The ArsA ATPase is the catalytic subunit of a novel arsenite pump, with two nucleotide-binding consensus sequences in the N- and C-terminal halves of the protein. The single tryptophan-containing Trp^159 ArsA was used to elucidate the elementary steps of the ATPase mechanism by fluorescence stopped-flow experiments. The binding and hydrolysis of MgATP is a multistep process with a minimal kinetic mechanism (Mechanism 1). A notable feature of the reaction is that MgATP binding induces a slow transient increase in fluorescence of ArsA, which is independent of the ATP concentration, indicative of the build-up of a pre-steady state intermediate. This finding, coupled with a phosphate burst, implies that the steady-state intermediate builds up subsequent to product release. We propose that the rate-limiting step is an isomerization between different conformational forms of ArsA. k_{cat} is faster than the phosphate burst, indicating that both nucleotide binding sites of ArsA are catalytic. Consistent with this interpretation, approximately 2 mol of phosphate are released per mole of ArsA during the phosphate burst.

ArS A^1-AT P ⇌ ArS A^1 + Mg ATP ⇌ ArS A^1-Mg ATP ⇌ ArS A^1-Mg ATP ⇌ ArS A^1-Mg ATP

Mechanism 1

Resistance to arsenical and antimonial salts in 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 metabolites is the membrane anchor for ArsA and the oxyanion-transporting sector of the pump (3). ArsA can be purified as a soluble ATPase in the absence of ArsB (4). ArsA is arranged into two homologous halves, the N-terminal (A1) (residues 1–282) and C-terminal (A2) (residues 321–583) domains, which are connected by a flexible linker (residues 283–320) (5, 6). Each domain contains the consensus sequence for the phosphate binding loop (P-loop) of an ATP-binding site (7). Site-directed mutagenesis of these sequences indicates that both of the putative nucleotide binding sites are required for catalysis and resistance (8, 9). Intergeneric complementation and intragenic suppression studies on ArsA were suggestive of a model in which a single catalytic site was formed at the interface of an A1 and an A2 ATP binding site, possibly within a homodimer (10, 11).

To investigate the interaction of nucleotides with the A1 and A2 sites, we have conducted stopped-flow fluorescence experiments on ArsA. We have previously shown that intrinsic tryptophan fluorescence can be used to investigate the interaction of ArsA with its ligands (12). However, the presence of multiple tryptophan residues in ArsA decreased the signal-to-noise response to ligand binding, leading us to construct single tryptophan derivatives of ArsA that are optically responsive to the binding of substrates or products (13). Mutant arsA genes were constructed containing single tryptophan codons (13). Tryptophan residues 253, 522, and 524 were changed to tyrosine and resistance by the inorganic phosphate generated by the ATPase activity was monitored (15). The phosphorolysis reaction was catalyzed by purified nucleotide phosphorylase. The components of the assay were provided as part of an EnzCheck phosphate assay kit (Molecular Probes, Eugene, OR) and used according to the manufacturer’s recommendations. Assays were performed in 40 mM Tris-HCl (pH 7.5); 2 mM MgCl_2, contain-

MATERIALS AND METHODS

Purification of His6-tagged ArsA ATPase—ArsA W159H6 ArsA was purified as described previously (13), 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 coefficient for W159H6 ArsA was calculated to be 20,250 M⁻¹ cm⁻¹ (14).

ATPase Assays—A continuous assay was used to monitor phosphate production by ArsA. Essentially, the absorbance change at 360 nm associated with the phosphorolysis of 2-aminomethylphospho-d-ribose-7-phosphate by the inorganic phosphate generated by the ATPase activity was monitored (15). The phosphorolysis reaction was catalyzed by purified nucleotide phosphorylase. The components of the assay were provided as part of an EnzCheck phosphate assay kit (Molecular Probes, Eugene, OR) and used according to the manufacturer’s recommendations. Assays were performed in 40 mM Tris-HCl (pH 7.5); 2 mM MgCl_2, contain-
The Kinetics of ATP Binding and Turnover—Previously we established that the tryptophan fluorescence of ArsA (W159H6) was sensitive to the binding of MgATP (13). Fig. 1A shows the change in tryptophan fluorescence when 5 mM ArsA was manually mixed with 1 mM MgATP; the reaction was initiated by the addition of 5 mM MgCl₂ to 5 μM ArsA, 1.0 mM ATP. There was a rapid biphasic increase in the tryptophan fluorescence, to reach a plateau, before the fluorescence decayed back to the baseline level (as defined by the fluorescence of 5 μM ArsA, 1.0 mM ATP), suggesting that little product MgADP remains bound to the ArsA. However, the addition of EDTA at the end of the reaction, to chelate the Mg²⁺, caused a further decrease in tryptophan fluorescence, suggesting that some product MgADP remained bound at the end of the reaction (Fig. 1A). A potentially plausible explanation for the fluorescence profile of the reaction is that the binding of MgATP to the ArsA induces an enhancement in the tryptophan fluorescence, which remains constant until the ATP has been depleted, at which point the fluorescence decays as the products are released. To test this hypothesis, the mixing experiment was repeated with higher concentrations of ArsA and ATP. The period during which the enhanced fluorescence remained constant should have been diminished for higher ArsA and extended for higher ATP if this phase was determined by the time taken for the steady-state turnover of the ATP. As shown in Fig. 1B and C, no significant effect was observed upon this phase with either higher ATP or ArsA concentrations, respectively. This suggested that the enhancement in tryptophan fluorescence was due to a transient build up and decay of a reaction intermediate. The addition of excess EDTA rapidly reduced the ArsA fluorescence, presumably due to the dissociation of ATP in the absence of Mg²⁺ (Fig. 1), thus suggesting that the intermediate was an ArsA-Mg²⁺ nucleotide complex because MgCl₂ alone did not produce a change in the fluorescence of the ArsA (data not shown). The addition of EDTA to ArsA or ArsA/Mg²⁺ did not produce any change in the fluorescence of the ArsA and only a small decrease with ArsA/MgADP (data not shown). This behavior suggests that the intermediate was ArsA-MgADP²⁻.

We have investigated the kinetics of formation of this intermediate in detail using stopped-flow fluorescence spectroscopy. A typical stopped-flow trace for the mixing of 5 μM ArsA, 0.5 mM ATP with 5 mM MgCl₂; B, 5 μM ArsA, 4 mM ATP with 5 mM MgCl₂; and C, 20 μM ArsA, 1 mM ATP with 5 mM MgCl₂.

Fig. 1. ATP-induced conformational changes in ArsA. ArsA was mixed with MgATP, in a Jasco FP777 fluorimeter, and the tryptophan fluorescence changes monitored (excitation = 292.5 nm and emission = 340 nm). The conditions were as follows: A, 5 μM ArsA, 1 mM ATP with 5 mM MgCl₂; B, 5 μM ArsA, 4 mM ATP with 5 mM MgCl₂; and C, 20 μM ArsA, 1 mM ATP with 5 mM MgCl₂.

1 The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.

2 These data also indicate that no residual ADP remains bound to the ArsA protein following its purification.
**Transcript Kinetics of ArsA**

The overall $K_d$ is a function of both $K_a$ and $K_f$: $K_d = K_a(1 + K_f)$. Accordingly, $K_a$ can be calculated, from the overall $K_a$ and $K_f$, as 3.33, indicating that 77% of the ArsA is in the ArsA$^1$ conformation before the binding of MgATP. Because the rate of phase 1 increased with the ATP concentration but was independent of the Mg$^{2+}$ concentration, this implies that we were monitoring the binding of MgATP to the ArsA. A possibility is that the ArsA$^1$ state is stabilized by the nonproductive binding of MgATP in the absence of Mg$^{2+}$, which must dissociate before MgATP can bind.

Neither the rate constant for, nor the amplitude of, the slow increase in fluorescence (e.g. phase 3) had any apparent dependence upon the ATP concentration (data not shown). The rate constant varied nonsystematically between 0.025 and 0.05 s$^{-1}$ (data not shown). The rate constant for the slow decay in fluorescence back to the baseline (phase 4) occurred with a rate constant of $2.3 \times 10^{-3}$ s$^{-1}$ that was also independent of the ATP concentration (data not shown). Consequently suggesting that these phases are attributable to a further isomerization of the ArsA-nucleotide complex (e.g. the formation and decay of ArsA$^3$).

In a parallel set of experiments, the binding of MgATP to ArsA was investigated. A typical stopped-flow trace for the mixing of 5 mM ArsA with 0.5 mM ATP, 5 mM MgCl$_2$ is shown in Fig. 2B. The binding of MgATP to ArsA was characterized by a slow, apparently monophasic, fluorescence enhancement that occurred with a rate constant of 0.0334 s$^{-1}$. Thus, this phase occurred at a rate comparable with the slow fluorescence enhancement that occurred when the reaction was initiated by mixing ArsA/MgATP with Mg$^{2+}$ (cf. 0.026 s$^{-1}$). However, the fast increase (phase 1) and decrease (phase 2) in fluorescence, which occurred within 4 s of initiating the reaction with Mg$^{2+}$, were not apparent. There was an indication of a hyperbolic increase in the rate constant, between 0.022 s$^{-1}$ and 0.058 s$^{-1}$, for the slow enhancement in fluorescence when ArsA was mixed with MgATP. This behavior is consistent with a two-step binding mechanism. The rapid equilibrium binding of the ATP, which is rate limited by a slow isomerization of the ArsA$^1$-ATP complex (e.g. $k_1 k_{-1} \gg k_2 k_{-2}$) is shown in Scheme 3.

The data could be fitted to a hyperbolic equation for such a model: $k_{obs} = k_{-2} + k_2 ([\text{MgATP}]/[[\text{MgATP}]+K_f])$, yielding values for $K_f$, $k_{-2}$, and $k_{-2}$ of 624, 0.038 s$^{-1}$ and 0.022 s$^{-1}$, respectively (Fig. 6A). The overall $K_f$ would be given by the following equation: $K_f^{(overall)} = K_f(1 + k_f k_{-2})$, indicating a $K_f$ of 232 ($\pm 75.5$) M$^{-1}$. The amplitude of the ATP-induced fluorescence enhancement increased in a hyperbolic manner with the ATP concentration, indicating an overall dissociation constant ($K_f$) of 427 ($\pm 62.3$) M$^{-1}$ (Fig. 6B). Hence, there is a small discrepancy between the predicted and measured overall $K_f$, which could readily be reconciled by postulating a further conformational transition of the ArsA-MgATP complex. However, this seems unwarranted at this time considering the difficulty in measuring the small increase in the rate constants $k_2$ and $k_{-2}$ and the consequent error in $K_f$. This behavior contrasts with that for the Mg$^{2+}$ initiated reaction, where the amplitude of the slow increase in fluorescence was apparent independent of the ATP concentration. A plausible explanation is that the MgATP initially binds to an ArsA state that does not produce any change in fluorescence but which is followed by a slow transition to a state with an enhanced fluorescence. When the ArsA was pre-equilibrated with Mg$^{2+}$ before initiating the reaction by mixing with ATP, the traces were similar to those generated by mixing ArsA with MgATP (data not shown). This indicates that if Mg$^{2+}$ can bind to ArsA in the absence of nucleotides then this must be a rapid equilibrium process.

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$^3$ A possible interpretation of this behavior is that there are two fluorescent transients, one of which becomes too fast to measure at high ATP concentrations. At intermediate ATP concentrations, a single exponential fit of the data is biased by a contribution from the first transient, whereas at higher ATP concentrations, the observed change reflects the second transition alone.

$^4$ This is a minimal scheme because we have assumed that the MgATP only binds to the ArsA$^2$ conformation.
These differences in the kinetics of MgATP binding indicate that ATP can bind to ArsA in the absence of Mg" to induce the protein to adopt a different conformation.

The Binding of ATP under Single Turnover Conditions—As shown in Fig. 7A, when 25 μM ArsA (a 50 μM nucleotide binding site concentration) was mixed with 25 μM MgATP the stopped-flow trace generated was similar to those obtained under multiple turnover conditions (e.g. 5 μM ArsA with 500 μM MgATP). There was an increase in fluorescence, which occurred with a rate constant of 3.6 ± 10^2 s^{-1}, and a subsequent decay in fluorescence that occurred with a rate constant of 7.8 ± 10^2 s^{-1}. The protein fluorescence decayed back to a level lower than the start of the trace, indicating that there is a very fast increase in fluorescence probably due to the binding of ATP. Indeed, we consistently noted a rapid increase in fluorescence at the start of the single turnover traces that occurred with a rate constant of approximately 20 s^{-1}. In comparison with multiple turnovers, the amplitude of the decay phase was greater in relation to that of the enhancement phase, suggesting that ADP remained bound to the ArsA after multiple turnovers.

In contrast, when 25 μM ArsA was incubated with 25 μM ATP before mixing with 5 mM MgCl_2 in the stopped-flow instrument, there was a rapid increase in the protein fluorescence (phases 1 and 2), followed by a slow decay (phase 4) (Fig. 7B). There was no slow increase in fluorescence (phase 3). It was possible to fit the data to a triple exponential function with the increase and the biphasic decrease in fluorescence occurring with rate constants of 12.3 s^{-1} (phase 1), 6.22 s^{-1} (phase 2), and 2.0 x 10^{-3} s^{-1} (phase 4), respectively. The rate constants for phases 1, 2, and 4 were comparable with the calculated rate of ATP binding (cf. 14 s^{-1} for 25 μM ATP) and with the rate of fluorescence decay under multiple turnover conditions (cf. 4.5–6.5 s^{-1} for
phase 2 and $2.3 \times 10^{-3}$ s$^{-1}$ for phase 4), respectively. Although there was no slow enhancement in fluorescence (e.g. phase 3) under these single turnover conditions, the amplitude of the decay phases (e.g. phase 4) when ArsA/ATP was mixed with Mg$^{2+}$ was similar to that when ArsA was mixed with MgATP (e.g. 3.0% versus 2.3% fluorescence change, respectively). This slow decay in fluorescence cannot be attributed to multiple turnovers, which depleted the concentration of this intermediate, during the latter stages of the reaction, because this phase was independent of the ATP concentration (cf. from measurements of single and multiple turnovers). The slow increase and decrease in fluorescence presumably represents the transient formation of an intermediate that forms and decays at rates of 6.5 s$^{-1}$ and $2.3 \times 10^{-3}$ s$^{-1}$, respectively. If this is an in-line intermediate then the pre-equilibration with ATP (in the absence of Mg$^{2+}$) must induce the ArsA to adopt a similar conformation.

**ADP Binding** — The binding of MgADP to ArsA was characterized by a small enhancement in tryptophan fluorescence (e.g. phase 3) under these single turnover conditions, the amplitude of the decay phases (e.g. phase 4) when ArsA/ATP was mixed with Mg$^{2+}$ was similar to that when ArsA was mixed with MgATP (e.g. 3.0% versus 2.3% fluorescence change, respectively). This slow decay in fluorescence cannot be attributed to multiple turnovers, which depleted the concentration of this intermediate, during the latter stages of the reaction, because this phase was independent of the ATP concentration (cf. from measurements of single and multiple turnovers). The slow increase and decrease in fluorescence presumably represents the transient formation of an intermediate that forms and decays at rates of 6.5 s$^{-1}$ and $2.3 \times 10^{-3}$ s$^{-1}$, respectively. If this is an in-line intermediate then the pre-equilibration with ATP (in the absence of Mg$^{2+}$) must induce the ArsA to adopt a similar conformation.

**ADP Binding** — The binding of MgADP to ArsA was characterized by a small enhancement in the tryptophan fluorescence, precluding a rigorous analysis of the kinetics of binding. However, when ArsA was mixed with a relatively high MgADP concentration (e.g. 10 mM), the stopped-flow traces generated were similar to those for the binding of MgATP to ArsA, which had been pre-equilibrated with ATP. There was a rapid increase and decrease in the fluorescence, followed by a slow increase but not a subsequent decrease (Fig. 8). Recently, we have established that the W141H6 ArsA mutant has greater optical sensitivity to the binding of ADP, and a detailed analysis of the ADP binding mechanism for this mutant will be presented elsewhere.

**ADP Dissociation** — To test whether the dissociation of MgADP was a rapid or slow process, the ArsA-MgADP complex was chased with excess MgATP. Clearly, if MgADP dissociation was a rapid process then we would have expected to observe an increase in the ArsA fluorescence as the MgATP bound. On the other hand, if dissociation of the MgADP was slow, then we would have expected to observe a decrease in the fluorescence as the MgADP dissociated. When 5 μM ArsA was equilibrated with 50 mM ADP, 5 mM MgCl$_2$, and mixed with 500 μM ATP in a stopped-flow device, there was a relatively slow decrease in fluorescence, presumably as the ADP was displaced, followed by a slow increase in fluorescence, presumably as the ATP was hydrolyzed (Fig. 9). The amplitude of the decrease in fluorescence increased in a hyperbolic manner with the ArsA/MgADP incubation time (Figs. 9 and 10), indicating that the ADP induced a slow conformational change in the ArsA, which occurred with a rate constant of 1.62 $\times$ 10$^{-4}$ s$^{-1}$. This behavior is consistent with the increase in ArsA fluorescence that we have noted upon the binding MgADP to ArsA. This conformational change was to a form that allowed more rapid product release because the rate constant for the dissociation process increased from 0.076 to 0.131 s$^{-1}$ over the 8-h incubation time (Fig. 10).

**Phosphate Burst** — A burst in phosphate production was identified using a continuous assay to monitor phosphate release from ArsA. As shown in Fig. 11, when 1 μM ArsA was mixed with 50 μM ATP there was an exponential increase in the phosphate concentration during the first 400 s, followed by a
FIG. 7. A single turnover of ATP by ArsA. Stopped-flow records that show the change in ArsA fluorescence under single turnover conditions. 25 μM ArsA (a 50 μM nucleotide binding site concentration) was mixed with 25 μM ATP, 5 mM MgCl₂ (A), and 25 μM ArsA/25 μM ATP was mixed with 5 mM MgCl₂ (B). The smooth curve through each record is the best-fit to a double-exponential function with rate constants of 3.6 × 10⁻² (±9.5 × 10⁻⁴) s⁻¹ and 7.8 × 10⁻⁴ (±5.0 × 10⁻⁵) s⁻¹ (A), and a triple exponential function with rate constants of 11.0 (±0.95) s⁻¹, 7.6 (±0.90) s⁻¹, and 2.1 × 10⁻³ (±1.2 × 10⁻⁴) s⁻¹ (B), respectively. One vertical division represents an 0.25% change in the fluorescence of ArsA.

linear steady-state release of phosphate.⁵,⁶ The rate of the pre-steady-state phase only increased slightly with increasing ATP concentration to a value of 3.7 × 10⁻³ s⁻¹ for 300 μM ATP. The pre-steady-state phase could not be readily resolved from the steady-state phase for higher ATP concentrations. In conclusion, during the phosphate burst approximately 2 nmol⁷ of phosphate were released at a rate faster than 3.7 × 10⁻³ s⁻¹ (for near saturating ATP concentrations).

The steady-state rate, as determined from the linear part of the phosphate release time course, increased in a hyperbolic manner with the ATP concentration to a maximal level around 200 μM ATP and thereafter was subject to substrate inhibition (Fig. 12). Hence, values for \( V_{\max} \), \( K_m \), and \( K_i \), the substrate inhibition constant, were determined from a fit of the data to the following equation for substrate inhibition: \( v = \frac{V_{\max}[\text{ATP}]}{K_m + [\text{ATP}] + [\text{ATP}]^2/K_i} \). This analysis yielded values for \( V_{\max} \), \( K_m \), and \( K_i \) of 5.4 × 10⁻³ nmol s⁻¹·nmol⁻¹ ArsA, 72 μM and 792 μM, respectively, and indicated a \( k_{cat} \) of 5.4 × 10⁻³ s⁻¹ (single site catalysis) or 2.7 × 10⁻³ s⁻¹ (two-site catalysis).

⁵ Less than 10% of the ATP was hydrolyzed over the studied time course.

⁶ The data was best-fitted to an exponential plus a linear term with a nonzero intercept (e.g. a nonzero absorbance at the start of the experiment) as judged by a 400% decrease in residual variance of the fit. We have found a small upward drift in the absorbance of the reactants before the addition of the ArsA to initiate the reaction.

⁷ Eight measurements gave an average burst of 1.7 (±0.28) nmol.

FIG. 8. The binding of ADP to ArsA. A representative stopped-flow trace for the mixing of 5 μM ArsA, 10 mM MgCl₂ with 10 mM ADP, 10 mM MgCl₂. The smooth curve through the data is the best-fit to a triple exponential function with rate constants of 10.5 (±1.27) s⁻¹, 9.28 (±1.31) s⁻¹, and 0.16 (±0.011) s⁻¹, respectively. One vertical division represents an 0.5% change in the fluorescence of ArsA.

FIG. 9. ADP dissociation. 5 μM ArsA was equilibrated with 50 μM ADP, 5 mM MgCl₂ for 1 min (A) or 120 min (B) and then mixed with 500 μM ATP in a stopped-flow device to displace the bound ADP.

DISCUSSION

ArsA is the catalytic subunit of the arsenite transporter and is thought to couple the hydrolysis of ATP to the movement of arsenicals and antimonials through the membrane-spanning ArsB protein. Consequently, knowledge of the ArsA ATPase mechanism will provide information of fundamental importance in understanding the energy transduction processes common to many transporters that are driven by ATP hydrolysis, such as those belonging to the ABC superfamily. Utilizing a derivative of ArsA that contains only a single tryptophan residue, Trp₁₅⁹, which is optically responsive to the binding of ATP, the ATPase mechanism of ArsA was investigated.

The kinetics of the binding of MgATP to ArsA were indicative of a multistep mechanism. When ArsA was pre-equilibrated with ATP before mixing with Mg²⁺ to initiate the reaction, the binding of MgATP to the ArsA could be monitored as an increase in ArsA fluorescence. The rate constant for the binding step increased hyperbolically, indicative of a two-step process for the sequential formation of ArsA₅·MgATP and ArsA₄·MgATP. The forward and reverse rate constants for the formation of these intermediates were calculated as \( k_3 > 0.34 \times 10^6 \text{ s}^{-1} \), \( k_2 = 7.3 \text{ s}^{-1} \), \( k_4 = 53.9 \text{ s}^{-1} \) and \( k_3 = 7.3 \text{ s}^{-1} \) (Schemes 1, 2, and 4 nomenclature). Formation of the ArsA₄·MgATP complex was followed by a further isomerization to the less fluorescent ArsA₅·MgATP state, at a rate of 4.5–6.5 s⁻¹. There then followed a further conformational change in which an intermediate with a higher fluorescence was formed at a rate of 0.025–0.05 s⁻¹. In comparison, when ArsA was mixed directly with MgATP only a slow monophasic increase in the fluorescence was observed, which occurred at a similar rate.
to the formation of this intermediate. However, both the rate constant for, and the concentration of, this intermediate increased in a hyperbolic manner with the ATP concentration. This behavior is consistent with a two-step binding process: the rapid equilibrium binding of ATP, to produce an ArsA-MgATP complex suggesting that it is the ArsA-MgADP complex. An alternative possibility is that it is the ArsA-MgADP-P complex. An alternative possibility is that it is a

Irrespective of the mixing order, the slow formation of an intermediate that builds up and decays with rate constants of 3.8 \times 10^{-6} \text{s}^{-1} and 1\text{–}3 \times 10^{-3} \text{s}^{-1}, respectively (e.g. phases 3 and 4). This cannot be the steady-state intermediate for the reaction; otherwise it would only decay after depletion of the ATP. Accordingly, its formation must precede the rate-limiting step of the reaction. This intermediate has greater fluorescence than the ArsA-MgADP complex suggesting that it is the ArsA-MgADP complex.

**Scheme 4**

\[
\begin{align*}
\text{ArsA}^1\text{-ATP} &\rightarrow \text{ArsA}^1 + \text{MgATP} &\rightarrow &\text{ArsA}^1\text{-MgATP} &\rightarrow &\text{ArsA}^1\text{-MgATP} \\
&\rightarrow &\text{ArsA}^1\text{-MgADP} &\rightarrow &\text{ArsA}^1\text{-MgADP} &\rightarrow &\text{ArsA}^1 + \text{MgADP} \\
&\rightarrow &\text{ArsA}^1 + \text{MgADP-P} &\rightarrow &\text{ArsA}^1\text{-MgADP} + P &\rightarrow &\text{ArsA}^1 + \text{MgADP-P} &\rightarrow &\text{ArsA}^1
\end{align*}
\]
state with a higher affinity for ADP relative to that of the final state. Consistent with the former interpretation, phosphate is released with a relatively slow rate constant (e.g., \( k_{\text{off}} = 3.7 \times 10^{-3} \text{ s}^{-1} \)) for the phosphate burst with 300 \( \mu \text{M} \) ATP compared with the rate of formation of this intermediate (e.g., \( k = 3.8 \times 10^{-2} \text{ s}^{-1} \)). The slow release of phosphate suggests that there would not be an appreciable build up of ArsA-MgADP during the time course of the build up of the intermediate with enhanced fluorescence. We tentatively conclude that this intermediate is the ArsA-MgADP-P\(_i\) complex. We have previously measured phosphate production during a 4-fold limited turnover of ATP by ArsA (13). A discontinuous assay was used to monitor the hydrolysis of 20 \( \mu \text{M} \) ATP by 5 \( \mu \text{M} \) ArsA, with the reaction terminated at set times with trichloroacetic acid to displace bound Pi. We found that during the period of enhanced fluorescence (phase 3, 100–200 s), there was a stoichiometric production of bound phosphate, consistent with the proposal that phase 3 is attributable to the production of ArsA-ADP-P\(_i\). The slow decay in fluorescence is presumably attributable to the product release steps. ADP is released at a much faster rate than the decay in fluorescence (e.g., for ADP \( k_{\text{off}} \geq 0.08 \text{ s}^{-1} \)), whereas \( k_{\text{diss}} \) for phase 4 is \( 1–3 \times 10^{-3} \text{ s}^{-1} \). On the other hand, the decay in fluorescence occurs at a rate similar to that for phosphate release (e.g., for Pi, \( k_{\text{off}} \geq 3.7 \times 10^{-3} \text{ s}^{-1} \), whereas \( k_{\text{diss}} \) for phase 4 is \( 1–3 \times 10^{-3} \text{ s}^{-1} \)). If this is the case, then hydrolysis of the ATP must be fast because the phosphate burst includes both the hydrolysis and phosphate release steps. An alternative possibility is that the fluorescence decay is due to a conformational change subsequent to the release of the phosphate. However, the fact that there is a phosphate burst implies that phosphate release is more rapid than the step that is rate-limiting in the steady-state. Because neither the hydrolysis nor product release steps are rate-limiting for the steady-state, we conclude that there is a rate-limiting conformational change in ArsA following product release.

The stoichiometry of the phosphate burst is 1.7 (±0.28), suggesting that both nucleotide sites of ArsA are catalytic. Moreover, the steady-state rate for a single site is apparently faster than the rate constant for the phosphate burst and phase 4 of the fluorescence profile, whereas these steps in the reaction mechanism must be faster than the rate-limiting step. However, if both sites were catalytic, this would in effect halve \( k_{\text{off}} \), which would then have a value reasonably consistent with the other kinetic data. Indeed, computer simulations revealed that the formation of the ArsA-MgADP-P\(_i\) intermediate (with enhanced fluorescence) need only be followed by the relatively fast formation of a further intermediate (e.g., \( 10 \times \) the rate constant for phase 4) that decays with a rate constant marginally slower than does ArsA-MgADP-P\(_i\). Under these conditions an intermediate would build up and decay at the rates of phases 3 and 4. Thus, there is kinetic evidence to support the supposition that both the A1 and A2 sites of ArsA bind and hydrolyze ATP. Previous studies have shown that the A1 and A2 sites can be covalently labeled with ATP and FSBA, respectively, indicating that both sites are available for nucleotide binding (17, 18). However, it was proposed that antimonite might act as a switch in regulating ATP binding to the A2 site (18). Our studies do not support this supposition; we find no evidence for two sites of differing affinity, and the data suggest that both sites are catalytically competent.

It was possible to simulate the time for the fluorescence changes associated with the binding of ATP to ArsA (e.g., 1 \( \mu \text{M} \) ATP to 5 \( \mu \text{M} \) ArsA) using the measured rate constants and the minimal kinetic mechanism shown in Scheme 4 with the kinetic constants, \( k_2 = 0.04 \mu \text{M}^{-1} \text{s}^{-1} \), \( k_{-2} = 7.3 \text{ s}^{-1} \), \( k_3 = 54 \text{ s}^{-1} \), \( k_4 = 6.5 \text{ s}^{-1} \), \( k_5 = 0.04 \text{ s}^{-1} \), \( k_{-5} = 0.02 \text{ s}^{-1} \), \( k_6 = 0.004 \text{ s}^{-1} \), \( k_7 = 6.5 \text{ s}^{-1} \).
Transcript Kinetics of ArsA

$= 0.08 \text{s}^{-1}, k_b = 0.001 \text{s}^{-1}, k_f = \text{fast}$. The model allows for the formation of an intermediate (e.g. ArsA-MgADP-P) during the first 100 s and its decay over the following 900 s and for a pre-steady state burst and subsequent steady-state release of phosphate (Fig. 13). The fluorescence of the intermediate decays to about 0.33-fold that of the maximum due to the formation of the subsequent steady-state intermediate (e.g. ArsA7), which only decays after all of the ATP has been depleted. Under single turnover conditions, there is a decay in the fluorescence of the ArsA-MgADP-P intermediate to near the baseline because there is no substantial build up in the steady-state intermediate (Fig. 13). The model incorporates a number of simplifications and assumptions; first, that hydrolysis occurs at step 5, whereas equally this could occur at step 4, which would then be followed by a slow isomerization between different conformations of the ArsA-ADP-P complex. In any event, the hydrolysis step is faster than the subsequent product-release steps and the overall mechanism would be similar. The build up of the ArsA-MgADP-P intermediate requires the subsequent formation (at moderate rate compared with $k_f$) and decay (at a slower rate than $k_f$) of a steady-state intermediate. To minimize the number of mechanistic steps, we propose the ordered dissociation of phosphate followed by ADP, leading to the build up of the ArsA7 steady-state intermediate. Both phosphate and ADP release precede the steady-state step, which we propose to be a conformational change in the ArsA protein. The model predicts a return to baseline fluorescence as the ATP is hydrolyzed irreversible. However, ArsA can bind MgADP, and this is a multistep process. We do not observe a decay in the fluorescence to the baseline under multiple turnover conditions probably because such behavior would be masked by the increase in fluorescence due to the binding of MgADP. As a further test of the validity of the model, we attempted to fit a stopped-flow fluorescence trace directly to kinetic Scheme 4. Clearly, for a single trace the problem would be too ill-posed to identify a unique solution in which all the parameters (e.g. the rates of interconversion of the different ArsA states and their relative fluorescence values) were allowed to simultaneously vary during the nonlinear fitting procedure. Instead, the rates were held constant, and the relative fluorescence values of the different ArsA states were optimized. The fitting procedure indicated relative fluorescence values for ArsA5, ArsA-MgATP, ArsA4-MgATP, ArsA3-MgATP, ArsA2-MgATP, ArsA-MgADP-P, ArsA-MgADP, and ArsA of 1.00, 0.995, 1.052, 1.041, 1.037, 1.797, and 1.019, respectively. This analysis suggested that the maximal fluorescence enhancement observed 100 s after mixing ArsA with MgATP was attributable to the build up of ArsA-MgADP-P (e.g. 3 μM) and ArsA-MgADP (e.g. 0.08 μM). Although there is only a slight build up of ArsA-MgADP, its high fluorescence enhancement (e.g. 79.7%) is a significant contribution to the overall enhancement. Fig. 14 shows the best-fit curve (e.g. curve A) superimposed on a semi-logarithmic plot of a stopped-flow trace. There is a deviation of the best-fit curve from the measured data toward the end of the trace that is probably attributable to the fact that the decay in fluorescence is biphasic (rather than monophasic). To test this hypothesis, we introduced a further step (e.g. an isomerization of ArsA4) and allowed a free fit of the rate constants for these last two steps (e.g. steps 8 and 9), indicating respective values of $1.3 \times 10^{-2}$ ($\pm 2.3 \times 10^{-5}$) s$^{-1}$ and $4.2 \times 10^{-5}$ ($\pm 1.7 \times 10^{-5}$) s$^{-1}$. Clearly the latter step is too slow relative to the steady-state rate to be an in-line intermediate but could be an isomerization to a different catalytic form of ArsA, such as ArsA2. We conclude that kinetic Scheme 4 provides a minimal model to account for both of the kinetics of the ArsA catalyzed ATPase reaction and of the fluorescence profile that results from the ATP-induced conformational changes in ArsA.

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\[a\] The second-order association rate constant \((k_f)\) for the binding of ATP to ArsA was calculated from the measured \(k_b\) and the apparent rate of dissociation. Both of these values were determined from a hyperbolic fit of \(k_{obs}\) for the binding of ATP (Fig. 4). The apparent dissociation rate constant was determined as the minimal rate of binding obtained by extrapolation of \(k_{obs}\) to zero ATP concentration.

\[b\] Attempts to fix the fluorescence of ArsA-MgADP to be greater than ArsA-MgADP were unsuccessful, in that the fitted fluorescence profile deviated substantially from the measured fluorescence data (resulting in a large increase in the residual variance).