. MgATP slowly produced a response that correlated with product formation, suggesting that the environment of Trp-141 is sensitive only to MgADP binding. Thus, during ATP hydrolysis the COOH-terminal end of the conserved domain moves into a less polar environment, whereas the NH2-terminal end moves into a more hydrophilic environment as product is formed. A hypothesis is presented in which the conserved domain of ArsA and homologs is an energy transduction domain involved in transmission of the energy of ATP hydrolysis to biological functions such as transport.

Resistance to arsenical and antimonal salts in the Gram-negative bacterium Escherichia coli is conferred by the ars operon of conjugative R-factor R773 (1). This operon encodes an ATP-coupled efflux pump that actively transports the trivalent arsenicals and antimonials out of the cell; reducing the intracellular concentration of those metalloid oxyanions to subtoxic levels produces resistance (2). The pump consists of two types of polypeptides, ArsA and ArsB. ArsA is the 63-kDa catalytic subunit. ArsB is a 45-kDa integral membrane protein that is the membrane anchor for ArsA and the oxyanion-translocating sector of the pump (3). In the absence of ArsB, ArsA can be purified as a soluble ATPase (4). The NH2-terminal (A1) and COOH-terminal (A2) halves of ArsA ATPase are homologous to each other, likely the result of an ancestral gene duplication and fusion (5). Both the A1 and A2 halves contain a consensus sequence for the phosphate binding loop (P-loop) of an ATP binding site (6), and both sites are required for catalysis and resistance (7, 8). From the results of intergenic complementation and intragenic suppression, a model was proposed in which a single catalytic site was formed at the interface of an A1 and an A2 ATP binding site (9, 10).

Construction of single tryptophan-containing proteins has proven of value in the study of other E. coli transport enzymes. For example, the environments of regions of the mannitol enzyme II were investigated from the fluorescence of strategically placed tryptophan residues (11). Senior and colleagues have used tryptophan fluorescence as a direct probe of nucleotide binding in the noncatalytic sites of E. coli F1-ATPase (12, 13) and to discriminate between binding of substrate and product in the catalytic sites (14). We have shown previously that intrinsic tryptophan fluorescence can be used to investigate the interactions with its ligands (15). However, the presence of multiple tryptophan residues in ArsA decreased the signal-to-noise ratio, indicating that this common motif may have a conserved function. Thus, to examine the microenvironment of the conserved sequence, an F141W substitution was introduced into the tryptophan-containing ArsA, producing a His-tagged ArsA containing only Trp-159 (W159H6); a His-tagged tryptophan-free ArsA was also constructed. The single tryptophan-containing W159H6 ArsA gave approximately a 4-fold increase in the signal-to-noise ratio in response to MgATP/mol of tryptophan residue compared with the wild type enzyme. The sequence D142TAPTGHTIRLL153 in A1 is highly conserved in ArsA homologs from every kingdom, implying that this common motif may have a conserved function. To examine the microenvironment of the conserved sequence, an F141W substitution was introduced into the tryptophan-free ArsA, producing a W141H6 enzyme. The fluorescence spectra indicate that Trp-141 is located in a hydrophilic region, whereas Trp-159 is found in a relatively hydrophobic environment. Binding of MgADP moved Trp-141 into a more hydrophilic region; in contrast, the addition of MgATP shifted Trp-159 into a more hydrophobic environment.

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To examine the effect of mutation in the nucleotide binding sites, substitutions in the A1 (G18R) and A2 (G337R) P-loops were introduced into single tryptophan-containing ArsAs. The results showed that neither binding of substrate nor product were introduced into single tryptophan-containing ArsAs. The sites, substitutions in the A1 (G18R) and A2 (G337R) P-loops, were the products of Life Technologies, Inc. and Promega. Mutagenic oligonucleotides were synthesized by the Macromolecular Core Facility at the University of Washington. Restriction enzymes and nucleic acid-modifying enzymes were from Promega, Inc. Plasmid DNA for sequencing was prepared with a miniprep kit (QIAGEN). A column containing 5 ml of ProBond Ni²⁺ affinity resin was obtained from Invitrogen. All other chemicals were purchased from commercial sources.

**Materials**—Restriction enzymes and nucleic acid-modifying enzymes were the products of Life Technologies, Inc. and Promega. Mutagenic oligonucleotides were synthesized by the Macromolecular Core Facility of the Department of Biochemistry and Molecular Biology at Wayne State University School of Medicine. The ProBond Ni²⁺ affinity resin was obtained from Invitrogen. All other chemicals were purchased from commercial sources.

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FIG. 1. 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-1-thiogalactopyranoside, and growth was measured after 6 h at 37°C. Cells had the following plasmids: ●, pALTER-AB (arsA); ●, pT73H6 (arsA, W235Y, W327Y, W328Y, E312H); ▲, pW141H6 (arsA, W141Y, W159Y, W235Y, W322Y, W328Y, E312H); □, pT73H6G18R (arsA, W235Y, W327Y, W328Y, G18R, E312H); ○, pW141H6G18R (arsA, W141Y, W159Y, W235Y, W322Y, W328Y, G18R, E312H); △, pW141H6G337R (arsA, W141Y, W159Y, W235Y, W322Y, W328Y, G337R, E312H); □, pALTER-1.

30K (Millipore) at 2,000 × g. Purified ArsA was quickly frozen and stored in small aliquots at −80°C. The concentration of purified ArsA was determined by UV absorbance at 280 nm. The extinction coefficients for W159H6 ArsA and W141H6 ArsA were calculated to be 20,250 and 21,530 M⁻¹ cm⁻¹, respectively (17).

ATPase Assays—Routine ATPase assay was carried out as described (18). To correlate the rate of ATP hydrolysis with the rate of change of tryptophan fluorescence, ATPase activity was estimated from the release of phosphate by the method of Fiske and SubbaRow (19). ArsA and ATP at the indicated concentrations were incubated at room temperature for 5 min in buffer containing 50 mM MOPS-KOH, pH 7.5. One portion was used to monitor the fluorescence change, and the remainder was used to assay ATPase activity. The reaction was initiated by the addition of MgCl₂ to a final concentration of 0.5 mM. At indicated times, portions of 0.2 ml were withdrawn and mixed with 0.2 ml of 20% trichloroacetic acid to terminate the reaction. Precipitated protein was removed by centrifugation, and 0.3 ml of the supernatant solution was combined with 0.2 ml of 20% H₂SO₄. After 10 min at 37°C, the absorbance at 740 nm was measured, and phosphate concentrations were determined from the absorbance of known amounts of phosphate.

Fluorescence Measurements—Fluorescence measurements were performed on a SLM-800C spectrophotofluorometer at room temperature. Samples were stirred continuously in a 1.0 × 1.0-cm quartz cuvette. The excitation wavelength was set at 295 nm for selective excitation of tryptophan fluorescence. The bandwidths for both emission and excitation monochromators were 4 nm. Spectra were corrected for background and Raman scattering by subtracting buffer spectra. Free tryptophan was used as an external standard for comparison of different spectra. To monitor the fluorescence intensity change with time or addition of quenching ligands, the emission wavelengths were set to 322 nm for monitoring the fluorescence intensity change with time or addition of MgCl₂ to a final concentration of 0.5 mM. At indicated times, portions of 0.2 ml were withdrawn and mixed with 0.2 ml of 20% H₂SO₄. After 10 min at 37°C, the absorbance at 740 nm was measured, and phosphate concentrations were determined from the absorbance of known amounts of phosphate.

RESULTS

Characteristics of Altered ArsA Enzymes—The arsenite resistance phenotype of cells of strain JM109 bearing plasmids with mutated arsA genes was analyzed (Fig. 1). Sequential substitution of tyrosines for Trp-253, Trp-524, and Trp-524 and addition of the six histidine residues at the COOH terminus produced an ArsA with only Trp-159 (W159H6). Substitution of tyrosine for Trp-159 in W159H6 produced a tryptophan-free ArsA. Subsequent substitution of tryptophan for Phe-141 resulted in the single tryptophan ArsA W141H6. Each mutated gene conferred wild type resistance to arsenite. Introduction of a G18R substitution in the A1 P-loop (8) or G337R in the A2 P-loop (7) in either W159H6 or W141H6 produced an arsenite-sensitive phenotype.

Each ArsA was purified by Ni²⁺ affinity chromatography and ATPase assay. Although ArsA with a COOH-terminal histidine tag had slightly less activity than the wild type enzyme, both W159H6 and W141H6 are as active as the wild type protein with a COOH-terminal histidine tag (data not shown). Combination of the G18R or G337R substitution with W159H6 or W141H6 resulted in inactive proteins. The results indicate that none of the tryptophan residues nor Phe-141 is essential for activity, allowing tryptophan fluorescence to be used as an intrinsic probe of ArsA catalysis.

Fluorescent Properties of W159H6 and W141H6 ArsA ATPases—The polarity of microenvironment of tryptophan residues in a protein can be assessed from their fluorescence emission spectra (21). In a less polar environment the maximum emission wavelength (λₓᵧₘₓ) of tryptophan shifts to a lower wavelength, with an increase in the fluorescence yield (21). When excited at 295 nm, free tryptophan in an aqueous solution has a λₓᵧₘₓ at 353 nm (Fig. 2, curve 5), whereas the λₓᵧₘₓ of Trp-159 and Trp-141 were 337 nm (Fig. 2, curve 2) and 322 nm (Fig. 2, curve 1), respectively. At the same concentration of protein the fluorescence yield of Trp-141 was nearly twice that of Trp-159. The λₓᵧₘₓ of each shifted to 353 nm upon denaturation of the proteins with 6 M guanidine HCl (Fig. 2, curves 3 and 4). These results demonstrate that Trp-141 is in a relatively less polar environment than Trp-159.

The environment of a specific tryptophan can also be evaluated by its accessibility to collisional fluorescence quenchers (20). The Stern-Volmer constants (Kₛ) for Trp-141, Trp-159, and free tryptophan quenching with acrylamide and I⁻ under native and denatured conditions were determined (Table I). In native ArsA Trp-141 is in an environment 3.3-fold less accessible to either I⁻ or acrylamide than Trp-159, even though both
Trp-159 and Trp-141 are almost equally accessible in denatured proteins by I⁻ or acrylamide. Compared with free tryptophan, Trp-141 was 37-fold and 13-fold less accessible to I⁻ and acrylamide, respectively, indicating that it is extremely shielded from the solvent. In denatured ArsA, Trp-141 was 11-fold and 6-fold more accessible by I⁻ and acrylamide, respectively, than in the native enzyme, whereas accessibility of Trp-159 was increased by a factor of 3.6 and 1.5 for I⁻ and acrylamide, respectively. However, the K_D values for the denatured proteins were still lower than the K_D values for free tryptophan, which may indicate that the proteins are not completely unfolded by guanidine HCl. Consistent with the results from fluorescence emission spectra presented above, the fluorescence quenching data also suggest that Trp-141 is located in a less polar region than Trp-159, possibly by proximity to negatively charged amino acid residues.

**Effect of MgATP and MgADP on Fluorescence of Trp-141 and Trp-159**—In the presence of both 1 mM ATP and 0.5 mM MgCl₂, the emission spectrum of W159H6 ArsA exhibited a shift of about 5 nm, with an increase in fluorescence intensity (Fig. 3A, curve 1) compared with the absence of nucleotide (Fig. 3A, curve 3). Neither ATP nor MgCl₂ alone produced a change in fluorescence (data not shown), indicating that binding of the MgATP complex induced a conformational change that moves Trp-159 into a relatively more hydrophobic region. MgADP produced no change in the emission spectrum of W159H6 (Fig. 3A, curve 2). In control experiments, MgATP was shown to have no effect on the fluorescence of free tryptophan or on denatured protein (data not shown). ArsA neither aggregates nor exhibits a change in light scattering properties upon addition of nucleotides (22), so the fluorescence increase cannot be due to light scattering produced by aggregation.

In contrast, when W141H6 was incubated with MgADP, the λ_max exhibited a red shift of about 5 nm, with a decrease in fluorescence intensity (Fig. 3B, curve 3) compared with the absence of nucleotide (Fig. 3B, curve 1). Neither MgCl₂ nor ADP alone produced a response (data not shown). Incubation of W141H6 with the nonhydrolyzable ATP analog AMP-PNP produced no change in the emission spectrum (Fig. 3B, curve 2). The response of W141H6 to MgATP was more complicated. With time λ_max gradually red shifted, while the fluorescence intensity decreased (Fig. 3C). After 20 min the spectra of the samples with MgATP resembled that of W141H6 exposed to MgADP. The facts that the effect of ATP was time-dependent and that a nonhydrolyzable ATP analog gave no response suggest that only MgADP, the product of hydrolysis, produces a change in the intrinsic fluorescence of Trp-141. Since the effect of MgADP is a red shift with a decrease in fluorescence intensity, the data suggest that binding of MgADP induces a movement of Trp-141 into a relatively more polar environment.

**Enhancement of Trp-159 Fluorescence Reports a Transient State during ATP Hydrolysis**—Although MgATP induces an enhancement of the fluorescence of Trp-159, in wild type ArsA the enhancement was relatively low compared with the total tryptophan fluorescence. The signal-to-noise ratio was increased considerably in the single tryptophan containing W159H6 (Fig. 4A, curve 1) compared with the wild type histagged enzyme (Fig. 4A, curve 2) when the amount of each protein was adjusted to equivalent concentrations of trypto-
phan residues. This enhancement allowed a more sensitive and detailed characterization of the MgATP-responsive signal.

Although a sustained fluorescence enhancement was observed with a saturating concentration of MgATP (Fig. 4A), enhancement was transient at 5 μM ArsA with 20 μM MgATP (Fig. 4B, curve 1). If a second pulse of ATP was added following the decrease in fluorescence, a further transient increase was observed (Fig. 4B, curve 1). Moreover, the rate of decay of the fluorescence signal correlated with the rate of phosphate released during ATP hydrolysis (Fig. 5A). Addition of neither MgADP (Fig. 4C, curve 2) nor MgATP or the combination of MgADP and phosphate (Fig. 4C, curve 4) enhanced fluorescence. These results indicate that neither the binding of substrate nor the binding of products to ArsA is sufficient to cause the fluorescence change, suggesting that the increase in fluorescence was related to ATP hydrolysis rather than MgATP binding per se, reporting the conformation of an intermediate formed during the catalytic cycle.

Binding of MgADP was shown to result in movement of Trp-141 to a more polar environment. Addition of MgADP to W141H6 produced a red shift in the λmax and a quenching of fluorescence, whereas MgATP produced a similar effect only at later times (Fig. 3C).
From the kinetics of the fluorescence quenching it appears that the addition of MgADP produced a rapid decrease in fluorescence intensity (Fig. 6, curve 1), whereas MgATP affected a slow change that took approximately 20 min to reach completion (Fig. 6, curve 2). Fluorescence quenching did not occur in the absence of MgCl₂ and was reversed by the addition of EDTA (data not shown), indicating a requirement for Mg²⁺.

The nonhydrolyzable ATP analogs AMP-PNP (Fig. 6, curve 3) and FSBA (Fig. 6, curve 4) had no effect on the fluorescence of W141H6. Moreover, FSBA inhibited the response to ATP (Fig. 6, curve 4). These results suggest that Trp-141 responds to the binding of the product of the reaction, MgADP, and not to MgATP. The rate of quenching produced by MgATP correlated with the rate of ADP formation (Fig. 5B). There was no effect of MgADP or MgATP on the fluorescence of W141H6 ArsA proteins with a G18R substitution in the A1 P-loop or a G337R substitution in the A2 P-loop (Fig. 6, curves 5–8). These proteins would still be expected to bind ADP at the nonsubstituted site, so occupancy of both sites may be required to produce the conformational change reported by Trp-141. However, the data clearly show that Trp-141 moves into a more polar environment upon binding of MgADP and is a sensitive probe for the binding of the product of hydrolysis.

**DISCUSSION**

The R773 ars operon confers arsenite and antimonite resistance to *E. coli* cells by encoding an ATP-coupled oxyanion pump that extrudes those toxic compounds out of the cell, lowering their intracellular concentration to subtoxic levels. The catalytic subunit of the pump, ArsA, is a 63-kDa protein that hydrolyzes ATP in the presence of Mg²⁺. It has a basal hydrolytic activity that is allosterically activated by antimonite or arsenite, which is accompanied by formation of a homodimer (4, 22). ArsA has two consensus ATP binding motifs (6), one in each of the A1 and A2 halves (5). Both the A1 and A2 binding sites are required for resistance and catalysis (7, 8). Based on results from biochemical analysis (22), genetic complementation (9), and intragenic suppression (10), a model has been proposed in which the interface of an A1 and an A2 binding site forms a single catalytic unit (9, 10). However, it is not known whether the interface forms from the A1 and A2 domains of a single ArsA monomer or from intersubunit interactions.

Wild type ArsA has four tryptophans located at positions 159, 253, 522, and 524 (5). We have shown previously that intrinsic tryptophan fluorescence can be used to investigate the interactions of ArsA with its ligands and that the fluorescence of Trp-159 is responsive to the interaction of ArsA with MgATP (15). In this study single tryptophan-containing ArsA enzymes were used as sensitive probes of ligand interaction. An ArsA with a single tryptophan at residue 159 exhibited a large enhancement of fluorescence upon addition of MgATP, with a concomitant shift in λ_max to lower wavelength. This increase of fluorescence intensity and the blue shift of emission spectrum of Trp-159 indicate that the interaction of ArsA with MgATP causes a specific conformational change that moves Trp-159 into a more hydrophobic environment. However, the addition of nonhydrolyzable MgATP analogs or the product, MgADP, did not produce a fluorescence change, nor did MgATP produce a change in inactive enzymes unable to bind nucleotide at either the A1 or the A2 site. Moreover, the fluorescence enhancement was transient and correlated with the rate of ATP hydrolysis. Thus it is likely that the fluorescence of Trp-159 is reporting a transient state of the enzyme formed during ATP hydrolysis. In addition, the loss of fluorescence response following inactivation of either nucleotide binding site supports our previous hypothesis that the A1 and A2 sites must interact to form a single catalytic unit (10, 22).

A second single tryptophan-containing ArsA was constructed in which Phe-141 was altered to Trp-141. The substitution was neutral, having no effect on ATPase activity. Comparison of the emission spectra of Trp-141 and Trp-159 suggests that the former is in a more hydrophobic environment than the latter. In accordance with this idea, the fluorescence of Trp-159 was nearly unaffected by the collisional quencher KI, whereas Trp-159 was quenched readily. In contrast to Trp-159, the fluorescence of Trp-141 was quenched by MgADP. Although MgATP produced quenching, the response was slow and paralleled formation of ADP by hydrolysis, suggesting that Trp-141 responds only to the product of the reaction. Consistent with this, nonhydrolyzable ATP analogs produced no quenching, nor did MgATP affect the fluorescence of catalytically inactive enzymes.

Thus two aminoacyl residues separated by only 17 residues (F₁₄₁,D₁₅₉,G₁₆₀,P₂₅₃,G₂₅₄) respond quite differently to nucleotides. This sequence is conserved in the A2 half of ArsA and in homologs from every kingdom, including bacteria, archaea, fungi, plants, and animals (Fig. 7). Although most of these homologs have been identified solely as open reading frames by nucleotide sequencing, the conservation of this sequence suggests that it is a functional domain. Interestingly, the human homolog has been implicated in arsenite resistance (23). In ArsA this sequence may be involved in ATP hydrolysis, since none of the conformational changes occurs with nonhy-
drolyzable ATP analogs or with catalytically inactive ArsA enzymes. By analogy, we would propose that this conserved domain is involved in ATP hydrolysis in homologs, which would, therefore, be ATPases. During ATP hydrolysis the COOH-terminal end of the domain moves into a more hydrophobic environment. As product is formed, the NH 2-terminal end moves into a more hydrophilic environment. At the NH 2-terminus of this domain is a conserved aspartate residue, corresponding to Asp-142 in the A1 half of ArsA. In preliminary experiments we have found that a D142N substitution results in a arsenite-sensitive phenotype, suggesting that Asp-142 is responding to Asp-142 in the A1 half of ArsA. In preliminary experiments we have found that a D142N substitution results in a arsenite-sensitive phenotype, suggesting that Asp-142 is responding to Asp-142 in the A1 half of ArsA. We hypothesize that the conserved domain of ArsA and homologs is an energy transduction domain that might be involved in transmission of the energy of ATP hydrolysis to other functions such as transport of arsenite through the ArsB subunit of the oxyanion pump.

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