Nucleotide Binding to the C-terminal Nucleotide Binding Domain of ArsA

STUDIES WITH AN ATP ANALOGUE, 5′-p-FLUOROSULFONYLBENZOYLADENOSINE (FSBA)*

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ArsA protein, the catalytic component of the plasmid-encoded anion-translocating ATPase in Escherichia coli, contains two consensus nucleotide binding domains, A1 and A2, that are connected by a flexible linker. ATP has previously been shown to cross-link to the A1 domain upon activation with UV light but not to the A2 domain. The ATP analogue, 5′-p-fluorosulfonylbenzoyladenosine (FSBA) was used to probe the nucleotide binding domains of ArsA. The covalently labeled protein was subjected to partial trypsin proteolysis, followed by Western blot analysis of the fragments with the anti-FSBA serum. The N-terminal amino acid sequence of the labeled fragment showed that FSBA binds preferentially to the C-terminal domain A2 both in the absence and the presence of antimonite. Occupancy of the two nucleotide binding sites was determined by protection from trypsin proteolysis. Trypsin cleaved the ArsA protein at Arg290 in the linker to generate a 32-kDa N-terminal and a 27-kDa C-terminal fragment. The 32-kDa fragment is compact and largely inaccessible to trypsin; however, the 27-kDa was cleaved further. Incubation with FSBA, which binds to the C-terminal domain, resulted in significant protection of the 27-kDa fragment. This fragment was not protected upon incubation with ATP alone, indicating that A2 might be unoccupied. However, upon incubation with ATP and antimonite, almost complete protection from trypsin was seen. ATP and FSBA together mimicked the effect of ATP and antimonite, implying that this fully protected conformation might be the result of both sites occupied with the nucleotide. It is proposed that the A1 site in ArsA is a high affinity ATP site, whereas the allostERIC ligand antimonite is required to allow ATP binding to A2, resulting in catalytic cooperativity. Thus antimonite binding may act as a switch in regulating ATP binding to A2 and hence the ATPase activity of ArsA.

ArsA protein is the catalytic component of the plasmid-encoded anion-translocating ATPase in Escherichia coli. In conjunction with the integral membrane protein ArsB, ArsA brings about ATP-dependent efflux of oxyanions, such as antimonite (Sb(III)) and arsenite (As(III)) (1). The purified ArsA protein shows oxyanion-stimulated ATPase activity (2). It consists of two homologous halves, the N-terminal domain A1 (residues 1–282) and the C-terminal domain A2 (residues 321–583), connected by a flexible linker (residues 283–320; 1). Each half contains one nucleotide binding site with significant homology to the consensus P-loop sequence found in most nucleotide binding proteins (3). It is of interest to determine the role of multiple nucleotide binding domains in ArsA and in other ATPases involved in transport. Site-directed mutagenesis studies with ArsA indicate that both of the sites are required for function of the protein (4, 5). In vivo complementation studies carried out with mutants in the A1 or A2 domain (6) as well as the suppressor analysis (7) suggested that an interaction between the A1 and A2 domains might occur in trans between subunits in a homodimer of ArsA. It has been proposed earlier that it is binding of the oxyanion that brings about dimerization of the protein (8). Upon UV cross-linking in the presence of [α-32P]ATP, only the A1 domain was shown to form an ATP adduct. The site of the ATP adduct lies between residues 283 and 320 of the protein (9), which forms a flexible linker region between the N- and C-terminal domains. ATP cross-linking studies carried out with the truncated peptides N28, N35, C35, and the in vitro reconstituted active complex of N28 and C35 also suggested that a molecule of ATP binds to the A1 nucleotide binding site; the phosphate group of this molecule interacts with the P-loop (residues 15–23), whereas the adenine ring of this same molecule binds to a region about 250 residues away, indicating that the A1 nucleotide binding pocket consists of residues far apart in the primary sequence of the protein (9). These experiments, however, provided no evidence for ATP binding to the C-terminal nucleotide binding domain of ArsA or its role in catalysis.

To understand the role of the C-terminal domain, we investigated binding of an ATP analogue, FSBA1 to the ArsA protein. ATP analogues have the potential of providing significant insights into the workings of an ATPase, since they allow us to look at certain conformations not otherwise accessible. FSBA, which can be considered an analogue of both ATP and ADP, contains an unmodified adenosine group; however, the phosphoryl groups in FSBA are replaced by an alkylating fluorosulfonylbenzoyl group (10). It has been used to analyze nucleotide binding sites in enzymes such as pyruvate kinase (11) and the F1-ATPase (12).

FSBA has previously been shown to inhibit the ATPase activity of the ArsA protein in a specific manner (8). However, binding site(s) for FSBA or its mechanism of inhibition have not been characterized. This study provides evidence for preferential binding of FSBA to the A2 site in ArsA. The results

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suggest a role for the allosteric activator arsenite or antimonite in allowing ATP binding to A2, although FSBA, having a higher affinity for ArsA than ATP in the absence of the oxyanion, can access the A2 site in its absence. Based on the results of this study, it is proposed that the two nucleotide binding sites in ArsA have different conformations and affinity for the nucleotide. ATP binding to the A2 site occurs only in the presence of the oxyanion, and this binding acts as a switch for the catalytic mode of ArsA.

**EXPERIMENTAL PROCEDURES**

**Purification of the ArsA Protein**—The arsA gene was subcloned into the pET16b vector (Novagen Inc., Madison, WI) to create a fusion of the His6 tag at the N terminus of ArsA. The pET16b vector (Novagen Inc., Madison, WI) was ligated to pET16b vector similarly digested with these two enzymes. Cells containing the plasmid pET16b-arsA were grown to mid-log phase and induced with 1 mM isopropyl-1-thio-

β-D-galactopyranoside. The cell lysate was prepared by a single passage through the French pressure cell at 20,000 p.s.i. The ArsA protein was purified by passing the cell lysate through a nickel-nitrilotriacetic acid-agarose (Qiagen Inc., Chatsworth, CA) column according to the manufacturer’s instructions.

**FSBA Labeling of the ArsA Protein**—Purified ArsA protein (0.4 mg/ml) was incubated with different concentrations of FSBA at 37 °C for 30 min in the presence or the absence of 0.5 mM antimonite. The reaction was carried out in a 100-μL reaction volume containing 50 mM MOPS-KOH, pH 7.5. ArsA protein containing dimethyl sulfoxide (final concentration 5%, v/v) was used as a control. Where indicated, the samples were preincubated with the substrates at 37 °C for 20 min. The samples were analyzed by SDS-PAGE on 10% polyacrylamide gels, followed by Western blotting with anti-FSBA serum (13). The Immunelite kit from Coomassie Blue staining. FSBA-labeled fragments were detected by Western blotting using anti-FSBA serum (13). The Immunelite kit from Coomassie Blue staining. FSBA-labeled fragments were detected by Western blotting using anti-FSBA serum (13). The Immunelite kit from Coomassie Blue staining. FSBA-labeled fragments were detected by Western blotting using anti-FSBA serum (13). The Immunelite kit from Coomassie Blue staining. FSBA-labeled fragments were detected by Western blotting using anti-FSBA serum (13). The Immunelite kit from Coomassie Blue staining. FSBA-labeled fragments were detected by Western blotting using anti-FSBA serum (13).

**CNBr-Cleavage**—120 μg of total ArsA protein was labeled with 1 mM FSBA in the presence or the absence of antimonite and subjected to partial trypsin proteolysis as described above. After electrophoresis on an SDS 10% polyacrylamide gel to separate the fragments, gel slices containing the desired fragments were excised, and the CNBr cleavage was carried out in gel slices as described earlier (9). The reaction was carried out in a 2% (w/v) solution of cyanogen bromide for 30 or 60 min at room temperature. To separate the peptides, the gel slices were applied onto a Tricine-SDS gel (16) containing 12% polyacrylamide. The cleaved fragments were visualized by Coomassie Blue staining of the gel. FSBA-labeled fragments were detected by using anti-FSBA serum.

**Amino Acid Sequencing**—Trypsin- or CNBr-digested fragments were transferred onto a polyvinylidene difluoride membrane at 50 V for 2 h. The fragments were visualized by staining the membrane briefly with 0.1% Coomassie Blue in methanol. The bands were excised and subjected to N-terminal sequencing using a Beckman model LF3000 solid phase amino acid sequencer in the core facility of the Department of Biology at the Georgia State University.

**RESULTS**

**FSBA Binding to the ArsA Protein**—FSBA has previously been shown to inhibit the ATPase activity of the ArsA protein (8). Inhibition by FSBA was increased in the presence of the oxyanion antimonite. A complete protection from inhibition was seen upon preincubation with ATP and antimonite, indicating that FSBA binding is specific for the nucleotide binding site(s) of ArsA (8). In this study, we characterized covalent binding of FSBA to ArsA by using polyclonal antibodies to FSBA raised in rabbits (13). Wild type ArsA protein was incubated with 0.5 mM or 1 mM FSBA in the presence or the absence of antimonite, and the Western blots were probed with the anti-FSBA antibody. Data in Fig. 1 show cross-reactivity of FSBA-treated ArsA with the FSBA antibody (lanes 1–8). This FSBA-reactive band is absent in control ArsA sample not treated with FSBA (lane 9). A band of faster mobility that cross-reacted nonspecifically with the antibody was seen in all the lanes, including the control (lanes 1–9). FSBA binding to ArsA was seen both in the presence (lanes 2 and 6) or the absence of antimonite (lanes 1 and 5). Preincubation with ATP resulted in a decrease in labeling with FSBA (lanes 3 and 7). Preincubation with ATP and antimonite further decreased incorporation of FSBA into ArsA (lanes 4 and 8).

**Identification of the FSBA Binding Site(s) in ArsA**—To determine whether FSBA inhibition results from binding to the N- or the C-terminal nucleotide binding site in ArsA, FSBA binding to the truncated N- or C-terminal peptides was investigated. Data in Fig. 2A show that FSBA binds predominantly to the C-terminal peptide C35 (lanes 4–6). This binding was diminished upon preincubation with ATP and antimonite (lanes 7 and 8).
The N-terminal peptide N28 and N35 showed very faint signals with the anti-FSBA antibody (lanes 1–3 and 7–9). Upon in vitro reconstitution of the N28 and C35 peptides to yield an active complex (15), FSBA binding was still predominantly seen on the C35 peptide (data not shown). A C-terminal clone, C31, that starts at amino acid residue 320 of ArsA and lacks the linker region was also tested for FSBA binding. Data in Fig. 2B (lanes 2 and 3) show that C31 was able to react with FSBA as C35 peptide (lane 1), indicating that the linker region is not involved in FSBA binding to the C-terminal domain. The linker region has previously been identified to be the site where the adenine ring of the ATP molecule bound to the A1 domain of ArsA forms an UV-activated adduct (9).

To identify the FSBA binding site in the wild type ArsA protein, purified protein was treated with different concentrations of FSBA in the presence or the absence of antimonite, followed by partial proteolysis with trypsin. The analysis of the peptides upon SDS-PAGE, followed by Coomassie Blue staining, showed three major fragments: roughly 54, 32, and 27 kDa (Fig. 3A). Western blot analysis of a similar gel using anti-FSBA antibodies showed that the major species that cross-reacted with the FSBA antibody was the 27-kDa fragment (Fig. 3B). The labeling was found to be dose-dependent so that it increased with increasing concentrations of FSBA. The presence of antimonite in the reaction was seen to enhance FSBA binding to the 27-kDa fragment slightly (lanes 4, 6, 8, and 10). The 32-kDa fragment showed very weak labeling although the amount of the 32-kDa fragment was significantly more than the 27-kDa fragment as seen from the Coomassie Blue-stained gel (Fig. 3A).

The origin of the 32- and 27-kDa fragments was determined by a combination of techniques. Antibodies to the N18 (residues 1–166; Ref. 9), C31 (residues 320–583) or the C46 (residues 167–583; Ref. 9) peptide of ArsA were used to determine if these fragments originated from the N or the C terminus of the protein. The data in Fig. 3C show that the 32-kDa fragment reacted with the anti-N18 (lane 3) and anti-C46 (lane 9) but not...
with the anti-C31 antibodies (lane 6), whereas the 27-kDa fragment reacted with the anti-C31 (lane 6) and the anti-C46 antibody (lane 9) but not the N18 antibody (lane 3). These data indicate that the 32-kDa fragment belongs to the N terminus of the protein and that it does not extend beyond the linker region, whereas the 27-kDa fragment starts after amino acid residue 166.

To determine the exact start point for these fragments, the fragments were transferred to polyvinylidene difluoride as described under “Experimental Procedures,” and N-terminal amino acid sequencing was undertaken. The N-terminal analysis of the 27-kDa trypsin fragment gave the sequence 291XXSTQPVAXP (X represents the residue that could not be definitively deciphered) indicating that the start point of the 27-kDa fragment lies within the linker region (residue 283–320) that connects the A1 and A2 halves of ArsA. Analysis of the N terminus of the 32-kDa trypsin fragment indicated that it started at the N terminus of the ArsA protein. It gave the sequence 1MQFLQNIPPY. CNBr cleavage of this fragment was carried out (Fig. 4A, lane 3). The N-terminal amino acid sequence of an internal 12-kDa fragment (marked with an arrow) yielded the sequence 157AGLEKQREQY. This CNBr fragment would extend up to residue Met282 (for a linear map of ArsA, see Fig. 6B). Hence, the 32-kDa trypsin fragment is probably the result of a cleavage event at the C terminus of Arg290 that also produces the C-terminal 27-kDa fragment. Since FSBA bound very weakly to the 32-kDa fragment, as seen from the reaction with anti-FSBA antibody, further analysis of this fragment was not carried out.

To narrow down the FSBA binding site, the 27-kDa fragment was cleaved further with CNBr (Fig. 4A, lane 5). Immunoblotting of a similar gel with the anti-FSBA antibody showed four bands that cross-reacted with the antibody (Fig. 4B, lane 1). The top band in both lanes 1 and 2 in Fig. 4B is the undigested 27-kDa peptide. Of the four FSBA-cross-reactive bands in lane 1, the lower three bands are about 1 kDa apart in size. All of the FSBA cross-reactive bands disappeared upon extension of the reaction time with CNBr from 30 to 60 min (Fig. 4B, lane 2). The C-terminal domain of ArsA contains 10 methionines, eight of which occur between residues 331 and 482. The fact that the FSBA-reactive bands disappeared upon longer incubation with CNBr might indicate that the FSBA binding site lies in this region rich in methionines. The smallest two bands marked with an asterisk were subjected to N-terminal amino acid sequencing. Both of these fragments were found to start at amino acid residue 355 with the amino acid sequence 355GFDVHLT. Since these fragments are between 14 and 15 kDa in size, the data suggest that the FSBA binding site in the A2 domain lies between residues 355 and residue 482. Greater resolution of the A2 binding site using the anti-FSBA antibody has not been possible.

Protection of ArsA from Trypsin Proteolysis in the Presence of ATP, FSBA, and Antimonite—To determine occupancy of the two nucleotide binding domains in ArsA, protection from proteolysis by trypsin in the presence of ATP and/or FSBA was investigated. Conditions of trypsin proteolysis defined in an earlier experiment (Fig. 3) were employed to examine the effect of occupancy of the A1 or A2 domain on protection. To decipher the role of the allosteric ligand on nucleotide binding, protection experiments were carried out in the presence or the absence of antimonite. FSBA was included in the protection experiments, since the binding site for FSBA had already been identified (Figs. 3 and 4), and it was expected that the FSBA bound form of ArsA might allow us to look at a conformation or a step in the sequence of events that it would not be otherwise possible to study in isolation. The experiment consisted of an incubation of ArsA with ATP and/or FSBA in the presence or the absence of antimonite, followed by trypsin treatment. The following combinations of ATP, FSBA, and antimonite were tested: ATP (A); FSBA (F); ATP plus antimonite (AS); FSBA plus antimonite (FS); ATP followed by FSBA (AF); ATP plus antimonite followed by FSBA (ASF); FSBA followed by ATP (FA); and FSBA plus antimonite followed by ATP (FSA). The abbreviations in parentheses are used here to discuss the effect of different conditions. The protection of the protein from proteolysis was determined by analysis of the fragments on SDS-PAGE. The data are shown in Fig. 5. The major fragments in both F and FS samples upon the addition of trypsin were 54, 32, and 27 kDa (lanes 2 and 4) as seen in Fig. 3A. The 32-kDa fragment comes from the N terminus of ArsA, and the 27-kDa fragment originates from the C terminus starting at residue 291 as shown above by N-terminal amino acid sequencing. Also, the 27-kDa fragment was shown above to contain the FSBA binding site (Fig. 3B). Interestingly, the 27-kDa fragment originating from the C terminus is seen only in F- or FS-treated samples. In the ATP-treated samples, only the N-terminal 32-kDa fragment is seen (Fig. 5, lane 16). Since the 32-kDa fragment is also seen in control samples (Fig. 5, lane 18), it appears that the N-terminal domain is very compact and...
is largely inaccessible to trypsin. Hence, under the proteolysis conditions used in this experiment, the most accessible trypsin cleavage site is in the linker region at Arg290, and it cleaves the protein into N and C domains. If the A2 site in the C terminus is occupied as in F or FS-treated samples, the C-terminal 27-kDa fragment is protected (Fig. 5, lanes 2 and 4). If the C-terminal site is unoccupied as in control and ATP-treated samples, the 27-kDa fragment is completely digested (Fig. 5, lanes 16 and 18). Interestingly, complete protection from trypsin was seen in FA, FSA, AF, or AS samples such that even the first cleavage at Arg290 did not occur (Fig. 5, lanes 6, 8, 10, 12, and 14). A comparison of the A and AS samples versus F and FS is also very revealing. The proteolysis pattern of the A and AS samples was significantly different. The A-treated ArsA was cleaved into the 32- and 27-kDa fragments, and the 27-kDa fragment was further digested completely, but the AS sample was completely protected. By comparison, no difference between the F and FS samples was observed. Both generate 32- and 27-kDa fragments, and the 27 kDa-fragment is protected.

**DISCUSSION**

In the present study, we characterized binding of an ATP analogue, FSBA, to the ArsA protein. FSBA has previously been shown to inhibit the ATPase activity of ArsA with a stoichiometry of 1 mol of FSBA/mol of ArsA in the absence of antimonite and 0.5 mol of FSBA/mol of ArsA in the presence of antimonite (8). These studies led to the hypothesis that each monomer of ArsA has a single binding site for FSBA. In the present study, we have shown that only the A2 domain in ArsA is covalently modified with FSBA (Fig. 3), implying that the two nucleotide binding sites in ArsA have distinct conformations. It is likely that both the sites in ArsA bind FSBA; however, binding to the A1 domain may not lead to covalent incorporation as has also been shown for FSBA binding to pyruvate kinase (10). The addition of FSBA to pyruvate kinase initially results in partial inactivation due to modification of a cysteine residue; however, this reaction does not lead to covalent incorporation. Complete inactivation of pyruvate kinase then results from modification of a tyrosine residue at a different location (18).

The FSBA binding site in the A2 domain of ArsA is different from the previously characterized A1 site that forms an adduct with ATP in the linker region on UV cross-linking (9). FSBA binding to the A2 site was seen both in the presence or the absence of antimonite. Earlier studies have shown that antimonite enhances the inhibition of ArsA by FSBA (8). Perhaps the role of antimonite is only to cause tighter binding of FSBA.
to the A2 domain in a dimer. FSBA binding to the truncated peptide C31 (A2) or C35 (linker +A2) suggests that these peptides acquire at least a partially native conformation like the N-terminal peptide N35 that also forms an independent domain capable of forming an UV-activated adduct with [α-32P]ATP (6, 9).

Although FSBA binding to A2 was seen in the absence of antimone, the data presented in this paper suggest that binding of ATP to the A2 site occurs predominantly in the presence of antimone. Trypsin protection experiments provided an invaluable tool to probe the occupancy of the two nucleotide binding sites in ArsA. A model based on the available data is shown in Fig. 6B. The ArsA protein consists of two homologous halves, A1 and A2, which are connected by a flexible linker. The catalytic activity of ArsA is stimulated by the oxyanion antimone or arsenite. Hence, ArsA is an allosteric protein that contains binding site(s) for the oxyanion in addition to the two nucleotide binding sites. In this model, conformations I–IV of ArsA show the temporal sequence of events leading to catalysis. Conformation IV is the active conformation and can be achieved via conformation II or III as a result of antimone binding. These conformations are based on their trypsin accessibility and on the trypsin accessibility of additional conformations (F, FS, FA, AF) seen in the presence of FSBA. I, II, and III are trypsin-sensitive conformations, and this is indicated by the availability of residue Arg290 in the linker. Cleavage at this residue results in an N-terminal 32-kDa and a C-terminal 27-kDa fragment (Fig. 5). FSBA can be added to conformation I or III, resulting in binding of FSBA to the A2 domain (Figs. 3 and 5). This is reflected in protection of the 27-kDa fragment derived from the A2 domain in F or FS conformations. Since this fragment is not protected (hatched lines in Fig. 6B) indicate complete cleavage) in conformation II, which is achieved in the presence of ATP, it is assumed that the A2 site under these conditions is unoccupied by the nucleotide. The addition of FSBA to conformation I or the addition of ATP to conformation F results in a completely trypsin-protected conformation AF or FA. In this conformation, Arg290 in the linker is not accessible to trypsin for the initial cleavage event. Hence, FSBA and ATP can be added to the ArsA protein in any order (samples FA and AF), retaining the same final trypsin-resistant conformation. Since FSBA binds to the A2 site, it appears that in AF or FA samples, ATP and FSBA are bound to the ArsA protein simultaneously (ATP to A1 and FSBA to A2). Hence, occupancy of both sites simultaneously by the nucleotide results in a change in conformation of the linker and a completely trypsin-protected conformation. This same conformation is also seen in the presence of ATP and antimone (AS or conformation IV). Hence, FSBA and ATP together mimic the effect of ATP and antimone, thus suggesting the role of antimone, which might be to induce ATP binding to the C-terminal site so that one ATP binds in the absence of antimone (to A1) and two molecules of ATP bind in its presence (to A1 and A2). Since FSBA binds to the A2 site irrespective of the presence or the absence of antimone, the effect of antimone on FSBA binding is not reflected in an increase in the number of sites occupied. FSBA, having a higher affinity for ArsA than ATP in the absence of the oxyanion, can access a site in ArsA that is accessible to ATP only in the presence of antimone. Accordingly, no difference is observed in trypsin-digested F or FS samples, whereas the difference between the trypsin-digested A and AS samples is significant (Fig. 5). ATP has previously been shown to form an UV-activated covalent adduct only to the A1 domain of ArsA (6, 9). If ATP binds to the A2 domain in the presence of antimone, one might expect ATP to UV cross-link to the A2 domain in the presence of antimone and to A1 in either the presence or the absence of antimone. Although UV-activated adduct was seen to form only to the A1 domain either in the presence or in the absence of antimone (data not shown), the absence of UV cross-linking to the A2 site does not necessarily imply the absence of ATP binding. It is possible that UV cross-linking might require certain specific residue(s) in the vicinity of the ATP binding site, and such a residue might be absent from the A2 domain.

The implications of the model presented in Fig. 6 are the following. The A1 site in ArsA is a high affinity ATP site (based on the fact that the AF or FA conformation, distinct from the F conformation, can be isolated), whereas the A2 site has low affinity and binds ATP only in the presence of antimone. Hence, antimone binding acts as a switch that regulates the binding of ATP to A2. It is likely that antimone does so by inducing a conformation, such as dimerization, that opens up the A2 site. When both sites are occupied by ATP, positive catalytic cooperativity results in promotion of catalysis. Asymmetry between the nucleotide binding sites and positive catalytic cooperativity resulting from occupancy of more than one site is well documented in the F1-ATPase (19). Each β-subunit in F1, at any one time has a different conformation and hence a different binding affinity for the nucleotide, but each subunit goes through the binding changes in a cyclic manner (17). This asymmetry in F1, is brought about by rotation of the α-β head relative to the Λ-subunit (17). In ArsA, the asymmetry between the sites and the difference in affinity for ATP appears to be built into the structure, perhaps to prevent futile cycles of ATP hydrolysis. Hence, in ATPases like ArsA, a switch is essential, and the switch in ArsA may be the binding of the allosteric ligand antimone or arsenite that allows ATP binding to the low affinity A2 site, resulting in a 10–15-fold stimulation of hydrolysis. The mechanism appears to be an "on/off" effect rather than an increase in affinity, since the effect of the oxyanion cannot be bypassed by increasing the concentration of ATP. Whether each site in ArsA is capable of unisite catalysis remains to be determined. Experiments are under way to directly test ATP binding to both nucleotide binding sites in ArsA in the presence of antimone and to one site in its absence. Structural information on different conformations of ArsA is also expected to greatly help in achieving a better understanding of the mechanism of this ATPase.

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