Interaction of Substrate and Effector Binding Sites in the ArsA ATPase†

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ABSTRACT: The *ars* operon of plasmid R773 confers resistance to antimonials and arsenicals in *Escherichia coli* by encoding an ATP-dependent extrusion system for the oxyanions. The catalytic subunit, the ArsA protein, is an ATPase with two nucleotide binding consensus sequences, one in the N-terminal half and one in the C-terminal half of the protein. The ArsA ATPase is allosterically activated by tricoordinate binding of As(3+) or Sb(3+) to three cysteine thiolates. Previous measurements suggested that the intrinsic fluorescence of tryptophans might be useful for examining binding of Mg^{2+}ATP and antimonite. In the present study an increase in intrinsic tryptophan fluorescence was observed upon addition of Mg^{2+}ATP. This enhancement was reversed by addition of antimonite. The ArsA protein contains four tryptophan residues: Trp159, Trp253, Trp522, and Trp524. The first two were altered to tyrosine residues by site-directed mutagenesis. Cells expressing both the $arsA_{W159Y}$ and $arsA_{W253Y}$ mutations retained resistance to arsenite, and the purified W159Y and W253Y proteins retained ATPase activity. While the intrinsic tryptophan fluorescence of the W253Y protein responded to addition of Mg^{2+}ATP, intrinsic tryptophan fluorescence in the purified W159Y protein was no longer enhanced by substrate. These results suggest that Trp159 is conformationally coupled to one or both of the nucleotide binding sites and provides a useful probe for the interaction of effector and substrate binding sites.

Plasmid-encoded arsenical and antimonial resistance in *Escherichia coli* results from the action of an ATP-coupled efflux pump that lowers the intracellular concentration of arsenite or antimonite (Rosen *et al.*, 1995). The pump is a complex of two proteins, the *arsA* and *arsB* gene products. The 45 kDa ArsB protein is an integral membrane protein that by itself can function as a potential-driven anion porter (Dey & Rosen, 1995). When the ArsA subunit is present, the complex is an obligatory ATP-coupled system. When the *arsA* gene is highly expressed, the ArsA protein can be isolated from the cytosol of *E. coli* as a soluble ATPase (Rosen *et al.*, 1988).

The ArsA protein has two homologous halves, most likely as a result of a gene duplication and fusion (Chen *et al.*, 1986). Both the N-terminal (A1) and C-terminal (A2) halves of the protein contain a consensus sequence for a nucleotide binding site (Walker *et al.*, 1982). Both sites bind ATP (Karkaria & Rosen, 1991), and both are required for both catalytic activity and resistance (Karkaria *et al.*, 1990; Kaur & Rosen, 1992).

Binding of the nucleotide substrate is not sufficient for catalysis; the activity of purified ArsA protein is allosterically activated by arsenite or antimonite (Bhattacharjee *et al.*, 1995). Activation occurs via the formation of a tricoordinate As(3+)− or Sb(3+)−thiolate complex with cysteines 113, 172, and 422 of the ArsA protein. In this respect arsenic and antimony act as soft metals and not nonmetallic oxyanions. Metalloactivation is associated with formation of an ArsA homodimer (Hsu *et al.*, 1991).

From the nucleotide sequence of the *arsA* gene, the ArsA ATPase is predicted to have four tryptophans located at position 159, 253, 522, and 524, respectively (Chen *et al.*, 1986). The intrinsic tryptophan fluorescence of the protein was shown to undergo changes in anisotropy upon the interaction individually with Mg^{2+}, ATP, or antimonite (Karkaria *et al.*, 1991), suggesting that one or more of the tryptophans in the ArsA protein can serve as intrinsic probes. We have extended those studies to demonstrate an enhancement of tryptophan fluorescence by Mg^{2+} ATP. The response was specific for ATP: neither ADP nor AMP could substitute. The enhancement was specifically reversed by the effectors antimonite or arsenite. Using site-directed mutagenesis Trp159 was shown to be responsible for the substrate-dependent fluorescence enhancement. Trp159 was shown to be located on the surface of the protein by quenching of its fluorescence with potassium iodide. These results suggest that Trp159 is conformationally coupled to the Mg^{2+} ATP binding site or sites, and its fluorescence can serve to report communication between the effector and substrate binding sites.

MATERIALS AND METHODS

Materials. All restriction enzymes and nucleic acid modifying enzymes were obtained from GIBCO BRL. Oligonucleotides were synthesized in the Macromolecular Core Facility of Wayne State University School of Medicine. All other chemicals were obtained from commercial sources.

Bacterial Strains and Media. Cultures of *E. coli* strains JM109 (Sambrook *et al.*, 1989) bearing the indicated plasmids were grown at 37 °C in LB medium (Sambrook *et al.*, 1989). Antibiotics were added as required. Sodium

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arsenite, potassium antimonyl tartrate, or IPTG[1] was added at the indicated concentrations.

DNA Manipulation. Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described (Sambrook *et al.*, 1989; Chung *et al.*, 1989).

Oligonucleotide-Directed Mutagenesis. Mutations in the sequence of the *arsA* gene were introduced by site-directed mutagenesis using the Altered Sites *in vitro* Mutagenesis System (Promega). The *arsA* and *arsB* genes inserted into the multiple cloning site of pALTER −1 vector (Promega) was used as the template (Bhattacharjee *et al.*, 1995). The mutagenic oligonucleotides used and the respective changes (underlined) introduced were AATAAAGCTACTGTAGGCGCCCGGC to produce W159Y and CTGTTCACGCTCGTATATTGCAGCA for W253Y. The identity of the mutations was confirmed by DNA sequencing each mutant gene. Double-stranded plasmid DNA was prepared using the Wizard Minipreps DNA Purification System (Promega) and denatured with 0.2 M NaOH and 0.2 mM EDTA for 30 min at 37 °C. Sequencing was done using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical) according to Sanger *et al.* (1977).

Purification and Assay of the ArsA Protein. Altered ArsA proteins were purified from cultures of *E. coli* strain JM109 bearing pALTER-1 derivatives containing the mutated *arsA* genes. Single clones were inoculated into and grown at 37 °C with aeration for 8 h in 5 mL of LB medium containing 50 μg/mL ampicillin and 5 mM sodium arsenite. A portion of the culture (0.5 mL) was inoculated into 0.25 L of LB medium containing 50 μg/mL ampicillin and grown overnight. The 0.25 L culture was then diluted to 2.5 L of prewarmed medium containing 50 μg/mL ampicillin and grown up for about 4 h. At an $A_{600nm} = 0.6-0.8$, production of the mutant protein was induced by addition of 0.1 mM IPTG. The ArsA proteins were purified essentially as described (Hsu & Rosen, 1989) and stored at −70 °C until use. Each ArsA protein was judged to be >95% homogeneous by Coomassie blue staining of samples separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Laemmli, 1970). The concentration of ArsA protein in purified preparations was determined using a modification of the method of Lowry *et al.* (1951). The ATPase activity of the ArsA protein was performed using the coupled assay of Vogel and Steinhart (1976).

Measurements of Fluorescence. Fluorescence measurements were performed using an SLM 48000s spectrofluorometer. The excitation wavelength was 295 nm. The slit widths for excitation and emission were 4 mm. Reagents were added in 1−2 μL volumes with a microliter syringe through a light-protected port to the cuvette containing 2 mL samples, and the solutions were continuously stirred during the measurement. Dilution effects were negligible. Tryptophan fluorescence quenching experiments with KI were performed by adding small volumes of a 5 M KI stock solution which was used within a few days of preparation and stored in the dark at 4 °C. $Na_2S_2O_3$ was added at 0.1 mM to the iodide stock solution to prevent I_3^- formation (Lehrer, 1971). Fluorescence intensity changes were recorded at 338 nm and corrected for dilution with NaCl (Eftink & Ghiron, 1976). Quenching data were analyzed by the modified Stern−Volmer relationship (eq 1)(Lakowicz,

$$F_o/\Delta F = 1/(f_a K[Q]) + 1/f_a \quad (1)$$

1983), assuming the existence of two tryptophan populations, one accessible to quenching, and the other inaccessible or buried, where F_o is the fluorescence intensity in the absence of KI, and ΔF is difference between the fluorescence intensities in the absence and presence of KI. Q is the concentration of KI, and f_a is the fraction of total fluorescence quenchable by KI. K is the dynamic quenching constant of the accessible fraction. A plot of $F_o/\Delta F$ against 1/[Q] yields $1/f_a$ as the intercept.

RESULTS

Isolation of $arsA_{W159Y}$ and $arsA_{W253Y}$ Mutations and Properties of the Gene Products. Residues Trp159 and Trp253 in the ArsA protein were individually changed to tyrosinyl residues, producing derivatives W159Y and W253Y. Cells bearing the mutated *arsA* genes and wild-type *ars*B gene exhibited normal levels of resistance on liquid or solid media containing 5 mM sodium arsenite (data not shown).

The steady-state level of production of the altered ArsA proteins was found to be the same as the wild-type ArsA protein as judged by Coomassie blue staining following electrophoresis of equivalent amounts of cell protein on sodium dodecyl sulfate−polyacrylamide gels, and no abnormal degradation of the altered proteins was observed (data not shown). Both proteins eluted from chromatographic matrices in the same way as the wild-type protein, and both were purified to >95% homogeneity as described under Materials and Methods. The antimonite-stimulated ATPase activity of the wild-type enzyme was 990 nmol of ATP hydrolyzed per min per mg of protein. The W159Y and W253Y proteins had slightly lower specific activities of 590 and 860, respectively. These values are all within the range found for wild-type enzyme, with similar variability noted between different preparations of the same protein. These results indicate that alteration of tryptophan to tyrosine residues at positions 159 or 253 does not have a large effect on the physiological or catalytic properties of the ArsA ATPases.

Emission Spectra of Tryptophans in Wild-Type and Altered ArsA Proteins. The emission spectra of tryptophan residues can reflect the polarity of their environment (Lehrer, 1971). The observed maximum emission wavelength (λ_{max}) of the wild-type ArsA protein was 338 nm (Figure 1, curve a), consistent with the tryptophans being in a relatively nonpolar environment. When the wild-type ArsA was denatured by addition of 6 M guanidine hydrochloride, the λ_{max} was shifted to 353 nm (Figure 1, curve d). Free tryptophan likewise had a λ_{max} of 353 nm (Figure 1, curve e), demonstrating that all four tryptophans in the denatured enzyme are exposed to the aqueous medium. Compared to the fluorescence emission spectrum of wild-type ArsA, the maximum emission wavelengths of the W159Y and W253Y proteins were slightly blue-shifted, 1.5 nm for W159Y and 2 nm for W253Y (Figure 1, curves b and c), respectively. Although the spectral shifts observed for these two proteins are slight, they may indicate that the tryptophan fluorescence in the wild-type ArsA protein is heterogeneous, with Trp159 and 253 exposed to a more polar environment than Trp522 and 524.

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; IPTG, isopropyl β-D-thiogalactopyranoside.

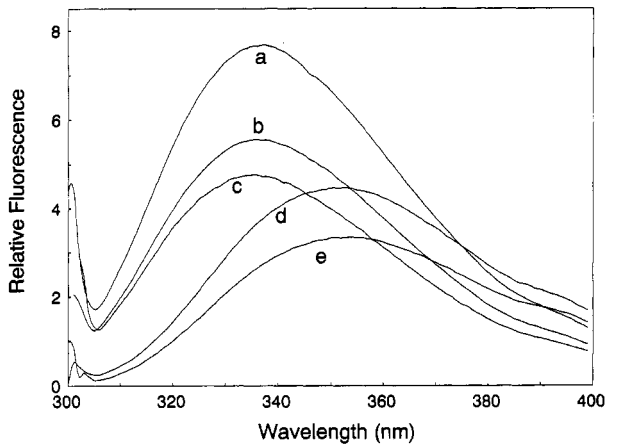

FIGURE 1: Emission scans of the wild-type and altered ArsA proteins. (a) Wild-type ArsA protein; (b) W159Y protein; (c) W253Y protein; (d) wild-type + 6 M guanidine HCl; (e) 2.5 μM tryptophan. The concentration of each protein was 0.625 μM. The excitation wavelength was 295 nm.

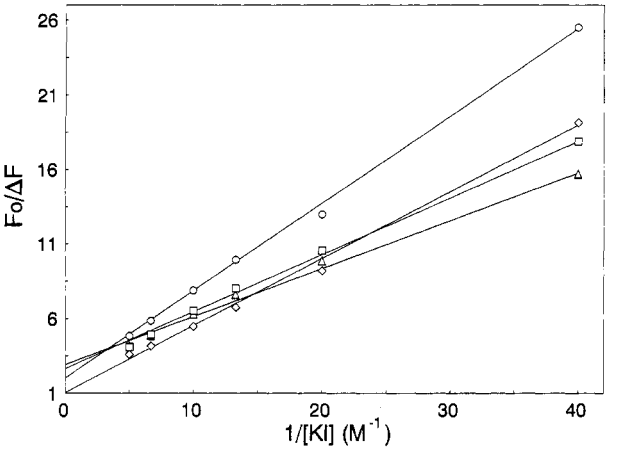

FIGURE 2: Quenching by potassium iodide of the fluorescence of the wild-type and altered ArsA proteins. Each protein was present at 0.625 μM. Protein: (●) wild-type ArsA; (■) W159Y; (▲) W253Y; (◆) wild-type + 6 M guanidine HCl. Excitation and emission wavelengths were 295 and 338 nm, respectively.

Quenching of Tryptophan Fluorescence with Potassium Iodide. Fluorescence quenching measurement provides a practical means to determine the surface accessibility of tryptophan residues in proteins (Lehrer, 1971). The degree of solvent exposure of the tryptophan residues in wild type and altered ArsA proteins was evaluated by quenching with potassium iodide, which is unable to penetrate hydrophobic regions of proteins. The data were analyzed on a modified Stern−Volmer plot with the assumption of two classes of tryptophan residues, KI accessible and inaccessible (Figure 2). In the wild-type enzyme, the value at the intercept indicated that half of the four tryptophan residues were accessible to KI (Table 1). Denaturation with guanidine HCl resulted in all four residues becoming KI accessible. In both altered enzymes approximately two-thirds of the remaining three tryptophans were shielded from KI. These results confirm that both Trp159 and 253 are either on the surface of the enzyme or in a polar environment accessible to KI, while Trp522 and 524 located in less polar regions of the protein.

Response of Intrinsic Tryptophan Fluorescence to Addition of Ligands. Addition of the nucleotide ATP, ADP, or AMP to the wild-type ArsA protein each resulted in a decrease in tryptophan emission (Figure 3, curves a, b, and c, respectively). This is most likely the result of an inner filter effect, since ATP had the same effect on free tryptophan (Figure 3, curve d). Addition of Mg^{2+} to the enzyme following ATP produced a marked increase in fluorescence (Figure 3, curve a). This increase was not observed with AMP or ADP (Figure 3, curves c and b), nor was there any increase in the fluorescence of free tryptophan (Figure 3, curve d). Mg^{2+} addition had no effect in the absence of ATP, and the order of addition made no difference in the final level of fluorescence (data not shown). The fluorescence enhancement could be reversed by removal of Mg^{2+} with EDTA (Figure 4). Since both Mg^{2+} and ATP are required, it is likely that it is the binding of the Mg^{2+} ATP complex, which is probably the true substrate of the ArsA ATPase (Hsu & Rosen, 1989), that results in the increase in fluorescence, although binding of Mg^{2+} to an additional site or sites cannot be ruled out.

The increase in tryptophan fluorescence could be reversed by addition of the allosteric activator antimonite (Figure 3, curve a, and Figure 5). Arsenite, also an activator, similarly decreased the Mg^{2+} ATP-dependent fluorescence enhancement. On the other hand, arsenate, which is neither an activator nor substrate of the Ars pump, had no effect. Neither Mg^{2+} ATP nor antimonite affected the fluorescence of guanidine HCl-denatured ArsA protein above the inner filter effect of the nucleotide (Figure 5). The level of Mg^{2+} ATP-dependent fluorescence enhancement was measured as a function of concentration of antimonite (Figure 6). From these data, the apparent K_d for antimonite was 5 μM (Figure

Table 1: Surface-Accessible Tryptophan Residues in Wild-Type and Altered ArsA Proteins

protein	intercept[a]	1/intercept	total tryptophans[b]	KI accessible tryptophans
wild type	1.89	0.53	4.00	2.12
wild type + 6 M guanidine HCl	1.00	1.00	4.00	4.00
W159Y	2.50	0.40	3.00	1.20
W253Y	2.91	0.34	3.00	1.03

[a] Values are least-square fits of the data from Figure 2. [b] Total number of tryptophans from the deduced sequence.

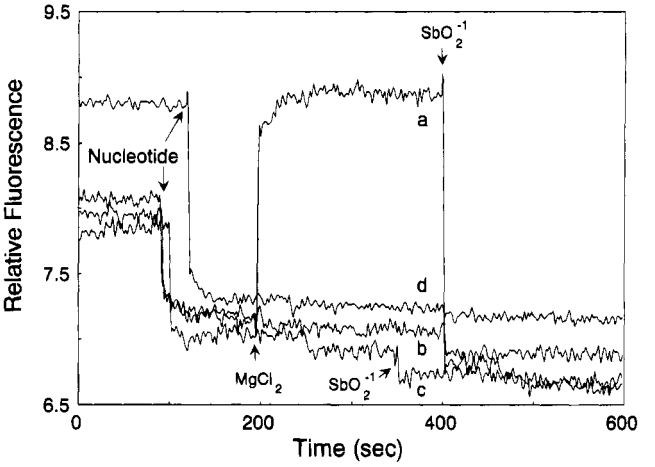

FIGURE 3: Effect of ligands on the intrinsic tryptophan fluorescence of the ArsA protein. At the indicated times the nucleotides were added at 5 mM, followed by 2.5 mM $MgCl_2$ and 0.1 mM potassium antimonial tartrate. Nucleotides: (a) ATP; (b) ADP; (c) AMP. In spectrum (d) ATP was added to a solution of 25 μM tryptophan. Other conditions were as described in the legend to Figure 2.

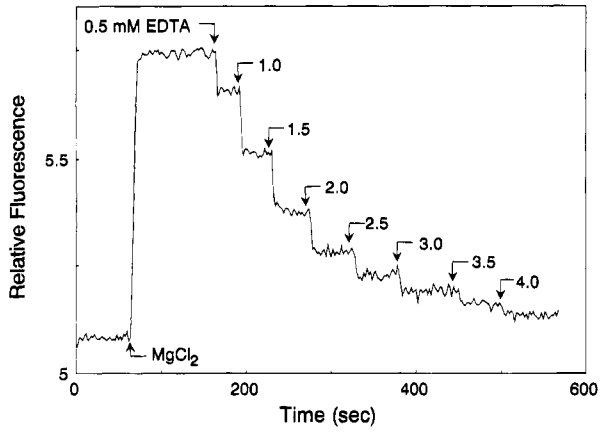

FIGURE 4: Effect of chelation of Mg^{2+} on the Mg^{2+} ATP-dependent enhancement of tryptophan fluorescence. ArsA protein was preincubated with 5 mM ATP. At the indicated time 2.5 mM $MgCl_2$ was added to produce fluorescence enhancement. Small amounts of 0.5 M EDTA were then added to produce the indicated concentrations. Other conditions were as described in the legend to Figure 2.

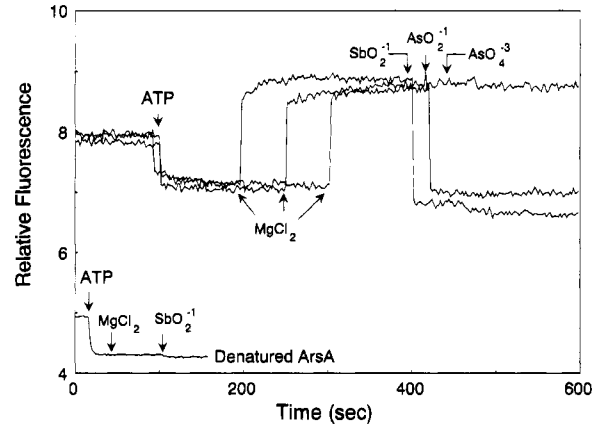

FIGURE 5: Effect of oxyanions on the Mg^{2+} ATP-dependent enhancement of tryptophan fluorescence. At the indicated times ATP and $MgCl_2$ were added to 5 and 2.5 mM, respectively. At the times indicated 0.1 mM potassium antimonial tartrate, 2.5 mM sodium arsenite, or 2.5 mM sodium arsenate was added. Other conditions were as described in the legend to Figure 2.

6, inset), consistent with the concentration of 2 μM antimonite required for half-maximal activation of the enzyme (Bhattacharjee *et al.*, 1995).

Trp159 Is the Substrate-Responsive Residue. To determine which tryptophan residue or residues reported the binding of ligands, the Mg^{2+} ATP-dependent fluorescence enhancement and reversal by antimonite were examined in the purified W159Y and W253Y proteins (Figure 7). The W253Y protein responded essentially as the wild type. In contrast, the W159Y protein lost the Mg^{2+} ATP-dependent fluorescence enhancement. Thus Trp159 is most likely the ligand-responsive residue. A less likely possibility is that the substrate binding site is conformationally coupled to one of the other tryptophan residues but that the alteration of Trp159 in some way alters that interaction. Construction of an *arsA* gene with no tryptophan codons will allow discrimination between these possibilities.

DISCUSSION

The plasmid-encoded Ars pump catalyzes extrusion of arsenite and antimonite from cells of *E. coli*, thus affording

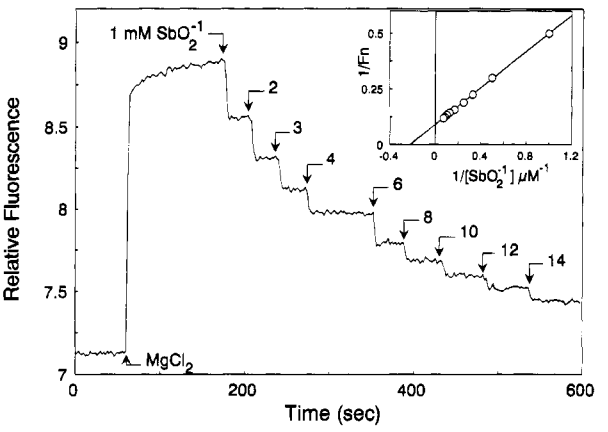

FIGURE 6: Effect of antimonite concentration on the Mg^{2+} ATP-dependent enhancement of tryptophan fluorescence. ArsA protein was preincubated with 5 mM ATP, and 2.5 mM $MgCl_2$ was added at the indicated time. At various times small amounts of 1 mM potassium antimonial tartrate were added to give the indicated concentrations. Other conditions were as described in the legend to Figure 2. (Inset) The average fluorescence at each concentration of antimonite was determined. An apparent K_d of 5 μM for antimonite was calculated from the double-reciprocal plot of the antimonite concentration against the difference in fluorescence in the presence and absence of antimonite normalized to the net increase in fluorescence produced by Mg^{2+} addition (F_n).

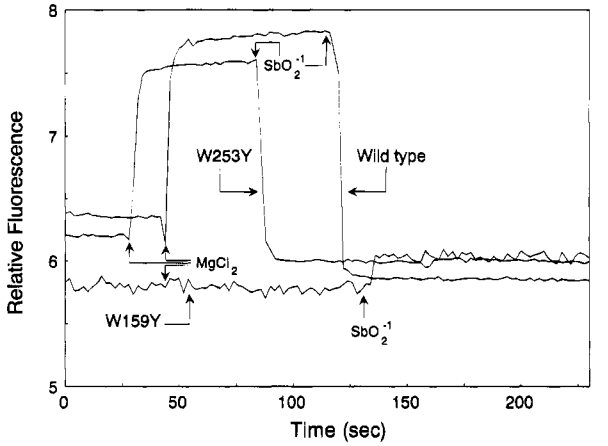

FIGURE 7: Effect of mutational alteration of Trp159 and 253 on the Mg^{2+} ATP-dependent enhancement of tryptophan fluorescence. Purified wild-type, W159Y, and W253Y proteins were each preincubated with 5 mM ATP. 2.5 mM $MgCl_2$ and 0.1 mM potassium antimonial tartrate were added at the indicated times. Other conditions were as described in the legend to Figure 2.

protection from those toxic oxyanions (Rosen *et al.*, 1995). The pump is composed of a membrane sector, the ArsB protein, and a catalytic sector, the ArsA protein. The ArsA protein is an ATPase that is activated by arsenite or antimonite. When the *arsA* gene is more highly expressed than the *arsB* gene, the ArsA ATPase can be isolated and purified as a soluble protein. The enzyme has two nucleotide binding sites, both of which are required for ATPase activity, for arsenite transport, and for resistance (Karkaria *et al.*, 1990; Kaur & Rosen, 1992). Mg^{2+} is required for ATP binding and hydrolysis (Hsu & Rosen, 1989); most likely the Mg^{2+} ATP complex is the substrate. Arsenite and antimonite are allosteric effectors (Rosen *et al.*, 1995). Activation requires three residues, Cys113, 172, and 422, suggesting that the chemical form of the arsenic(antimony)–ArsA complex is as the tricoordinate As(3+)$(S)_3$ or Sb(3+)-$(S)_3$ (Bhattacharjee *et al.*, 1995). Thus activation requires

formation of covalent metal−thiol bonds and not through a noncovalent oxyanion binding site. The information from As(3+) or Sb(3+) binding must be transmitted to the ATP sites to promote catalysis.

This communication between binding sites has been difficult to examine because of the limited assays available. Fluorescence provides a rapid and sensitive means to observe ligand interactions, and we had previously shown that it might be possible to use the intrinsic fluorescence of the four tryptophans in the ArsA protein in this way (Karkaria *et al.*, 1991). In that study the time decay of fluorescence anisotropy was used to show that the rapid rotational mode corresponding to localized motion of the fluorophores could be largely suppressed in the presence of the ligands, suggesting a more rigid conformation of the enzyme.

In this report we extend those studies to include the effect of substrate and effector on the intrinsic fluorescence. More importantly, we demonstrate a substrate-dependent enhancement of fluorescence that can be modulated by the allosteric effector. The properties of the fluorescent changes were consistent with the properties of activation and catalysis. The process was specific for ATP in that neither ADP nor AMP gave a response, even though ADP binds to protein (Rosen *et al.*, 1988). Mg^{2+} was also required for fluorescence enhancement with ATP, and the ArsA forms a light-activated adduct with [α-^{32}P]ATP only in the presence of Mg^{2+} (Rosen *et al.*, 1988), suggesting that Mg^{2+} ATP is the compound recognized. However, additional Mg^{2+} binding sites cannot be ruled out. Either arsenite or antimonite reversed the fluorescence enhancement produced by nucleotide binding, while arsenate, which is not an activator, did not. Denatured protein did not respond to ligands. Because of inner filter effects of the adenine ring it was difficult to quantify the nucleotide response.

However, by measuring the effect of various concentrations of antimonite on enhancement produced by the nucleotide, an apparent K_d of 5 μM could be calculated for antimonite. This is in good agreement with the concentration of antimonite required for half-maximal activation of catalysis, which was determined to be 2 μM (Bhattacharjee *et al.*, 1995). In this way the flow of information from allosteric site to catalytic site can be monitored.

The ArsA protein has only four tryptophan residues, Trp159, 253, 522, and 524. The intrinsic fluorescence is the sum of their individual signals, which may differ if the residues reside in different environments. From quenching experiments with potassium iodide it is clear that two of them are accessible to iodide and two are not, indicating a surface or solvent-accessible location for two and a more nonpolar environment for the other two. Using site-directed mutagenesis Trp 159 and 253 could be assigned to the polar location, while Trp 522 and 524 are in the nonpolar environment. The latter two are near the C-terminus of the protein, which suggests that the C-terminus may be buried in the interior of the enzyme. Only the W159Y protein lacked the substrate-dependent fluorescent enhancement, indicating that Trp159 is spatially proximate to one or both of the nucleotide binding sites and reports its occupancy. Alternatively, it could be near a Mg^{2+} binding site that itself is affected by nucleotide binding, or Trp159 could be at a distance but conformationally coupled to the nucleotide binding site. The reversal of substrate enhancement of fluorescence by antimonite could result from additional conformational changes. Interaction between binding of substrate and effector has also been observed as a synergistic protection of the enzyme from trypsin inactivation (Hsu & Rosen, 1989), indicating cooperative conformational changes upon binding of both. Binding of effector has also been shown to result in ArsA dimerization (Hsu *et al.*, 1991). The reversal of enhancement could be due to a more polar environment for Trp159 in the dimer. These are all testable hypotheses for which intrinsic tryptophan fluorescence will become a major tool for analysis of the ArsA protein.

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