Ligand Interactions in the ArsA Protein, the Catalytic Component of an Anion-Translocating Adenosinetriphosphatase†

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ABSTRACT: The *ars* operon of the conjugative R-factor R773 produces resistance to arsenicals in cells of *Escherichia coli*. The operon encodes an oxyanion pump which is composed of a membrane subunit, the 45.5-kDa ArsB protein, and a catalytic subunit, the 63-kDa ArsA protein. Purified ArsA protein is an arsenite(antimonite)-stimulated ATPase. From its amino acid sequence, as deduced from the nucleotide sequence, the ArsA protein has four tryptophanyl residues which could serve as intrinsic fluorescent probes for the study of substrate-induced conformational changes. Both static and dynamic measurements of tryptophan fluorescence were performed with the ArsA protein. Results from static anisotropy measurements indicated differences in molecular motion with addition of ATP, SbO_2^-, or Mg^{2+}. These results were supported by time decay measurements of fluorescence anisotropy. The results of time decay measurements indicated a shorter correlation time, reflecting localized motion in the vicinity of the probe, and a longer correlation time, which could have arisen from rotation of the major portion of the molecule. The longer correlation time changed with addition of the various effectors, especially $MgCl_2$, suggesting that binding of Mg^{2+} decreases probe mobility.

Resistance to antibiotics and toxic agents in bacteria is often mediated by extrachromosomal resistance factors (Foster, 1983). A frequent stratagem for resistance is extrusion of the toxic compound out of the cell, reducing the intracellular concentration to subtoxic levels (Tisa & Rosen, 1990). We have shown previously that the resistance determinant to arsenite, antimonite, and arsenate carried by plasmids of Gram-negative bacteria encodes an ATP-driven anion pump which produces resistance by extruding the toxic oxyanions out of the cell (Mobley & Rosen, 1982; Silver & Keach., 1982; Rosen & Borbolla, 1984). From sequence and structural analysis, this pump appears to be the first identified member of a new family of anion-translocating adenosine-triphosphatases (ATPases).[1]

The arsenical resistance operon of resistance factor R773 encodes four genes (Mobley et al., 1983; Chen et al., 1986; Hsu & Rosen, 1989a), of which only two, *arsA* and *arsB*, are required for arsenite(antimonite) resistance and transport. The 45.5-kDa ArsB protein is an inner membrane in *Escherichia coli* (San Francisco et al., 1989) and is the membrane anchor for the 63-kDa ArsA protein and is postulated to be the subunit responsible for anion conduction (Chen et al., 1986).

From the nucleotide sequence of the *arsA* gene (Chen et al., 1986), the ArsA protein is predicted to have two sequences that are potential nucleotide binding sites (Walker et al., 1982). Purified ArsA protein exhibits oxyanion-stimulated ATPase activity and can be photo-cross-linked with [α-^{32}P]ATP (Rosen et al., 1988). Although ATP hydrolysis required the oxyanionic substrate, nucleotide binding was independent of the presence of oxyanion. Partial proteolysis with trypsin of purified ArsA protein in the presence of antimonite and ATP conferred complete protection of the native 63-kDa species, while in the absence of antimonite and ATP, degradation of the 63-kDa species was much more rapid (Hsu & Rosen 1989b). These results indicate that both substrates together produce a significant conformational change which is different from the state of the ArsA protein in the absence of substrate or the presence of either substrate alone. These results suggest unique binding sites for antimonite and ATP. Although Mg^{2+} is required for activity, there are no data which directly demonstrated binding of the divalent cation.

Changes in fluorescence are a sensitive means for detection of ligand-induced conformational changes. From the deduced primary sequence of the ArsA protein, there are tryptophans at positions 159, 253, 522, and 524, each of which could serve as an intrinsic fluorescence probe. Static and dynamic measurements of tryptophan fluorescence in the ArsA protein were made with combinations of substrates. From the static measurements, it appears that each of the substrates, including Mg^{2+}, produces unique conformational changes.

Materials and Methods

Measurements of Static Fluorescence. ArsA protein was purified by the method of Rosen et al. (1988), as modified by Hsu and Rosen (1989b). Static fluorescence measurements were performed with an SLM 8000 spectrofluorometer. The excitation wavelength was either 295 or 302 nm, as indicated. Emission intensity as a function of wavelength was expressed as a ratio of the intensity of the rhodamine B internal standard. Static excitation anisotropy measurements were done on an SLM 48000 spectrofluorometer with 360 nm as the emission wavelength. The slit widths for excitation and emission were 4 mm, corresponding to a spectral resolution of 8 nm.

Tryptophan fluorescence quenching experiments with acrylamide were performed by adding small volumes of an

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[1] Abbreviation: ATPase, adenosinetriphosphatase.

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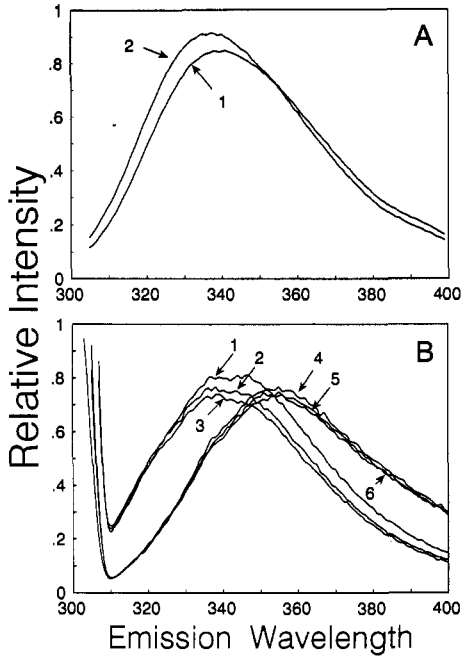

FIGURE 1: Emission scans of the ArsA protein in 50 mM MOPS–KOH in a total volume of 2 mL. The excitation wavelength was 295 nm in (A) and 302 nm in (B). (A) Curve 1, 5 μM ArsA protein; curve 2, 5 μM ArsA protein + 5 mM $MgCl_2$; (B) curve 1, 7 μM ArsA protein; curve 2, 7 μM ArsA protein + 25 μM ATP; curve 3, 7 μM ArsA protein + 50 μM ATP; curve 4, 40 μM tryptophan; curve 5, 40 μM tryptophan + 25 μM ATP; curve 6, 40 μM tryptophan + 50 μM ATP.

acrylamide stock solution in the presence of different substrates. No overall changes in the shape of the emission spectrum were observed on addition of acrylamide. Fluorescence intensity changes were recorded at 338 nm and corrected for dilution (Eftink & Ghiron, 1976). Quenching data were analyzed by the Stern–Volmer relationship (eq 1),

$$F_0/F = K_{SV}[Q] + 1 \quad (1)$$

where [Q] is the concentration of acrylamide and K_{SV} is the dynamic quenching constant. F_0 and F are unquenched and quenched fluorescence, respectively.

Time decay measurements of fluorescence intensity and anisotropy were performed with an Edinburgh nanosecond fluorometer (J. Lakowicz, Department of Biological Chemistry, School of Medicine, University of Maryland, Baltimore) using as a light source a mode-locked Nd-YAG laser driving a rhodamine laser. The frequency was 1 MHz. The excitation and emission and emission wavelengths were 298 and 360 nm, respectively.

Reagents. Potassium antimonyl tartrate was used as the source of antimonite and was obtained from Aldrich Chemical Co. Other reagents were of best grades available.

RESULTS

Static Fluorescence. In the fluorescence emission spectrum of the ArsA protein at pH 7.5, the observed emission maximum of 337 nm suggests that the chromophore is in a nonpolar environment (curves 1 in Figure 1A,B) (Burstein et al. 1973). Mg^{2+} caused a slight increase in quantum yield (Figure 1A, curve 2), suggesting that one or more of the tryptophans is near the binding site for the divalent cation but buried in a nonpolar region. An overall decrease of quantum yield was observed when ATP was added at 25 μM (Figure 1B, curve 2) or 50 μM (Figure 1B, curve 3) to the solution containing ArsA protein, suggesting an alteration in the microenvironment

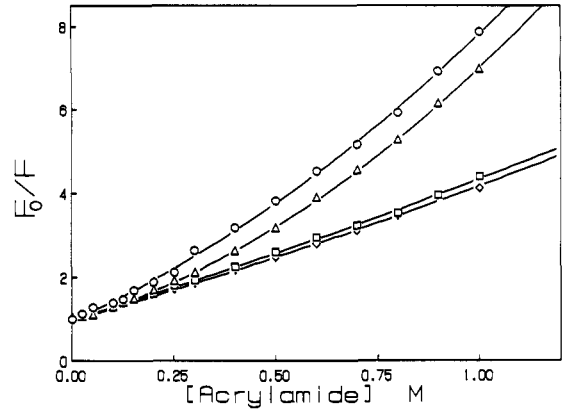

FIGURE 2: Quenching by acrylamide of the fluorescence of the ArsA protein (5 μM) in 50 mM MOPS–KOH. The excitation wavelength was 295 nm and the emission wavelength 337 nm, respectively. (○) Control; (▲) +5 mM $MgCl_2$; (□) +5 mM $MgCl_2$ + 1 mM ATP; (◇ +5 mM $MgCl_2$ + 0.1 mM potassium antimonyl tartrate + 1 mM ATP.

of at least one of the tryptophans. Inner filter effects were minimized by exciting at 302 nm; at that wavelength, the effect of ATP on the fluorescence of a tryptophan solution of equivalent initial fluorescence is minimal (Figure 1B, curves 4–6), suggesting that a significant portion of the effect arises from an induced conformational change. Addition of both ATP and Mg^{2+} produced an intermediate spectrum (data not shown). Antimonite had no effect on the spectrum either alone or in combination with the other two ligands. The fluorescence excitation spectrum of the ArsA protein with and without its substrates also gave similar differences in quantum yield (data not shown).

The results of acrylamide quenching experiments also indicate that one or more of the tryptophans was only partially exposed (Figure 2). The dynamic quenching constant, K_{SV}, provides a measure of solvent accessibility (Eftink & Ghiron, 1976, 1981). At pH 7.5, the limiting K_{SV} values determined for the ArsA protein with different substrates are the same at lower concentrations of acrylamide and are approximately 0.3 M^{-1}. At higher concentrations of acrylamide, the curves for ArsA protein with or without Mg^{2+} are concave upward with an increase in the apparent K_{SV} to 1 M^{-1}, suggesting that the conformational change taking place in the presence of Mg^{2+} is distinct from the change induced by addition of ATP or SbO_2^-. The reported values of the dynamic quenching constant K_{SV} vary over a wide range, ranging from 13 M^{-1} for highly exposed tryptophan side chains to 0 for residues buried in the interior (Eftink & Ghiron, 1976), again suggesting that some of the fluorescing tryptophans are at least partially located in the interior of the ArsA molecule. Most of the effect produced by ATP and antimonite occurs at the higher levels of quencher, suggesting that the influence of these ligands is exerted predominantly upon the less accessible tryptophans.

Static excitation anisotropy measurements of the ArsA protein were done with different combinations of substrates (Figure 3). As expected, the wavelength variation indicated multiple transitions, with an increase in anisotropy at wavelengths greater than 300 nm; a minimum occurs near 285 nm. Addition of SbO_2^- to ArsA protein increased the overall anisotropy over the spectrum scanned from 280 to 310 nm. Addition of ATP, Mg^{2+}, and SbO_2^- decreases the overall anisotropy.

Dynamic Fluorescence. The time decay of fluorescence intensity was multiexponential. Three components were sufficient to obtain an optimal fit (Table I). The observed heterogeneity of decay times could be explained by the com-

Table I: Time Decay of Fluorescence Intensity[a]

sample[b]	channel	α_1	τ_1[c] (ns)	α_2	τ_2[d] (ns)	α_3	τ_3[d] (ns)	$\langle\tau\rangle$[d,e] (ns)	shift	χ^2
ArsA	30–500	0.237	0.475	0.500	2.34	0.189	5.73	3.80	−0.701	3.01
ArsA + SbO_2^-	30–500	0.113	0.649	0.224	2.70	0.054	6.65	3.92	0.443	2.38
ArsA + Mg^{2+}	30–500	0.049	0.431	0.103	2.37	0.037	5.86	3.87	0.784	2.40
ArsA + Mg^{2+} + ATP	30–500	0.058	0.725	0.101	2.45	0.034	5.21	3.34	0.005	1.81
ArsA + SbO_2^- + Mg^{2+} + ATP	30–500	0.043	0.231	0.001	2.08	0.026	4.94	3.38	0.606	2.45

[a] The intensity is assumed to decay according to the relation $I(t)_i = \sum \alpha_i e^{-t/\tau_i}$ where $I(t)$ is the intensity as a function of time, t, and α_i and τ_i are the amplitude and decay time, respectively, of the ith decay mode. [b] Concentrations of reagents: ArsA protein, 10 μM; $MgCl_2$, 5 mM; potassium antimonyl tartrate, 0.1 mM; ATP, 1 mM. [c] Values are ±0.1. [d] Values are ±0.2. [e] The average decay time, defined by $\langle\tau\rangle = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i$.

Table II: Rotational Correlation Times[a]

sample[b]	channel	β_1[c]	σ_1[d] (ns)	β_2[d]	σ_2[e] (ns)	A_∞[f]	χ^2
ArsA	30–500	0.045	0.04	0.103	24.63	0	1.151
ArsA + SbO_2^-	30–500	0.015	0.38	0.090	28.08	0	1.032
ArsA + Mg^{2+}	30–500	0.018	4.97	0.077	35.29	0	1.102
ArsA + Mg^{2+} + ATP	60–500			0.12	26.63	0	1.040
ArsA + SbO_2^- + Mg^{2+} + ATP	30–500	0.004	0.06	0.097	31.03	0	1.186

[a] Correlation times, σ_i, were calculated from the anisotropy decay data as described by Steiner and Norris (1987a,b) where β_i and σ_i are the amplitude and rotational correlation time, respectively, corresponding to the ith rotational mode. [b] Concentrations of reagents as in the legend to Table I. [c] Values are ±0.002. [d] Values are ±0.02. [e] Values are ±5.0. [f] Assumed limiting value of anisotropy at infinite time.

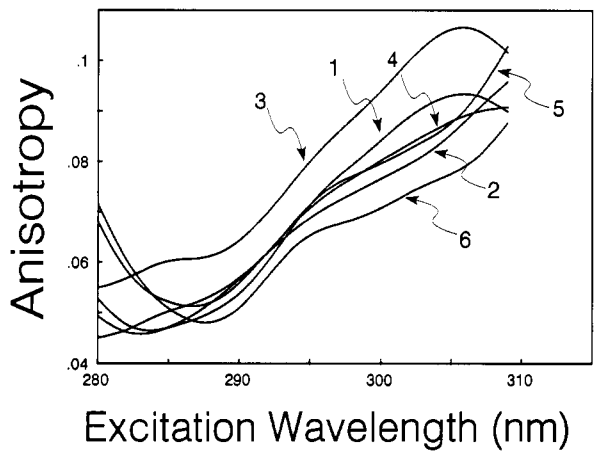

FIGURE 3: Fluorescence anisotropy excitation spectra of the ArsA protein (10 μM) in 50 mM MOPS–KOH, pH 7.5. The emission wavelength was 360 nm. Curve 1, control; curve 2, +5 mM $MgCl_2$; curve 3, +0.1 mM potassium antimonyl tartrate; curve 4, +5 mM $MgCl_2$ + 1 mM ATP; curve 5, +5 mM $MgCl_2$ + 0.1 mM potassium antimonyl tartrate; curve 6, +5 mM $MgCl_2$ + 0.1 mM potassium antimonyl tartrate + 1 mM ATP.

bined effects of the multiplicity of tryptophans and the sampling of different microenvironments by the probe as a consequence of its localized motion, as well as relaxation processes involving the solvent or adjacent residues on the protein surface. It is noteworthy that ATP produces a decrease in the average decay time in the presence of Mg^{2+}, whether or not antimonite is present. This is consistent with the observed decrease in quantum yield in the presence of ATP.

The time decay of fluorescence anisotropy at pH 7.5 was also multiexponential and could be adequately fitted in terms of two rotational modes with different correlation times (Table II). The longer of these has a magnitude of 25–35 ns, which probably represents rotational motion of the major portion of the ArsA molecule. The shorter correlation time presumably arises from the localized motion of the probe itself. However, in all cases the apparent limiting anisotropy, A_0 ($=\beta_1 + \beta_2$), was much smaller than the value of 0.29 expected for immobilized tryptophan at the present wavelengths of excitation and emission. The implication is that, under all conditions, rotational modes exist which are too rapid to be detected by the present time domain instrumentation; this would be the case if the corresponding correlation times were small in comparison with the half-width (60 ps) of the excitation pulse. The amplitude, β_2, corresponding to the longer correlation time is 0.10 ± 0.02 in all cases, indicating that most of the time-dependent decay of anisotropy arises from localized rotation. The magnitude of the longer correlation time, σ_2, in the absence of substrate is 25 ns, which is in the range expected for a spherical protein of molecular mass 63 kDa. There is thus no indication of a domainal rotation within the ArsA protein. While there is some indication of an increase in the value of σ_2 in the presence of substrates (Table II), the change is probably not in excess of experimental uncertainty. It should be noted that it is unlikely that any significant substrate-dependent dimerization would be detected at the very low concentrations of these measurements. Both the apparent amplitude and magnitude corresponding to the shorter correlation time are dependent upon conditions. As noted above, it is likely that it is an oversimplification to represent the anisotropy decay in terms of only two rotational modes. With this reservation, there appears to be a pattern in the reduction of the amplitude of the more rapid rotational mode detected in the presence of either substrate, suggesting a change in the localized motion of the fluorophores. However, since the quantum yield of tryptophan fluorescence is influenced by the presence of substrates, it is not possible to distinguish between an actual change in tryptophan immobilization and a change in the relative contribution of different tryptophans.

Discussion

The ArsA protein, the catalytic subunit of the arsenical pump, exhibits oxyanion-stimulated ATPase activity (Rosen et al., 1988). Activity requires the anionic substrate, antimonite, the nucleotide substrate, ATP, and a divalent cation, Mg^{2+}, pointing to specific binding sites for each of the three ligands. Addition of ATP and/or antimonite has been shown to affect accessibility of surface residues to trypsin, indicating substantial conformational alterations upon substrate binding (Hsu & Rosen, 1989b). There was no specific effect of Mg^{2+} on proteolysis by trypsin. However accessibility to trypsin is a rather gross measure of conformation. Since divalent cation specific conformational changes could be detected by alterations in the fluorescence intensity or anisotropy of calmodulin (Steiner & Norris, 1987a,b) and CheY (Lukat et al., 1990), it was reasonable to examine the effect of Mg^{2+}, as well as the anionic and nucleotide substrates, on the intrinsic tryp-

tophan fluorescence of the ArsA protein.

The results presented in this report provide compelling evidence for ligand- and/or substrate-induced conformational changes in the ArsA protein, suggesting that the protein has unique binding sites for Mg^{2+}, antimonite, and ATP. In the presence of either Mg^{2+} or the nucleotide substrate, ATP, a transition occurs to a conformation in which some of the tryptophans are substantially more shielded from solvent (Figures 1 and 2). Addition of the third ligand, antimonite, does not change the environment of the tryptophans. The transition produced by Mg^{2+} alone is different than ATP alone, suggesting that Mg^{2+} binding is independent of nucleotide binding. The results of static excitation anisotropy show an overall increase in anisotropy with antimonite, suggesting decreased mobility of the ArsA molecule (Figure 3). Since antimonite alone produces no change in the environment of the tryptophans (Figures 1 and 2), the anion binding site is probably not near the tryptophanyl groups affected by the other two ligands. This suggests that the binding site for the anion is distinct from other ligand binding sites.

Finally, while the time decay of fluorescence anisotropy is, under all conditions, dominated by the global rotation of the protein, in the case of the apoprotein there is a significant contribution of a rapid rotational mode corresponding to localized motion of the fluorophores. This is largely suppressed in the presence of the ligands, suggesting a more rigid conformation.

Registry No. ATPase, 9000-83-3; ATP, 56-65-5; Mg^{2+}, 7439-95-4; antimonite, 1317-86-8.

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