Conformational Changes in Four Regions of the *Escherichia coli*
ArsA ATPase Link ATP Hydrolysis to Ion Translocation*

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Structures of ArsA with ATP, AMP-PNP, or ADP-AlF₃ bound at the A₂ nucleotide binding site were determined. Binding of different nucleotides modifies the coordination sphere of Mg²⁺. In particular, the changes elicited by ADP-AlF₃ provide insights into the mechanism of ATP hydrolysis. In-line attack by water onto the γ-phosphate of ATP would be followed first by formation of a trigonal intermediate and then by breaking of the scissile bond between the β- and γ-phosphates. Motions of amino acid side chains at the A₂ nucleotide binding site during ATP binding and hydrolysis propagate at a distance, producing conformational changes in four different regions of the protein corresponding to helices H₄–H₅, helices H₉–H₁₀, helices H₁₃–H₁₅, and to the S₁–H₂–S₂ region. These elements are extensions of, respectively, the Switch I and Switch II regions, the A-loop (a small loop near the nucleotide adenine moiety), and the P-loop. Based on the observed conformational changes, it is proposed that ArsA functions as a reciprocating engine that hydrolyzes 2 mol of ATP per each cycle of ion translocation across the membrane.

Although bound to ArsB in vivo, ArsA can be expressed and purified as a soluble protein (4) whose ATPase activity is stimulated by As(III) or Sb(III) (5). ArsA is composed of two homologous domains, designated A₁ and A₂, connected by a linker of 23 amino acids; each domain contains a consensus sequence for a nucleotide binding site (NBS) (see Fig. 1A).

We have recently determined the crystal structure of the enzyme in complex with MgADP (6). The A₁ and A₂ halves of the protein are related by a pseudo-2-fold axis of symmetry. The two NBSs are located at the interface between A₁ and A₂, in close proximity of each other. Both NBSs are formed by residues from both A₁ and A₂. However, one NBS is contributed mostly by A₁ residues and is thereby named A₁ NBS; the other NBS is contributed mostly by A₂ residues and is named A₂ NBS.

Also at the interface between A₁ and A₂, but at the opposite end of the molecule with respect to the NBSs, is a site in which three distinct As(III) or Sb(III) ions bind (6). Three cysteines (Cys₁¹³⁻, Cys₁⁷², Cys₄²²), two histidines (His₁⁴⁹, His₅₅₃), and one serine (Ser₄⁹⁰) ligate the ions. Each As/Sb(III) is coordinated by one residue from A₁ and one residue from A₂. Thus, binding of each of the three metalloids tightens the interaction between A₁ and A₂, possibly triggering ATP hydrolysis.

One of the major unanswered questions in the ArsA mechanism is how the two NBSs work together to provide the energy necessary for ion transfer. It is clear that both NBSs bind nucleotides (7). Furthermore, pre-steady-state analyses of ATP hydrolysis by ArsA in the absence or presence of arsenite or antimonite show that both NBSs are catalytic, although not equivalent (8, 9). These kinetic data are consistent with binding studies using the ATP analog 5’-fluorosulfonylbenzoyladenosine, which suggest that nucleotides can be easily exchanged at the A₂ NBS but not at the A₁ NBS (10). A possible basis for these observations is found in the structure of ArsA in complex with MgADP, which shows the A₁ NBS in a “closed” conformation, while the A₂ NBS is in an “open” conformation (6).

ArsA is structurally similar to NiFH, the Fe-protein of bacterial nitrogenases (11; see also sequence alignment in Fig. 1A below). Like ArsA, NiFH has two NBSs facing each other and its iron-sulfur center is almost coincident with the As/Sb(III) cluster of ArsA. Because NiFH has long been recognized as a relative of G-proteins (12–14), it has become customary to identify specific regions of ArsA with definitions borrowed from G-protein terminology. For example, in G-proteins, as well as in ArsA, Mg²⁺ is coordinated (directly or via a water molecule) by an aspartic acid located in a strand-loop-helix structure that is referred to as the Switch I region (15). Likewise, the DTAPTH signature sequence of ArsA (6, 16, 17) has an exact counterpart in NiFH (Fig. 1A) and is believed to correspond to the Switch II region of G-proteins (14). The terms Switch I and Switch II are commonly used in reference to these regions throughout this report.

In *Escherichia coli* resistance to the metalloids arsenic and antimony is conferred by the arsen operon of plasmid R773 (1). The *arsA* and *arsR* genes of the operon encode, respectively, the catalytic subunit ArsA (ATPase) and the membrane subunit ArsB of a pump that extrudes arsenite (As(III)) and antimonite (Sb(III)) ions from the cytosol (2). Arsenic efflux in bacteria is catalyzed by either ArsB alone, functioning as a secondary transporter, or by the ArsAB complex, functioning as a transport ATPase (3). *E. coli* can utilize either mode physiologically; however, the ATP-coupled pump is more efficient, capable of producing concentration gradients as high as 10⁶, equivalent to a concentration of 1 nM intracellular arsenite (10⁻⁶M). In particular, the Switch I and Switch II regions, the A-loop (a small loop near the nucleotide adenine moiety), and the P-loop. Based on the observed conformational changes, it is proposed that ArsA functions as a reciprocating engine that hydrolyzes 2 mol of ATP per each cycle of ion translocation across the membrane.

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Also at the interface between A₁ and A₂, but at the opposite end of the molecule with respect to the NBSs, is a site in which three distinct As(III) or Sb(III) ions bind (6). Three cysteines (Cys₁¹³⁻, Cys₁⁷², Cys₄²²), two histidines (His₁⁴⁹, His₅₅₃), and one serine (Ser₄⁹⁰) ligate the ions. Each As/Sb(III) is coordinated by one residue from A₁ and one residue from A₂. Thus, binding of each of the three metalloids tightens the interaction between A₁ and A₂, possibly triggering ATP hydrolysis.

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To elucidate the structural basis of ATP hydrolysis at the NBSs of ArsA, we have determined the crystal structure of the enzyme in complex with ATP, the non-hydrolyzable ATP analog AMP-PNP, and the transition state analog of ATP hydrolysis, ADP-AIF₃. The results presented here in conjunction with previous studies of the pre-steady-state kinetics of ATP binding and hydrolysis (8, 9) suggest that the enzyme may function as a reciprocating engine.

MATERIALS AND METHODS

Crystals of ArsA in complex with MgADP were prepared by vapor diffusion in hanging drops at 30 °C (18). Drops were prepared by mixing equal amounts of a solution containing 20 mg/ml ArsA protein, 2 mM ADP, 1 mM MgCl₂, 2 mM NaAsO₂, 1 mM CdCl₂, 10 mM Bis-Tris-propane (pH 8.0) with the reservoir solution containing 4% (w/v) polyethylene glycol 3000, 100 mM Bis-Tris-Propane (pH 8.0). Crystals were harvested from the crystallization tray in a holding solution consisting of 20% glycerol (v/v) as cryoprotectant. Crystals of ArsA in complex with MgADP were initially obtained by soaking crystals of the MgADP enzyme in the presence of 5 mM ATP and 2.5 mM MgCl₂. Under these conditions the crystals undergo a space group change from P2₁2₁2₁ (a = 73 Å, b = 76 Å, c = 223 Å) to P2₂₁2₂ (a = 76.7 Å, b = 222.2 Å, c = 74.0 Å). The new unit cell is only apparently different from the original as shown by the observation that the choice of the unconventional space group P2₂₁2₂ instead of P2₁2₁2₁ would be associated with essentially unchanged unit cell dimensions (a = 74 Å, b = 77 Å, c = 222 Å) with respect to the original P2₁2₁2₁ space group. The asymmetric unit of P2₂₁2₂ contains two molecules related by a 2-fold axis of local symmetry. De novo crystallization of the enzyme, using ATP instead of ADP, also yielded crystals of space group P2₂₁2₂, which were indistinguishable from those obtained by soaking P2₁2₁2₁ crystals in the presence of MgCl₂ and ATP. Crystals of the enzyme with AMP-PNP also belong to space group P2₂₁2₂ and could be obtained by incubating crystals of the ADP complex with AMP-PNP but not by de novo crystallization in the presence of this ATP analog. Crystals of the ADP-AIF₃ enzyme were obtained using the same conditions that yield ADP crystals, except for the addition of 8 mM NaF and 2 mM AlCl₃. Crystals of the ADP-AIF₃ enzyme were obtained by incubating crystals of the ADP-AIF₃ enzyme in a manner identical to that at pH 6.2 containing MES as buffer instead of Bis-Tris-propane.

Data sets were collected at 100 K with a R axis IV image plate detector (Table I) and processed with HKL (19). The structure of ArsA in complex with MgATP was determined by molecular replacement using the enzyme from space group P2₁2₁2₁ (6) as the starting model. All the steps of the molecular replacement procedure were carried out using the CNS v. 1.0 suite of crystallographic programs (20). Self-rotation and cross-rotation function analyses clearly identified the presence of two molecules in the asymmetric unit, related by a 2-fold axis of local symmetry. Correct placement of the molecules was obtained with two consecutive translation searches. Model refinement was also carried out with CNS v. 1.0 using cross-validated maximum likelihood as the target function (21). Solvent molecules were added during the final stages of refinement after the protein model had stabilized. Because crystals of the ADP-AIF₃ enzyme are isomorphous to crystals of the MgADP enzyme, the structure of the former was obtained by simple crystallographic refinement.

Difference distance matrices were computed with CNS v. 1.0 and displayed with Mathematica (Wolfram Research). Least-square fits were carried out with LSQMAN written by Gerard Kleywegt.

RESULTS

Structure of the Enzyme in Complex with ATP—When ArsA is crystallized in the presence of ATP, or when crystals containing MgADP are soaked with ATP, the resulting crystals belong to space group P2₂₁2₂ and contain two molecules in the asymmetric unit (Table I). Several choices of a dimer are consistent with crystal symmetry: The dimer with the largest buried surface is shown in Fig. 2. It should be emphasized that, although there is some suggestion that ArsA may be a dimer in vivo (22), the crystallographic dimer shown in Fig. 2 may not reflect the actual structure of the enzyme when it is part of the ArsAB complex.

In both monomers, the A1 NBS is occupied by MgADP, while the A2 NBS is occupied by Mg-ATP. The local 2-fold axis that relates the monomers corresponds to a crystallographic 2-fold axis in the crystal of the I222 space group described previously (6), which contains ADP at both the A1 and A2 NBSs. A superposition of the A2 NBS with bound ADP (I222 crystals) and of the same site with bound ATP (P2₂₁2₂ crystals, Molecule B) is shown in Fig. 3. The most striking difference is observed in the region corresponding to residues 567–573. Throughout this report we adopt the convention that corresponding structural elements of A1 and A2 are referred to by a single denomination as helix H₆ or strand S₄ of A1 or A2, respectively (Fig. 1). Thus, for example, residues 567–573 form a small loop between strand S₈ and helix H₁₁ of A2, which wraps around the adenine moiety of the nucleotide. The equivalent loop between strand S₈ and helix H₁₁ of A1 encompasses residues 277–283 (see sequence alignment of Fig. 1A). We refer
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Each data set was collected from a single crystal at 100 K.

### Table I

Data collection and refinement statistics

|                  | ATP       | AMP-PMP   | ADP - AIF3 |
|------------------|-----------|-----------|------------|
| **Space group**  | P2₁2₁2    | P2₁2₁2    | I222       |
| **Cell dimensions (a/b/c) (Å)** | 76.0/222.7/274.1 | 76.0/222.5/274.0 | 73.9/75.9/222.6 |
| **Resolution range (Å)** | 28.6–2.4 | 37–2.6 | 25.7–2.15 |
| **Measurements** | 316,955   | 198,246   | 213,945    |
| **Unique reflections** | 46,952    | 37,265    | 34,275     |
| **(Redundancy)** | 1103      | 1094      | 540        |
| **Water molecules** | 350       | 269       | 192        |
| **Molecule B** | 40.2      | 46.0      | 42.8       |
| **Molecule A** | 0.31      | 0.27      | 0.31       |
| **αA coordinate error (Å)** | 0.006     | 0.009     | 0.010      |
| **r.m.s.d. bond lengths (Å)** | 1.6       | 1.8       | 1.6        |
| **r.m.s.d. bond angles (deg)** | 22.7      | 22.9      | 22.5       |
| **r.m.s.d. improper (deg)** | 0.97      | 1.06      | 0.94       |

* R<sub>merge</sub> = ΣΣ(h)|I(h) - <Σ(h)|I(h)|/ΣΣ|h|I(h)|, where I(h) is the i/th measurement.

* R<sub>free</sub> = ΣΣ(h)|I(h) - <Σ(h)|I(h)|/ΣΣ|h|I(h)|, where I(h) is the i/th measurement.

* Root mean square deviation.

**Fig. 2. Dimeric ArsA.** A dimer of ArsA in the asymmetric unit of P2₁2₁2 crystals (red trace) is shown together with a least-square superposition of the equivalent dimer in I222 crystals (ivory trace). Least square fitting was carried out to maximize the superposition of only one of the molecules. The two ArsA molecules in I222 crystals are derived from application of a 2-fold axis of crystallographic symmetry and are therefore identical. In contrast, the two ArsA molecules in P2₁2₁2 crystals (here labeled as Molecule A and Molecule B) are similar (root mean square deviation of 0.63 Å for 546 Ca values corresponding to the ordered regions of the protein) but not identical, which explains the space group difference.

The Switch II region contains the signature sequence G<sub>447</sub>TAP<sub>453</sub>, whose N-terminal end, Asp<sub>447</sub>, is involved in Mg<sup>2+</sup> coordination, and whose C-terminal end, His<sub>453</sub>, is one of the As/Sb(III) ligands (Fig. 1A). It has been proposed that this sequence may act as a signal transduction element connecting to this loop as the “adenine loop” or “A-loop.” The A-loop was shown to form a photo-adduct with [α<sup>32P</sup>]ATP (23). In the presence of ATP the A-loop is displaced producing a wider opening of the A2 NBS cavity (Fig. 3). The view presented in Fig. 3 clearly shows how Mg<sup>2+</sup> is located at the crossroad of the P-loop (green trace), the Switch I region (pink trace), and the Switch II region (cyan trace). When ADP is bound at the A2 NBS, Mg<sup>2+</sup> displays a distorted octahedral geometry with six ligands, two axial and four equatorial (Fig. 4A). The two axial ligands are a water molecule hydrogen bonded to a β-phosphate oxygen and the hydroxyl of Thr<sub>341</sub>. The four equatorial ligands are a Mg<sup>2+</sup> phosphate oxygen, a water molecule hydrogen bonded to an Asp<sub>447</sub> (Switch II) and to the carboxylate of Asp<sub>364</sub> (Switch I), a water molecule that is also hydrogen-bonded to the carboxylate of Asp<sub>447</sub> (Switch II) and to the hydroxyl of Ser<sub>363</sub> (Switch I), and a water molecule that is also hydrogen-bonded to the carbonyl of Asp<sub>447</sub>. By virtue of these interactions, the P-loop, the Switch I, and the Switch II elements are held tightly together around the Mg<sup>2+</sup> ion. When ATP binds at the A2 NBS, one of the γ-phosphate oxygens replaces water as an axial ligand to Mg<sup>2+</sup>, such that the metal now bridges the β- and γ-phosphates (Fig. 4, B and C). However, there are differences between the two ArsA monomers in the equatorial coordination of Mg<sup>2+</sup>. In one monomer (Molecule B) one of the water molecules that coordinates Mg<sup>2+</sup> is hydrogen-bonded to another more distant solvent, which in turn is hydrogen-bonded to Ser<sub>363</sub> and Asp<sub>447</sub> (Fig. 4B). In the other monomer (Molecule A) the equatorial coordination of Mg<sup>2+</sup> resembles more closely that observed in the presence of ADP, with a water ligand forming a direct hydrogen bond with Ser<sub>363</sub> and Asp<sub>447</sub> (Fig. 4C). In molecule B, Mg<sup>2+</sup> displays a perfect octahedral coordination (Fig. 4B), while in molecule A the coordination geometry is slightly distorted (Fig. 4C). In these various configurations of the A2 NBS with ADP or ATP bound, Mg<sup>2+</sup> appears to move back and forth among the nucleotide, the Switch I, and the Switch II elements. This motion is reflected by changes in the distance between Mg<sup>2+</sup> and nearby residues of the P-loop, the Switch I, and the Switch II region (Table II).
more similar to that of the ADP complex than to that of the ATP complex. This is also evident in the fact that crystals of both the ADP and ADP-AIF₃ complex belong to the same space group I222 (Table I). However, as was the case for ATP and AMP-PNP, ADP-AIF₃ binds only at the A2 NBS. At this site the aluminate is clearly recognizable as a planar trigonal molecule occupying a position equivalent to that of the γ-phosphate of ATP (Fig. 4D). Consequently, the hydroxyl moiety of the β-phosphate of ADP corresponding to the bridging oxygen between the β- and γ-phosphate of ATP is only 2.3 Å from aluminum. Mg²⁺ is 1.9 Å from one of the fluorine atoms and bridges the latter to one of the β-phosphate hydroxyls. On the other side of the aluminum atom, a water molecule occupies a position almost symmetrical with respect to the β-phosphate hydroxyl (Fig. 4D). This water molecule is stabilized by a hydrogen bond to another solvent molecule, which in turn is hydrogen-bonded to the hydroxyl of Ser₁⁶₃ (Switch I). There is no water near the γ-phosphate in the structures of the enzyme with ATP bound at the A2 NBS.

As previously mentioned, the structure of the enzyme in complex with ADP-AIF₃ at the A2 NBS is very similar to that of the ADP complex. In particular, the position of the A-loop (residues 567–573) is similar to that observed in the ADP complex, and the loop between helices H9 and H10, which is disordered in the ADP enzyme but visible when ATP binds at the A2 NBS, is also disordered in the ADP-AIF₃ complex.

It is worth emphasizing that the ADP-AIF₄ complex described above is of the ADP-AIF₃ form rather than of the ADP-AIF₄ form observed in similar experiments with other enzymes (24–26). Because the coordination number of the AIF₄ in transition state analogs of phosphoryl transfer is pH-dependent and the probability of the aluminate to be in the AIF₄ form increases with decreasing pH (27), we have sought to determine whether lowering the pH of the mother liquor of ArsA crystals would affect the aluminum coordination. In fact, when crystals of the ADP-AIF₄ complex are incubated at pH 6.2, four fluoride ions are present as square-plane ligands of the aluminum ion (not shown), suggesting that the aluminum coordination number has changed from 5 to 6 (including water and the β-phosphate hydroxyl as axial ligands). It is widely accepted that hydrolysis of ATP proceeds by attack of a water molecule on the γ-phosphorus (28–30) with formation of a trigonal bipyramidal transition state (31, 32). Thus, the ADP-AIF₄ complex (trigonal coordination) observed at pH 8.0 represents a better structural analog of the transition state of ATP hydrolysis than the ADP-AIF₄ complex (square-planar coordination) observed at pH 6.2.

Global Conformational Changes Associated with the Binding of Different Nucleotides at the A2 NBS—A quantitative analysis of the differences between structures can be achieved through the use of difference distance matrices (33). In a distance matrix, the differences between the coordinates of each Ca of a protein and every other Ca of the same protein are utilized to build a density plot in which residues that are close in space are represented as a point of high density. In a difference distance matrix the distance matrix of a first structure is subtracted from the distance matrix of a second structure. If the distance $A_n - A_m$ between two residues $A_n$ and $A_m$ of the A structure changes in the B structure, then the distance $B_n - B_m$ between the same two residues in the B structure will be either $>$ $A_n - A_m$ or $<$ $A_n - A_m$. In the first case a positive value will be associated with these two residues in the difference distance matrix between B and A; in the second case a negative value will be obtained. Thus, to evaluate whether changes have occurred between two structures, it is informative to look at both positive and negative values of the differ-

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![Image](image-url)

**Fig. 3. The A2 NBS.** Superposition of the A2 NBS with ADP bound (space group I222) and with ATP bound (molecule B in space group P₂₁₂₁₂). The Ca trace of ArsA with ADP bound is shown with solid ivory bonds, whereas its side chains, ADP and Mg²⁺, are transparent. The Ca trace of ArsA with ATP bound is shown with different colors representing the P-loop (green), the Switch 1 region (pink), the Switch II region (cyan), and the A-loop (red); side chains are shown with yellow bonds, Mg²⁺ as CPK in magenta, and ATP as bonds colored according to atom types: oxygen red; nitrogen, blue; carbon, ivory; phosphorus, orange.
A paral analog alternating at the A2 NBS. A, ADP complex; B, ATP complex (Molecule B); C, ATP complex (Molecule A); D, ADP-AlF3 complex. In all four panels the Ca trace of ArsA is shown with different colors representing the P-loop (green), the Switch I region (pink), and the Switch II region (cyan). Mg2 and solvents are shown as CPK in magenta and light blue, respectively; side chains appear with yellow bonds; ATP, ADP, and AlF3 are shown as bonds colored according to atom types: oxygen, red; nitrogen, blue; carbon, yellow; phosphorus, orange; aluminum, light gray; fluorine, aquamarine. Mg2 and AlF3 coordination is shown as transparent rods. Hydrogen bonds are shown as dashed lines.

TABLE II

| ATP        | ADP·AlF3 | ADP  |
|------------|----------|------|
| Mg2-Thr741 | 2.14     | 2.19 | 2.34 |
| Mg2-Ser663 | 4.99     | 6.10 | 4.96 | 4.74 |
| Mg2-Asp364 | 5.24     | 4.35 | 4.58 | 4.63 |
| Mg2-Asp447 | 4.46     | 4.75 | 4.81 | 4.77 |
| Mg2-Asp447 | 3.65     | 3.96 | 4.02 | 3.95 |

TABLE II: Distances (Å) between Mg2 and residues of the P-loop, Switch I, and Switch II regions at the A2 NBS. Distances for the ADP structure were derived from Ref. 6.

The findings reported in this report bear relevance to two fundamental aspects of the ArsA mechanism: how ATP is hydrolyzed, and how As/Sb(III) ions are translocated across the membrane. When crystals of ArsA are either formed or incubated in the presence of ATP, this nucleotide is found at the A2 NBS, while ADP is at the A1 NBS. One possible explanation for this observation is that the A1 NBS is catalytic and the A2 NBS is not. However, if ATP is rapidly hydrolyzed at the A1 NBS, such that only ADP is observed at this site, then, when crystals of the enzyme are incubated with the non-hydrolyzable analog AMP-PNP, this compound should be found at the A1 NBS. Instead, AMP-PNP is found only at the A2 NBS. Moreover, if de novo crystallization is attempted in the presence of AMP-PNP, no crystals are obtained, suggesting that crystals can be formed only if the A1 NBS contains ADP. This might occur because conformational changes associated with ATP binding and hydrolysis at the A1 NBS are not allowed in the crystal. Constraints imposed by the crystal lattice on catalysis are also likely to be the reason why ATP and not ADP is found at the A2 NBS, despite overwhelming evidence from pre-steady-state kinetic data showing that the A2 site is also hydrolytic (8, 9). With regard to this point, it may be worth noting that, when a number of intact crystals are incubated in a holding solution containing a regenerating assay mixture to monitor ATP hydrolysis, only minimal enzymatic activity is detected.
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Fig. 5. Difference distance matrices between structures of ArsA in complex with various nucleotides. For each pairwise comparison positive and negative values of the matrix are shown in the left and right panels, respectively. In each panel conformational changes that involve equivalent structural elements in A1 and A2 are marked with symbols of the same shape and color: pink rhombus, helices H4–H5; cyan circle, helices H9–H10; red triangle, helices H13–H15 and A-loop; yellow square, a loop and a strand located at the end of the Switch I region of A1; green star, region S1–H2–S2 encompassing the P-loop of A2. Completely clear areas of the density plots correspond to disordered regions of the structures. Visible pixels correspond to positional shifts between 1.0 and 3.5 Å. Residue numbers are labeled on both the X and Y axes of each panel.

shown), which is likely to originate from the small amount of free protein in equilibrium with crystalline ArsA. However, when ArsA crystals are dissolved, full enzymatic activity is restored. Thus, it can be argued that ArsA molecules are catalytically competent inside the crystals, but they cannot turn over because the conformational changes that occur in association with ATP hydrolysis are denied within the rigid frame of the crystal lattice.

Instead of being a drawback, this situation can be an advantage; it allows visualization of the initial changes that occur in the active site during the first turnover. In particular, the structures presented here draw attention to the role played in catalysis by residues from both the Switch I and Switch II region of the enzyme and by water molecules in the coordination sphere of Mg$^{2+}$ (Fig. 4). When ATP binds at the A2 NBS, in one molecule of the crystallographic dimer Asp$_{447}$ (Switch II) and Ser$_{365}$ (Switch I) form direct hydrogen bonds with a water ligand of Mg$^{2+}$, while in the other molecule an additional solvent molecule is intercalated between these residues and the ion. Thus, at least two different conformations of the A2 hydrolytic site are possible in the presence of ATP. When ADP and trifluoroaluminate bind together at the A2 NBS, they mimic the transition state of the hydrolysis reaction (Fig. 4D). This conclusion, which is in line with similar observations made with other enzymes that hydrolyze triphosphonucleotides (24–26, 34–43), is supported by the observation that a water molecule, which in this complex is located in proximity of the aluminum atom, mimics an in-line nucleophilic attack onto the γ-phosphate of ATP. Altogether, the four panels of Fig. 4 can be viewed (going clockwise from B to A through C and D) as consecutive movie frames displaying the scene of ATP hydrolysis. In this movie, the walls of the active site (P-loop, Switch I, and Switch II regions) are pulsing around the Mg$^{2+}$ ion, producing subtle distortions of its coordination geometry that may be essential for the breaking of the scissile bond.

The vibrations involving residues around the nucleotide and Mg$^{2+}$ appear to propagate to distant parts of the protein and are likely to provide the physical basis for the events that propel As/Sb(III) into ArsB. A comparison of the structure of the enzyme with ATP bound at the A2 NBS versus the structures in which this site is filled with ADP or ADP-AlF$_3$ (Fig. 5) identified conformational changes involving primarily helices H4–H5, H9–H10, H13–H15, a loop located at the end of the Switch I region, and a region encompassing strands S1, helix H2, and strand S2. Surprisingly, the largest positional shifts take place in A1 (for example, see right panel of row 4 in Fig. 5) despite the fact that nucleotides are being exchanged only at the A2 NBS. With regard to this point, it may be of relevance that the cavity of the A2 NBS is completed by residues at the end of strand S6 and the beginning of helix H12 of A1 (e.g. Gln$_{208}$ of A1 is hydrogen-bonded to the 3′-hydroxyl of the nucleotide ribose at the A2 NBS), two structural elements that follow immediately helices H4–H5 and H9–H10 of A1 (Fig. 6). Also in contact with the A-loop of the A2 NBS are helices H14–H15 of A1 (not shown). Conformational changes in these helices may carry information on the nucleotide type and occupancy from the A2 NBS to the A1 NBS via the A-loop of the latter (see below). Clearly, there is extensive cross-talk between A1 and A2, which is likely to be essential for catalysis.

Interestingly, helices H4–H5 are also connected to the Switch I region; helices H9–H10 are connected to the Switch II region; helices H13–H15 are connected to the A-loop; and the region S1–H2–S2 contains the P-loop. Thus, the structure of each half of ArsA can be dissected in four different components, each of which acts as a transducer of events occurring at the NBS (Fig. 7); all the ligands of As/Sb(III) are connected to residues of the hydrolytic site via either the Switch I pathway (Cys$_{113}$ is connected to Asp$_{45}$ in A1; Ser$_{420}$ and Cys$_{422}$ are connected to Asp$_{564}$ in A2) or the Switch II pathway (His$_{448}$ and Cys$_{172}$ are connected to Asp$_{142}$ in A1; His$_{453}$ is connected to Asp$_{447}$ in A2). In our study the largest conformational changes were observed between molecule A and molecule B of ArsA in complex with ATP (Fig. 5, row 4), and between either of these molecules and the enzyme in complex with ADP-AlF$_3$ (Fig. 5,
In contrast, no significant differences were observed between the structure with ADP·H18528·AlF₃ and that with ADP, suggesting that a large conformational change is associated with ATP hydrolysis at the A2 NBS but not with phosphate release. Finally, we have not been able to obtain a crystal structure of the enzyme with the A2 NBS unoccupied, even after prolonged incubation of the crystals with periodic changes of a holding solution devoid of any nucleotides. This fact suggests that release of ADP from the A2 NBS is also associated with a conformational change not allowed by the crystal lattice.

These observations are of considerable importance for understanding the mechanism of energy coupling to ion translocation. Although ArsB alone can form a channel for the diffusion of As(III) or Sb(III) ions across the membrane (3), by itself ArsA
cannot translocate ions. However, ArsA provides the ArsAB pump with the capacity to hydrolyze ATP to drive active transport of As/Sb(III) against a chemical gradient (44). One way this can occur is if the metalloid is first bound on the cytosolic side of ArsA and then transferred to a pocket at the interface with ArsB, from which it can travel along the channel. With regard to this point, pre-steady-state kinetics indicate that release of ADP from the A2 NBS is associated with release of As(III) or Sb(III) from the metal binding site, while binding of ATP favors the uptake of these ions (9). Based on these observations, the motions of helices H4–H5 and H9–H10 of both A1 and A2 concurrent with ATP hydrolysis at the A2 NBS (see above) might herald the formation of a "tense" state of ArsA in which bound ion moves from the cytosolic side of the enzyme into the protected pocket at the interface with ArsB. Release of ADP from the A2 NBS would trigger the release of the ion inside this pocket. ATP hydrolysis at the A1 NBS might then be required to bring ArsA back to the ground state. On this basis, the catalytic cycle of ArsA would be similar to that of a reciprocating engine (Fig. 8). Helices H9–H10 are expected to play a central role in this mechanism. The H9–H10 region of A1 fills the space between helices H4–H5 from both A1 and A2 and provides the ceiling of the cavity where As/Sb(III) ions bind (Fig. 6; see also Ref. 6). The H9–H10 region of A2 is disordered in the structures of ArsA reported to date, but it is reasonable to believe that it might assume the position of the equivalent region of A1 at some point of the catalytic cycle. Thus, helices H9–H10 of A1 and A2 could alternate at the interface with ArsB forming a gate for As/Sb(III) ions (Fig. 8).

An experimental determination of the number of ATP molecules hydrolyzed by ArsA per cycle of ion translocation has been hampered by difficulties in reconstituting a functional ArsAB pump. However, a number consistent with the model presented in Fig. 8 can be derived from evolutionary considerations about ArsA. As previously mentioned, ArsA displays significant structural similarity to NifH, the Fe-protein of nitrogenase, an enzyme that couples nucleotide hydrolysis to electron transfer (Ref. 11; see also Fig. 1A). The stoichiometry of ATP hydrolyzed per redox cycle by NifH is 2. If a reciprocating mechanism is the basis for ArsA function (Fig. 8), then in this enzyme 2 ATP molecules would also be hydrolyzed in each catalytic cycle. However, it is important to notice that the stoichiometry of ions translocated per ATP hydrolyzed may not be constant. The critical role played by the protein in this process is to expose one or more high affinity sites for As/Sb(III) to the cytosol (the "in" conformation) and to convert them to low affinity when they face the membrane channel (the "out" conformation). In particular, the differences in the affinities for these ions between the in and the out conformations are likely to be an invariant property of the enzyme, such that the stoichiometry of ion translocation would depend primarily on the relative ion concentrations in the two compartments and on the $K_d$ values for these ions in the in and out state. ArsA has a maximum capacity for three As/Sb(III) ions (6), and kinetic studies suggest the three ions bind with different affinities (9). Thus, the presence of three distinct metal sites might represent a special advantage for the enzyme allowing operation under different ranges of ion concentration or with variable stoichiometry.

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Conformational Changes in Four Regions of the *Escherichia coli* ArsA ATPase Link ATP Hydrolysis to Ion Translocation

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