Reaction of the ArsA Adenosinetriphosphatase with 2-(4′-Maleimidoanilino)naphthalene-6-sulfonic acid†

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ABSTRACT: The oxyanion-translocating ATPase encoded by the plasmid-borne *ars* operon catalyzes extrusion of antimonials and arsenicals from cells of *Escherichia coli*, thus providing resistance to those toxic oxyanions. The purified catalytic subunit of the ATPase, the ArsA protein, exhibits oxyanion-stimulated ATPase activity. The nature of the oxyanion binding site was probed by reaction with the fluorescent sulfhydryl probe 2-(4′-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS). Our results indicate that MIANS reacts with the ArsA protein in an antimonite-dependent manner. After the protein had been modified with MIANS, two of four cysteines in the ArsA protein had reacted with the probe in the absence of the oxyanionic substrate, and three in the presence of antimonite. The quantum yield of the MIANS–ArsA protein adduct was significantly higher if modification of the protein had occurred in the presence of oxyanionic substrates. Thus binding of the anionic substrate of the pump produces a conformational change in the ArsA protein such that a single additional cysteinyl residue reacts more readily with the sulfhydryl probe.

An anion-translocating ATPase, encoded by the arsenical resistance *ars* operon of R-factor R773, produces resistance in *Escherichia coli* cells to the toxic oxyanions arsenite and antimonite (Kaur & Rosen, 1992). This anion pump is composed of two subunits, the hydrophobic 45.5-kDa ArsB protein, located in the inner bacterial membrane (San Francisco et al., 1989), and the catalytic 63-kDa ArsA protein (Chen et al., 1986), a hydrophilic protein that binds to the membrane through protein–protein interactions with the ArsB protein (Tisa & Rosen, 1990).

When expressed in high amounts, the ArsA protein is found mainly in the cytosol, and it has been purified from cytosol as a soluble protein (Hsu & Rosen, 1989). Purified ArsA protein exhibited antimonite- and arsenite-stimulated ATPase activity (Hsu & Rosen, 1989). Both from the primary sequence and from properties such as inhibitor sensitivity the ArsA protein can be distinguished from members of other families of ion-transport ATPases (Hsu & Rosen, 1989; Chen et al., 1986). It appears to have evolved by duplication of a gene ancestral to a family of cytosolic ATPases, none of which have a transport function (Rosen et al., 1992).

Conformational changes in the ArsA protein have been observed upon substrate binding (Hsu et al., 1991). The results suggested that there are separate binding sites for the oxyanionic and nucleotide substrates on the ArsA protein. The requirement for oxyanion binding in catalysis appeared to be related to dimerization of the ArsA protein, where the equilibrium between inactive monomer and active dimer is shifted toward dimer when oxyanion is bound (Hsu et al., 1991). In this respect the ArsA protein can be considered an allosteric enzyme, with the oxyanion as a positive effector.

From the amino acid sequence the ArsA protein has four tryptophanyl residues potentially able to serve as intrinsic fluorescent probes for the study of the substrate-induced conformational changes. Both static and dynamic measurements of tryptophan fluorescence of the ArsA protein showed that binding of Mg^{2+} decreases probe mobility, while addition of ATP or antimonite produces only slight changes in the molecular motion of the protein (Karkaria et al., 1991). Since intrinsic fluorescent probes did not appear to be a promising tool for the investigation of substrate-induced conformational changes in the ArsA protein, extrinsic fluorescent probes were examined for one that might give a substrate-specific fluorescent signal.

The fluorescent probe 2-(4′-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS)[1] binds covalently to cysteinyl residues in proteins, and its fluorescence is sensitive to the polarity of its environment, requiring a hydrophobic site to develop appreciable fluorescence (Gupte & Lane, 1979; Haugland, 1983). Conformational changes in a number of membrane proteins have been examined by selective modification with MIANS, including Na^+,K^+-ATPase (Gupte & Lane, 1983; Forgac, 1980), sarcoplasmic reticulum Ca^{2+}-ATPase (Bigelow & Inesi, 1991), myosin (Hiratsuka, 1992a,b), pyruvate oxidase (Koland & Gennis, 1982), calmodulin (Mills et al., 1988; Kosk-Kosicka et al., 1990), ferrochelatase (Dailey, 1985), and crystallin (Mandal & Chakrabarti, 1988; Andley & Clark, 1988).

In this study MIANS was used to demonstrate conformation changes in the ArsA protein upon binding of the oxyanionic substrates. Reaction with MIANS inhibited the ATPase activity of the ArsA protein. The primary amino acid sequence of the ArsA protein deduced from the nucleotide sequence includes four cysteinyl residues. Two sulfhydryl groups were labeled in the absence of antimonite, and three cysteines

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[1] Abbreviations: MIANS, 2-(4′-maleimidoanilino)naphthalene-6-sulfonic acid; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MOPS, 3-[*N*-morpholino]propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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reacted in the presence of the oxyanion. Both the increase in quantum yield of MIANS fluorescence and the rate of the MIANS reaction were used to examine ligand-induced conformational changes in the ArsA protein. The results support the previous conclusion that binding of the anionic substrate causes specific conformational changes in the enzyme (Hsu et al., 1991). MIANS fluorescence should provide a sensitive tool for future investigations of the mechanism of binding of the anionic substrate to the ArsA protein and may also help to localize the reactive sulfhydryl groups of cysteinyl residues within the protein.

MATERIALS AND METHODS

Materials. MIANS was purchased from Molecular Probes, Inc. All other chemicals were obtained from commercial sources.

Purification of the ArsA Protein. ArsA protein was purified as described previously (Hsu & Rosen, 1989), except that phenyl-Sepharose column chromatography was performed without dithiothreitol (DTT). The concentration of purified ArsA protein was determined from the absorption at 280 nm, using a molar extinction coefficient of 33 480 (Hsu & Rosen, 1989).

Labeling of the ArsA Protein with MIANS and Fluorescence Measurements. MIANS was dissolved in water. The concentration of MIANS was determined from the absorbance at 322 nm, assuming a molar extinction coefficient of 20 000 (Haugland, 1983). Solutions of MIANS and MIANS-modified ArsA protein were stored in the dark at −20 °C, and the fluorescence was measured within 48 h. Fluorescence measurements were performed with an SLM Aminco 8000 spectrophotofluorometer at 23 °C. The emission spectra were acquired at an excitation wavelength of 340 nm with 4- and 8-nm slits for excitation and emission, respectively. Time-based measurements of fluorescence intensity were acquired at 440 nm with an excitation wavelength of 340 nm.

To determine the rate of MIANS reaction with the ArsA protein, the enzyme (0.1–0.35 mg/mL) was preincubated with the indicated ligands in a reaction buffer consisting of 50 mM MOPS–KOH, pH 7.5, and 0.25 mM Na_2EDTA for 10 min at 23 °C. The reaction was started by addition of varying concentrations of MIANS. For complete modification of the protein with MIANS, the reaction was carried out for 2 h with a minimum 4-fold molar excess of MIANS over the protein. Unreacted MIANS was removed by gel filtration in spin columns (Penefsky, 1977). A syringe (1 mL) was nearly filled with Sephadex G-25 and equilibrated with the reaction buffer. The sample was added to the top of the syringe, which was then centrifuged for 2 min at 1000 rpm using a swinging bucket rotor. The eluant contained the protein, and unreacted MIANS was retained in the spin column. Modification of the ArsA protein with NEM was performed as described for MIANS.

Determination of the Amount of MIANS and DTNB Bound to the ArsA Protein. The ArsA protein was modified with MIANS for 2 h, and unbound probe was removed with a spin column, as described above. The samples were mixed with 0.5% SDS, and the absorbance was measured at 322 nm. The absorbance of the same amount of unmodified ArsA protein after treatment with SDS was subtracted, and the concentration of bound MIANS was calculated from the difference in absorption.

The stoichiometry of DTNB binding to the ArsA protein and to the MIANS–ArsA protein complex was determined at pH 8 according to the method of Ellman (1959). DTNB (50 μM) was added to a solution containing 5 μM ArsA protein in reaction buffer containing 1% SDS and allowed to react for 2 min at 23 °C. The amount of DTNB incorporated was determined from the release of thionitrobenzoate at 412 nm, assuming a molar extinction coefficient of 13 600 (Ellman, 1959).

Fluorescence Lifetime Measurements. Fluorescence lifetimes were determined using an SLM48000S fluorometer. These measurements were derived using the phase/modulation procedure described by Lakowicz (1983). The frequency domain data were analyzed by the method of nonlinear least squares (Gratton et al., 1984; Lakowicz et al., 1984). The experimental data were fit to two- or three-exponential models by an interative process, so that a minimal x_R^2 was obtained.

Fluorescence Quenching Studies. Increasing amounts of freshly prepared KI were added to the MIANS–ArsA protein complex in reaction buffer. To correct for dilution and ionic strength effects, the same amounts of KCl were added in control assays. The data were analyzed by using the Stern–Volmer equation (Lakowicz, 1983):

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_D[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the bimolar quenching constant, τ_0 is the lifetime of fluorescence in the absence of quencher, [Q] is concentration of quencher, and K_D is the Stern–Volmer quenching constant. The ratio was normalized by dividing by the F_0/F value obtained when the same concentration of KCl was added to the probe–enzyme complex.

Proteolysis with Trypsin. Trypsin digestion was performed at 23 °C in 20 mM MOPS–NaOH buffer, pH 7.5, containing 20% (w/w) glycerol and 1.4 mg/mL ArsA protein. The protein/trypsin ratio was 100:1. Where indicated, ArsA protein was preincubated with 0.5 mM potassium antimonyl tartrate for 10 min at 23 °C. The protein was incubated with 0.1 mM MIANS for 2 h before addition of trypsin. Proteolysis was terminated at various times by addition of a 2-fold excess of soybean trypsin inhibitor. Samples were subjected to boiling in SDS sample buffer for 5 min and then were analyzed by SDS–PAGE using a 10% polyacrylamide gel, as described by Laemmli (1970). Fluorescent bands were visualized on a transilluminator, following which the gel was fixed and stained with Coomassie blue.

ATPase Activity. The coupled assay of Vogel and Steinhart (1976) was used to measure oxyanion-stimulated ATPase activity. The reaction mixture (1 mL) contained 50 mM MOPS–KOH buffer, pH 7.5 (unless otherwise noted), 0.25 mM Na_2EDTA, 5 mM ATP, 1.25 mM phosphoenolpyruvate (PEP), 0.25 mM NADH, and 10 units each of pyruvate kinase (Sigma Chemical Co.) and lactate dehydrogenase (Sigma Chemical Co.) with or without 0.1 mM potassium antimonyl tartrate (antimonite). ArsA protein was added to the cuvette in a final concentration of 10–50 μg/mL and preincubated in the assay mixture for 10 min at 37 °C. The reaction was initiated by addition of 2.5 mM $MgCl_2$ and was linear up to 15 min at 37 °C. The slopes of the curves were used to calculate the specific activity.

RESULTS

Spectral Properties of the MIANS–ArsA Protein Complex. MIANS, a fluorescent maleimide that binds covalently to sulfhydryl groups, is nonfluorescent until its maleimide group reacts with thiols (Gupte & Lane, 1979; Haugland, 1983). Reaction with a 5-fold molar excess of 2-mercaptoethanol

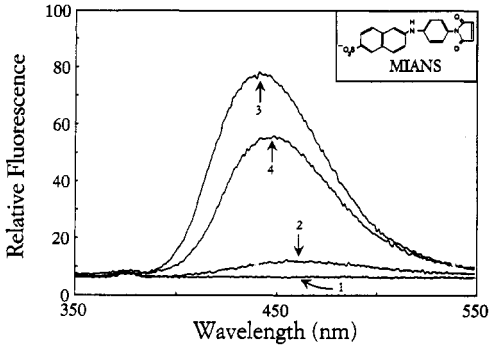

FIGURE 1: Spectral characteristics of MIANS–mercaptoethanol and MIANS–ArsA protein adducts. Either purified ArsA protein (9 μM) (curve 3) or 10 μM 2-mercaptoethanol (curve 2) was incubated for 2 h with 2 μM MIANS. Following reaction the ArsA protein was separated from the unbound MIANS on a spin column, and emission spectra were measured with an excitation wavelength of 340 nm. To the cuvette with the ArsA protein was added SDS to 1% to denature the protein (curve 4). Curve 1 is a superimposition of two curves: MIANS alone and ArsA protein pretreated with NEM before reaction with MIANS. ArsA protein (5 μM) was modified with 1 mM NEM, separated from unbound NEM on a spin column, and reacted with 25 μM MIANS for 2 h. Inset: The chemical structure of MIANS.

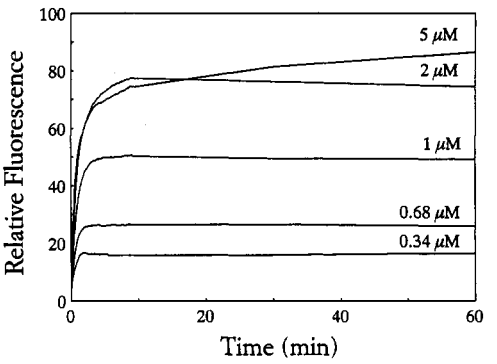

FIGURE 2: Time and concentration dependence of MIANS binding to the ArsA protein. The reaction was initiated by addition of MIANS at the indicated concentrations to 5 μM ArsA protein in reaction buffer. The fluorescence emission of MIANS was measured at 440 nm.

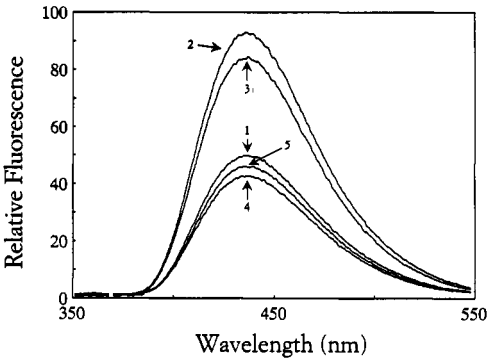

FIGURE 3: Effect of substrates on the fluorescence emission spectra of the MIANS–ArsA protein adduct. Purified ArsA protein (5 μM) was preincubated for 10 min at 23 °C with ligands, followed by addition of 25 μM MIANS. Following reaction the ArsA protein was separated from the unbound MIANS on a spin column, and emission spectra were measured with an excitation wavelength of 340 nm. Additions (final concentrations): none (curve 1), 0.3 mM sodium arsenite (curve 2), 0.5 mM potassium antimonial tartrate (curve 3), 5 mM MgATP plus 2.5 mM $MgCl_2$ (curve 4), and 3 mM sodium arsenate (curve 5).

resulted in an increase in MIANS fluorescence intensity, with a λ_{max} at 460 nm (Figure 1). *N*-Acetylcysteine gave the same fluorescence increase as 2-mercaptoethanol (data not shown). Addition of MIANS to the purified ArsA protein resulted in an increase in fluorescence intensity, with a λ_{max} for emission at 444 nm (Figure 1). In the presence of a 4.5-fold molar excess of protein the quantum yield of the MIANS–ArsA protein adduct was 6-fold higher than the quantum yield of the MIANS–mercaptoethanol adduct, and the emission maximum was blue shifted by 18 nm. The quantum yield and λ_{max} of MIANS depend on the polarity of the solvent (Gupte & Lane, 1979; Haugland, 1983). The blue shift observed with the ArsA protein is intermediate between that of MIANS dissolved in butanol and that in hexane or benzene, indicating that the probe molecules are bound in a somewhat more polar environment than solvent alone. Denaturation of the MIANS–ArsA protein complex with 1% SDS decreased the fluorescence intensity about 40% and red shifted the emission maximum by 6 nm. Similarly, digestion of the MIANS–ArsA protein complex with trypsin reduced the fluorescence intensity by 80% and red shifted the emission maximum by 16 nm (data not shown). As described below, MIANS remained with the ArsA protein following gel filtration using a spin column and SDS polyacrylamide gel electrophoresis, indicating covalent modification of the protein. Thus the results suggest that the increase in MIANS fluorescence following reaction with the ArsA protein resulted from covalent binding to the protein and movement of the probe into a more hydrophobic environment. Moreover, pretreatment of the ArsA protein with *N*-ethylmaleimide, which reacts selectively with thiol groups under these conditions (Lundblad, 1991), completely prevented MIANS binding (Figure 1), indicating that the probe reacted selectively with sulfhydryl groups in the ArsA protein.

Concentration Dependence of MIANS Binding. The relationship between fluorescence intensity and MIANS concentration was examined with a constant amount of ArsA protein (Figure 2). The rate of reaction was rapid, reaching a plateau in approximately 3 min. When MIANS was present in molar excess over ArsA protein, the final level of fluorescence remained unchanged for at least 1 h, indicating relative stability of the MIANS–ArsA protein complex. The final level of fluorescence intensity at 440 nm increased with increasing concentrations of MIANS up to 5 μM. With higher concentrations of MIANS the rate of binding increased, but there was a slight decrease in the plateau level of fluorescence intensity, possibly due to self-absorption (data not shown).

Effect of Substrates of the ArsA ATPase on the Reaction of the ArsA Protein with MIANS. In separate assays purified ArsA protein was pretreated for 10 min at 37 °C with 2.5 mM MgATP, its nucleotide substrate; with either 3 mM sodium arsenite or 0.5 mM potassium antimonyl tartrate, its oxyanionic substrates; or with 3 mM sodium arsenate, a nonsubstrate arsenic oxyanion. The protein was then incubated with an excess of MIANS for 2 h. Neither MgATP nor arsenate had any effect on the fluorescence of the ArsA–MIANS adduct (Figure 3). In contrast, preincubation with arsenite or antimonite increased the maximal fluorescence up to 2–3-fold over the fluorescence of the ArsA protein without substrates. Denaturation with SDS, trypsin digestion, and cyanogen bromide cleavage all reduced the quantum yield of the fluorescent MIANS–ArsA protein adduct obtained in the presence of antimonite (data not shown). There was no increase in MIANS fluorescence produced by reaction with arsenite or antimonite in the absence of protein. If the ArsA protein was incubated with MIANS before addition of oxyanion, neither arsenite nor antimonite produced an increase in MIANS fluorescence; neither did these oxyanions have an effect on the fluorescence of MIANS reacted with 2-mer-

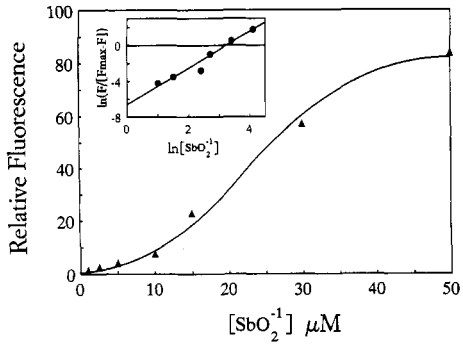

FIGURE 4: Concentration dependence of antimonite on the fluorescence emission intensity of the MIANS–ArsA protein adduct. ArsA protein (5 μM) was preincubated with the indicated concentrations of potassium antimonial tartrate for 10 min at 23 °C, followed by addition of 25 μM MIANS. Following reaction the ArsA protein was separated from the unbound MIANS on a spin column, and emission spectra were measured with an excitation wavelength of 340 nm. The fluorescence intensity at 440 nm of the MIANS–ArsA protein adduct obtained in the absence of antimonite was subtracted from the intensity at 440 nm at each concentration of antimonite. Inset: Hill plot of the data; a Hill coefficient of 2.1 was determined by computer analysis using nonlinear regression (Enzfitter, Elsevier-BIOSOFT, Cambridge, U.K.).

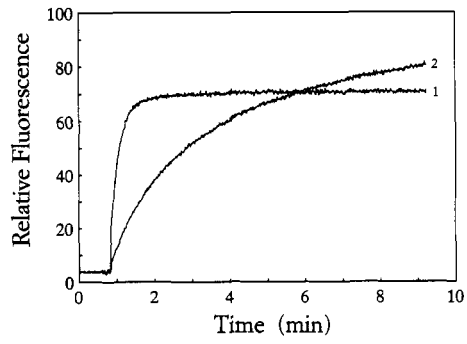

FIGURE 5: Effect of antimonite on the rate of the MIANS reaction with the ArsA protein. ArsA protein (5 μM) was incubated for 10 min at 23 °C with (curve 1) and without 0.5 mM potassium antimonial tartrate (curve 2). The reaction was initiated by addition of 25 μM MIANS. Fluorescence was measured with an excitation wavelength of 340 nm and an emission wavelength of 440 nm.

captoethanol, dithiothreitol, or bovine serum albumin (data not shown). The oxyanion-dependent increase in fluorescence could result from a conformation change that decreases the polarity of the probe environment, from additional cysteinyl residues reacting with MIANS, or from a combination of these factors.

The increase in fluorescence of the MIANS–ArsA protein adduct was measured as a function of the concentration of antimonite, with a half-maximal increase observed at approximately 20 μM antimonite (Figure 4). This is similar to the concentration of 10 μM antimonite required for half-maximal stimulation of ATPase activity for the ArsA protein (Hsu & Rosen, 1989) and indicates that the effect of antimonite on MIANS fluorescence reflects binding of oxyanion to a specific site on the ArsA protein. Between 50 μM and 0.5 mM antimonite there was little increase in fluorescence (data not shown). The data could be fit with a Hill coefficient of 2.1 (inset), implying positive cooperativity.

As shown above, in the absence of oxyanionic substrates the ArsA protein reacted rapidly with MIANS, indicating a comparatively high accessibility and reactivity of cysteinyl residues. However, following pretreatment of the ArsA protein with antimonite, the rate of increase in MIANS fluorescence at 440 nm was considerably slower (Figure 5), with 50% of the decrease in rate observed at 5 μM antimonite (data not shown). This effect could be explained by a decrease in the rate of reaction of the cysteinyl residues that react in the absence of oxyanion (perhaps by movement of one or more cysteinyl residues to a more hydrophobic environment), by one or more additional cysteinyl residues reacting at a slower rate, or by a combination of these.

The rate of MIANS binding to the ArsA protein exhibited pH dependence, with a maximal rate at pH 8.5 in the presence or absence of oxyanion (Figure 6A). Since cysteine is more reactive as the thiolate anion (Lundblad, 1991), MIANS would be expected to react more quickly at higher pH. Although the pK of cysteine in solution is 10.5, the pK of cysteinyl residues in proteins can be several orders of magnitude lower (Lundblad, 1991). There was no pH dependence of the maximal fluorescence at 440 nm of protein reacted with MIANS in the absence of oxyanion and only a slight effect

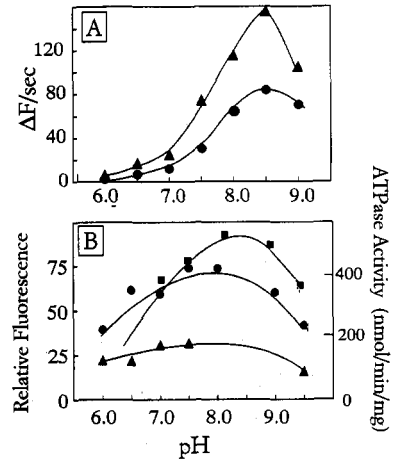

FIGURE 6: Effect of pH on the reaction of the ArsA protein with MIANS. ArsA protein (6 μM) was preincubated for 10 min at 23 °C with (●) or without 0.5 mM antimonite (▲) in solutions buffered with a combination of 15 mM 2-(*N*-morpholino)ethanesulfonate, 15 mM MOPS, 15 mM *N*-[tris(hydroxymethyl)methyl]glycine and 15 mM 2-(*N*-cyclohexylamino)ethanesulfonate, containing 0.25 mM EDTA. The buffer was adjusted to the appropriate pH values with concentrated HCl or KOH as required. The reaction was initiated by addition of 25 μM MIANS. (A) The initial rate of fluorescence increase was measured at λ_{ex} = 340 nm and λ_{em} = 440 nm. (B) After 2 h unbound MIANS was removed using spin columns, and the fluorescence emission at 440 nm was measured. Also shown (B) is the effect of pH on antimonite-stimulated ATPase activity of the ArsA protein (■).

in the presence of oxyanion (Figure 6B), indicating that the environment of the probe is largely unchanged by pH.

Effect of Oxyanion on the Protein Concentration Dependence of the Fluorescence of the MIANS–ArsA Protein Adduct. Varying concentrations of ArsA protein were modified with 50 μM MIANS in the presence and absence of 0.5 mM antimonite. The labeled protein was separated from unbound MIANS on spin columns, and the fluorescence emission of the MIANS–protein adduct was measured at 440 nm (Figure 7). In the absence of antimonite the protein bound MIANS in a nearly hyperbolic manner, with a Hill coefficient of 1.2 (Figure 7, inset). Binding in the presence of the oxyanion exhibited a distinct sigmoidicity, with a Hill coefficient of 2.1. Since the catalytic form of the ArsA protein is a dimer (Hsu et al., 1991), these results are consistent with a positive cooperativity between the subunits of the ArsA protein produced by binding of the oxyanion that influences the availability of cysteinyl residues for reaction with MIANS.

Stoichiometry of MIANS Binding to the ArsA Protein. From the primary sequence of the ArsA protein predicted

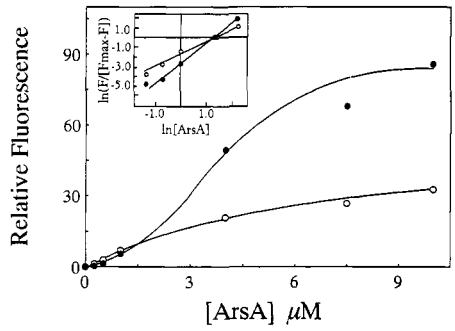

FIGURE 7: Protein concentration dependence of MIANS–ArsA protein adduct formation. ArsA protein was incubated at the indicated concentrations without (○) or with 0.5 mM potassium antimonial tartrate (●) in reaction buffer for 10 min at 23 °C. MIANS was then added to 50 μM, and the reaction continued for 2 h. Unbound MIANS was removed with spin columns, and the emission fluorescence at 440 nm was measured. Inset: Hill plots of the data determined using ENZFITTER. The Hill coefficients were 1.2 in the absence of antimonite and 2.1 in the presence of antimonite.

Table I: Stoichiometry of MIANS Binding to the ArsA Protein

ArsA protein concn (μM)	SbO_2^- (0.5 mM)	MIANS/ArsA ratio[a]	DTNB/ArsA ratio[b]
0.5	–	2.2 ± 0.3	
	+	2.2 ± 0.4	
5.0	–	2.0 ± 0.3	1.9 ± 0.1
	+	2.9 ± 0.3	1.2 ± 0.2

[a] ArsA protein was preincubated with or without 0.5 mM potassium antimonial tartrate for 10 min at 37 °C. MIANS was added in excess, to either 20 or 50 μM, and incubation continued for 2 h. The labeled protein was separated from unbound MIANS on spin columns containing Sephadex G-25 fine. The protein was denatured with 1% SDS, and the concentration of bound MIANS was determined from the absorbance at 322 nm. [b] The number of unreacted cysteinyl residues was determined using DTNB at 412 nm, as described under Materials and Methods.

from the nucleotide sequence, the 583-residue ArsA protein contains four cysteinyl residues: C26, C113, C172, and C422 (Chen et al., 1986). Thus 1 mol of protein is capable of binding 4 mol of MIANS. The ArsA protein was reacted with a saturating concentration of MIANS with and without a saturating concentration of antimonite. Two concentrations of ArsA protein were used, corresponding to the high and low points of the saturation curve shown in Figure 7. The labeled protein was separated from the unbound MIANS on spin columns. Since the absorption of MIANS is unaffected by the environment of the probe (Hiratsuka, 1992b), the stoichiometry could be determined spectrophotometrically using the known extinction coefficients of MIANS at 320 nm (ϵ = 20 000 M^{-1} cm^{-1}) and ArsA protein at 280 nm (ϵ = 33 480 M^{-1} cm^{-1}) (Table I). At low protein concentration 2 mol of MIANS was bound per 1 mol of ArsA protein independent of the presence of antimonite. At the higher concentration of the protein two MIANS molecules were bound per molecule of protein in the absence of oxyanion, while three were bound in the presence of antimonite. Titration of the remaining cysteinyl residues with DTNB showed that those residues remained in the reduced state. These results indicate that the effect of antimonite on the increase in the fluorescence of the ArsA–MIANS adduct can be explained by the reaction of an additional cysteinyl residue with MIANS. The reaction of MIANS with cysteinyl residues in a protein occurs with varying rates for each residue, depending on its environment (Haugland, 1983). Thus it is likely that each fluorescence signal represents an individual reacting cysteine, rather than an average of signals. However, identification of the labeled residues would be necessary to verify this assumption.

Table II: MIANS Fluorescence Lifetimes

ArsA protein concn (μM)	SbO_2^- (0.5 mM)	τ, ±5% (ns)	% of signal
0.5	–	2.3	34
	–	7.9	66
	+	2.0	24
	+	6.9	76
5.0	–	2.3	23
	–	7.4	77
	+	1.0	6
	+	4.4	57
	+	8.8	37

Lifetime Measurements of MIANS Bound to the ArsA Protein. Measurement of the fluorescence lifetimes of MIANS bound to the ArsA protein was used to characterize the local environment of the probe and determine the number of probe molecules bound to the protein (Table II). Although the number of bound probe molecules cannot be extracted solely from data analysis (Bigelow & Inesi,1991), a heterogeneous fluorescence decay implies the existence of multiple bound probe molecules (Bigelow & Inesi, 1991; Birmachu et al., 1989). The results are consistent with the stoichiometries obtained spectrophotometrically (Table I). In the absence of antimonite a two-exponential fit produced a minimal x_R^2, suggesting two fluorescence lifetimes of approximately 2 and 8 ns at both low and high protein concentration. In the presence of antimonite the same two fluorescence lifetimes were calculated at low protein concentration. At high protein concentration in the presence of oxyanion three exponentials were required to produce a minimal x_R^2, generating lifetimes of approximately 1, 4, and 9 ns. Thus binding of the oxyanionic substrate results in binding of a third probe molecule, although it is not possible to determine the correspondence between the lifetimes and which cysteines react. The relatively short fluorescence lifetimes indicate that none of the MIANS molecules are completely in a hydrophobic environment in the ArsA protein. However, two of the three lifetimes were longer when antimonite was present, suggesting that the microenvironments of those probe molecules had increased in hydrophobicity.

Solvent Accessibility of MIANS in the ArsA Protein. The accessibility of the protein-bound MIANS molecules to solvent was investigated by fluorescence quenching studies with KI (Figure 8). Similar results were obtained using acrylamide as a quenching agent (data not shown). In spite of the heterogeneity observed in the lifetime measurements, both quenching agents produced linear Stern–Volmer plots, indicating that within the concentration range of quenching agents used the bound MIANS molecules were all approximately equally accessible to the solvent (Lakowicz, 1983). From the results with KI (Figure 8), the Stern–Volmer constants for the MIANS–ArsA protein adducts obtained in the absence and presence of antimonite were 1.74 and 0.84 M^{-1}, respectively. This result suggests that binding of antimonite results in the movement of the reactive cysteinyl residues into a less solvent exposed environment, consistent with the increase in fluorescence lifetimes produced by oxyanion.

Inhibition of ATPase Activity of the ArsA Protein by MIANS. MIANS specifically inhibited the antimonite-stimulated ATPase activity of the ArsA protein, with half-maximal inhibition at approximately 1 μM MIANS (Figure 9). In this experiment, MIANS was added directly to the reaction mixture and ATPase activity was measured. Inhi-

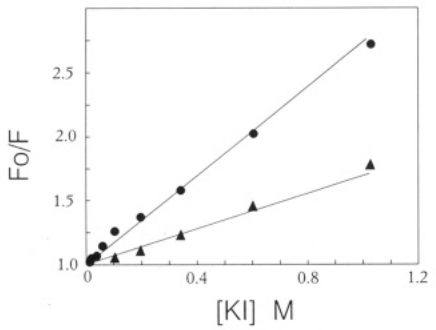

FIGURE 8: Solvent accessibility of MIANS in the ArsA protein. Quenching of the fluorescence of the ArsA protein with KI was done following complete modification of the ArsA protein with MIANS. The ArsA protein (4.4 μM) was modified with 0.1 mM MIANS in the presence (▲) or in the absence (●) of antimonite and purified on spin columns. Increasing amounts of 4 M KI were added to the cuvette containing the MIANS–ArsA protein complex, and fluorescence emission was measured at 440 nm.

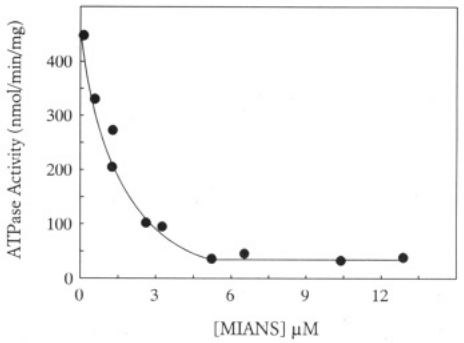

FIGURE 9: Effect of MIANS on the oxyanion-stimulated ATPase activity of the ArsA protein. ArsA protein (42 μM) was preincubated with 0.1 mM potassium antimonial tartrate for 10 min at 37 °C. MIANS was added to the indicated concentrations, following which the ATPase assay was immediately initiated by addition of $MgCl_2$, as described under Materials and Methods. The same inhibition by MIANS was observed if MIANS was added to the reaction mixture before the ArsA protein (data not shown).

bition did not depend on the order of addition of substrates or inhibitor and was more rapid than could be measured with the coupled assay. Although inhibition may be reversible in its early phase, ArsA protein recovered after the assay was covalently reacted with MIANS and was irreversibly inhibited. Thus there may be one or more sulfhydryl groups critical for enzymatic activity, although a direct participation in catalysis cannot be determined from these data. Alternatively, the addition of bulky ANS moieties may influence catalysis through steric effects.

Identification of MIANS-Labeled Polypeptides in the ArsA Protein. Following modification of the ArsA protein with MIANS, a fluorescent band corresponding to the MIANS–ArsA complex could be visualized upon SDS–PAGE, demonstrating covalent binding of the probe to the protein (Figure 10). In the presence of antimonite the fluorescence intensity of the band corresponding to the ArsA protein was greater, even though the protein was denatured, consistent with part of the increase in fluorescence resulting from incorporation of additional probe. The ArsA protein can be cleaved by brief trypsin treatment to produce a characteristic band of approximately 50 kDa that subsequently is cleaved to 30 kDa (Hsu et al., 1991). Both bands were fluorescent following digestion of the MIANS–ArsA adduct, with the amount of fluorescence increased when the protein was labeled in the presence of oxyanion.

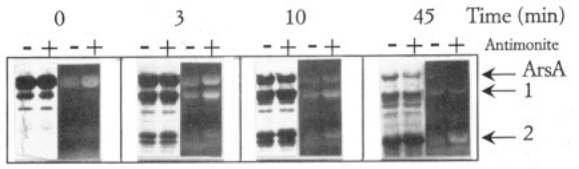

FIGURE 10: Fluorescence of tryptic products of the MIANS–ArsA protein adduct. ArsA protein (1.4 mg/mL) was reacted with 0.1 mM MIANS in the absence or presence of 0.5 mM potassium antimonial tartrate. Trypsin treatment was performed as described under Materials and Methods, and the polypeptide products were resolved by SDS–PAGE on a 10% gel. In the right panels are the fluorescent bands visualized on an ultraviolet transilluminator prior to fixation and staining. In the left panels are polypeptides visualized following fixation and staining with Coomassie blue. Indicated are the positions of the native 63-kDa ArsA protein, the 50-kDa tryptic product (arrow 1), and the 30-kDa tryptic product (arrow 2), as determined by the migration of standard proteins. The lower bands in the control lanes appear from immunoblotting experiments to be degradative products of the ArsA protein that are formed during long-term storage of the protein (data not shown). They are present before MIANS treatment and do not form fluorescent adducts.

DISCUSSION

The ArsA protein, the catalytic subunit of the oxyanion pump, exhibits antimonite- and arsenite-dependent ATPase activity (Hsu & Rosen, 1989). To have this activity, there must be one or more binding sites for the nucleotide substrate and one or more oxyanion binding sites. Moreover, the oxyanion dependency of catalysis implies communication between the two types of binding sites. Direct assays of radioactive arsenite and antimonite binding have proven difficult because of high background produced by the extreme reactivity of these oxyanions. The fluorescent maleimide MIANS has proven to be a useful probe of ligand binding and conformational changes (Gupte & Lane, 1979, 1983; Haugland, 1983; Forgac, 1980). The effect of the oxyanionic substrates of the ArsA protein on the fluorescence of MIANS has provided an indirect assay for oxyanion binding. Moreover, the effect of maleimides on the ATPase activity of the ArsA protein (Figure 9) suggests a role for cysteinyl residues in catalytic activity.

In the absence of oxyanion two of these residues react with the sulfhydryl probe MIANS (Table I). In the presence of antimonite either two or three cysteinyl residues react, depending on the concentration of ArsA protein. Thus MIANS labeling does not show a simple dependence on the concentration of either oxyanion (Figure 4) or protein (Figure 7). In both cases the data indicate cooperativity. In solution the ArsA protein has been shown to self-associate, forming an active dimer (P. Kaur and B. P. Rosen, unpublished results), with the equilibrium between monomer and dimer strongly dependent on the presence of the oxyanionic substrate (Hsu et al., 1991). One possible explanation for the apparent cooperativity of MIANS reaction is that either binding of the oxyanion or self-association at high protein concentration shifts the monomer–dimer equilibrium in favor of the dimer, and the dimer binds one additional MIANS molecule per molecule of ArsA subunit. Thus a monomer of ArsA would bind two MIANS molecules, and a dimer would bind six, three per subunit. Moreover, the decrease in the Stern–Volmer constants from fluorescence quenching with KI (Figure 8) implies movement of the the bound MIANS molecules to a less solvent accessible environment, consistent with a change in the conformation of one or more of the cysteinyl residues, perhaps as a result of dimer formation.

The ArsA protein contains four cysteinyl residues at C26, C113, C172, and C422 (Chen et al., 1986). As discussed,

from the direct measurements of MIANS binding, two react under conditions that favor monomer, and three with dimer (Table I). The data from the decay of fluorescence anisotropy indicate two lifetimes in the absence of oxyanion and three lifetimes in the presence of antimonite (Table II), but no assignment of the signals can be made with specific residues. To determine which of the four cysteinyl residues react will require isolation and sequencing of labeled peptides and/or mutagenesis, and both types of studies are in progress.

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