Biochemical Evidence for Interaction between the Two Nucleotide Binding Domains of ArsA

INSIGHTS FROM MUTANTS AND ATP ANALOGS*

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ArsA, the peripheral membrane component of the anion-translocating ATPase ArsAB, consists of two nucleotide binding domains (A1 and A2), which are connected by a linker sequence. Previous studies on ArsA have focused on the function of each nucleotide binding domain and the role of the linker, whereas the present study looks at the interactions between the binding domains and their interactions with the linker. It has previously been shown that the A1 domain of ArsA carries out unisite catalysis in the absence of antimonite, while A2 is recruited in multisite catalysis by antimonite in the presence of a functional A1 domain. Multisite catalysis thus seems to result from an interaction between A1 and A2 brought about by antimonite. In the present study, we provide direct biochemical evidence for interaction between the two nucleotide binding domains and show that the linker region acts as a transducer of the conformational changes between them. We find that nucleotide binding to the A2 domain results in a significant, detectable change in the conformation of the A1 domain. Two ATP analogs, FSBs and ATPγS, used in this study, were both found to bind preferentially to the A2 domain, and their binding resulted in changing the otherwise compact A1 domain into an open conformation. Point mutations in the A2 domain and the linker region also produced a similar effect on the conformation of A1, thus suggesting that events at A2 are relayed to A1 via the linker. We propose that nucleotide binding to A2 produces a two-tiered conformational change. The significance of these changes in the mechanism of ArsA is discussed.

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† The abbreviations used are: NBD, nucleotide binding domain; ATPγS, adenosine 5′-O-(thiotriphosphate); FSBs, 5′-γ-fluorosulfonfylbenzoyladeninosine; MOPS, 4-morpholinepropanesulfonic acid.

The studies discussed above point to a sequence in the catalytic mechanism of ArsA, and at the same time suggest interaction between the A1 and the A2 domains, which may be responsible for catalytic co-operativity. The present work provides direct biochemical evidence for interaction between the domains. We find that events at the A2 domain result in a significant conformational change in the A1 domain, thus providing strong evidence for interaction. Our results also indicate that the linker region acts as the mediator of the conformational changes between A1 and A2 and that events from A2 are transduced to A1 via the linker.

MATERIALS AND METHODS

Purification of ArsA

His-tagged wild type and mutant ArsA proteins were purified by Ni-nitrilotriacetic acid chromatography according to the procedure described earlier (6).

Trypsin Sensitivity Assay

ArsA (10 μM) in a 100-μl reaction volume containing MOPS-KOH, pH 7.5 was incubated with the indicated substrates for 10 min at 37 °C. The
Inhibition of the ATPase Activity of ArsA by ATPγS

To determine the inhibitory effect of ATPγS on the ATPase activity of ArsA, either the coupled assay (5) or the 32PγP release assay (7) was employed, as described below.

Coupled Assay—ArsA (15 μg) was incubated with the reaction components of the coupled assay and the indicated concentrations of ATPγS in a 1-ml cuvette. Antimonite (0.5 mM) and ATP (1 mM) were added, and the samples were preincubated at 37 °C for 10 min. The reactions were started by the addition of magnesium and decrease in absorbance of NADH was monitored at 340 nm. A control experiment showed that ATPγS has no effect on the components of the coupled assay system itself. To determine the protective effect of ATP on ATPγS inhibition, ArsA was incubated with different concentrations of ATP, antimonite (0.5 mM), and a fixed concentration of ATPγS in the coupled assay system for 10 min. The reaction was started by the addition of magnesium.

32PγP Release Assay—ArsA (1 μg) was preincubated with [γ-32P]ATP (2.5 μCi) and different concentrations of ATPγS in a 40-μl reaction volume in 50 mM MOPS-KOH, pH 7.5 for 10 min at 37 °C. Antimonite was added to the preincubation mixture where indicated. The reaction was started by the addition of magnesium, and the samples were withdrawn at different times, followed by TLC.

Labeling of ArsA with γ35S-ATP

Purified ArsA (10 μM) was incubated with γ35S-ATP (10 μCi) in 100 μl of buffer A (50 mM Tris-CI, pH 7.5, 10% glycerol, 1 mM EDTA) for 10 min at room temperature. The samples were placed in a microculture plate and subjected to UV cross-linking by placing a hand-held UV lamp (254 nm) directly above the microtitration plate for 30 min on ice. The plate and subjected to UV cross-linking by placing a hand-held UV lamp (254 nm) directly above the microtitration plate for 30 min on ice. The samples were precipitated with 10% trichloroacetic acid on ice for 30 min at room temperature. The samples were placed in a microtitration plate and subjected to UV cross-linking by placing a hand-held UV lamp (254 nm) directly above the microtitration plate for 30 min on ice. The plate was withdrawn at different times, followed by TLC.

Identification of the Site of Labeling with γ35S-ATP in ArsA

ArsA, UV cross-linked with γ35S-ATP in buffer A, as described above, was directly mixed with trypsin at a trypsin/protein ratio of 1:1000. The samples were incubated at 25 °C for 60 min, followed by addition of 2-fold excess trypsin inhibitor. The samples were then analyzed by SDS-PAGE on a 10% polyacrylamide gel, followed by autoradiography.

Amino Acid Sequencing of the Peptide Fragments

Trypsin-digested fragments were transferred onto a polyvinylidene difluoride membrane. 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

Previous studies have shown that Arg-290 in the linker region (residues 282–320) of ArsA is a readily accessible trypsin site (6). At a ratio of trypsin to ArsA of 1:1000, cleavage at Arg-290 divides the protein into two halves, a 32-kDa N-terminal fragment (residues 1–290) and a 27-kDa C-terminal fragment (starting at residue 290). The N-terminal 32-kDa fragment is very compact and is resistant to further cleavage by trypsin, whereas the 27-kDa fragment is cleaved further into smaller fragments (6). This digestion pattern is seen in the control sample (no substrate) as well as in the presence of ATP. Interestingly, however, in the presence of ATP and antimonite, ArsA acquires a trypsin-resistant conformation, which has been designated AS as compared with the A conformation obtained in the presence of ATP alone (6). Most ArsA in the AS conformation is found in the 63-kDa species, indicating that Arg-290 is now buried in the structure and is inaccessible to trypsin. In this study, we looked at the ability of the point mutants in the nucleotide binding sites of ArsA to acquire the AS conformation. Two point mutants were chosen, G20S with a mutation in the A1 NBS (4) and K340E with a mutation in the A2 NBS (5). The data in Fig. 1 suggest that, unlike in the wild type ArsA (lanes 1–3), neither G20S nor K340E can acquire the AS conformation in the presence of ATP and antimonite. The digestion pattern of K340E is exactly the same under three different conditions, which include the control (no substrate, lane 7), A (ATP, lane 8), or the AS (ATP and antimonite, lane 9). The N-terminal 32-kDa fragment, which results from cleavage at Arg-290, is the predominant fragment in all situations (Fig. 1, lanes 7–9). The AS conformation is also completely absent in G20S (Fig. 1, lanes 4–6).

Since the AS conformation of ArsA is resistant to trypsin at a concentration of 1:1000, trypsin concentrations of 1:100 and 1:50 were employed to analyze the AS conformation further. The data in Fig. 2A show that Arg-290 in AS is relatively inaccessible even at high trypsin concentrations, however a new 40-kDa fragment is produced exclusively in the AS sample (lanes 3 and 5) and not in the A sample (lanes 2 and 4). Western blot analysis (Fig. 2B) showed that this band cross-reacts with both anti-N18 (residues 1–176) (lane 2) and anti-C30 (residues 320–583) (lane 4) antibodies, thus suggesting that it originates within the A1 domain and extends into the A2 domain. This fragment was designated the NC40 fragment. An N-terminal amino acid sequence analysis of this fragment showed that it originates at residue Arg88 in the A1 domain and it bears the amino acid sequence DEQDVPIKGVL (Table I). Since this site is inaccessible to trypsin in the control (no substrate) or in the A (with ATP) conformation, it suggests that an alternate trypsin site within the A1 domain becomes available in the AS conformation, albeit only at high concentrations of trypsin. (See Fig. 3 for a linear depiction of ArsA.)

To determine if availability of the alternate trypsin site (Arg-88) in A1 depends on nucleotide binding to A2, analogs of ATP, including FSB and ATPγS, were used. FSBA inhibits the ATPase activity of ArsA in a specific manner (10). It was chosen for this study, in particular, because it binds preferentially to the A2 domain of ArsA, and its binding results in protection of the C27 fragment from proteolysis at a trypsin concentration of 1:1000 (6). In the present work, we find that binding of FSB to ArsA results not only in the protection of the C27 fragment but also produces another fragment of about 25 kDa in size, which is present exclusively in the FSBA-treated ArsA samples (Fig. 4A, lane 3) and not present in the

FIG. 1. AS conformation in wild type and mutant ArsA proteins. Purified wild type or mutant (A1 mutant G20S and A2 mutant K340E) ArsA proteins (10 μM) were incubated with 5 mM ATP and 0.5 mM antimonite for 10 min at 37 °C and subjected to trypsin proteolysis at a trypsin/protein ratio of 1:1000 for 60 min, as described under “Materials and Methods.” The samples were analyzed by 12% SDS-PAGE, followed by staining with Coomassie Blue. Lanes 1–3, wild type ArsA; lanes 4–6, G20S; lanes 7–9, K340E; lanes 1, 4, and 7, no substrate; lanes 2, 5, and 8, 5 mM ATP; lanes 3, 6, and 9, 5 mM ATP and 0.5 mM antimonite.
Effect of binding of another ATP analog, ATPγS, on the function of ArsA was also studied. Initial experiments focused on determining if ATPγS inhibits the ATPase activity of ArsA and in localization of its binding site in ArsA. To determine if ATPγS inhibits the ATPase activity, ArsA was preincubated with the indicated concentrations of ATPγS in the presence of antimonite and ATP, and its effect on the ATPase activity was determined by the coupled assay, as described under “Materials and Methods.” The ATPase activity of ArsA was inhibited in a dose-dependent manner (Fig. 5). Half-maximal inhibition was observed at 20–40 μM ATPγS. ATP was found to protect against the inhibitory effect of ATPγS. Complete protection from inhibition was observed at 8 mM ATP (data not shown), suggesting that ATPγS binds more tightly to one or both sites in ArsA as compared with ATP.

To understand the mechanism of ATPγS inhibition, its effect on the kinetic parameters was determined (Fig. 6). ArsA was preincubated in the presence of antimonite, ATP, and different concentrations of ATPγS. In the control reaction carried out in the absence of ATPγS, a $K_m$ of 131 μM and a $V_{max}$ of 966 nmol/min/mg ArsA was observed. These values are consistent with the values reported earlier (1, 5). In the presence of increasing concentrations of ATPγS, an increase in $K_m$ was observed, whereas $V_{max}$ was found to remain unchanged (Fig. 6). These data show that in the presence of ATPγS, affinity of ArsA for ATP is significantly decreased, suggesting that ATPγS is a competitive inhibitor that binds with a higher affinity than ATP.

ArsA has previously been shown to exhibit unisite catalysis from the A1 site in the absence of antimonite and multisite catalysis involving A1 and A2 in the presence of antimonite (7, 8). The effect of different concentrations of ATPγS on the initial rate of unisite and multisite catalysis was determined by the $32^P$ release assay. Preincubation with ATPγS was carried out in the presence of ATP and either the presence (multisite conditions) or the absence of antimonite (unisite conditions). Data in Fig. 7 show that the unisite activity of ArsA (squares) is unaffected even at concentration as high as 100 μM ATPγS, thus suggesting that ATPγS either does not bind to the A1 site or it does not inhibit the unisite activity of A1. However, a significant decrease in the initial rate of activity in the presence of antimonite was seen (triangles), which may suggest binding of ATPγS primarily to the A2 domain of ArsA.

To identify the ATPγS binding site, ArsA was mixed with γ-35S-ATP and exposed to UV light as described under “Materials and Methods.” Wild type ArsA was seen to form a UV-activated adduct with γ-35S-ATP (Fig. 8, lane 1). The addition of antimonite or magnesium was not found to increase the efficiency of labeling (data not shown). UV-induced photolabeling in the presence of γ-35S-ATP was also studied in the A1 (G20S) or the A2 (K340E) mutants. The data in Fig. 8 show that the A1 mutant, G20S, forms adduct with γ-35S-ATP (lane 2), while no labeling is seen in the A2 mutant, K340E (lane 3). These results thus suggest that labeling with γ-35S-ATP most likely occurs to the A2 domain.

To localize the binding site of γ-35S-ATP in ArsA, partial trypsin proteolysis of the labeled proteins was carried out. Under the trypsin conditions employed (described under “Materials and Methods”), ArsA is cleaved at Arg-290 into an

**Table 1**

| Peptide fragment | N-terminal sequence | Putative length of fragment | Source |
|------------------|---------------------|----------------------------|--------|
| NC40 (A5)        | VTDPFKGVL           | 89–422                     | This study |
| N25 (FSBA)       | VTDPFKGVL           | 89–290                     | This study |
| NC40 (ATPγS)     | VTDPFKGVL           | 89–422                     | This study |
| N25 (D303G mutation) | VTDPFKGVL       | 89–290                     | This study |
| C18 (ATPγS-binding peptide) | LXSXQPV    | 291–424                    | This study |
| C27 (FSBA-binding peptide) | LXSXQPV    | 291–536                    | (6) |
| C14 (FSBA-binding peptide) | GFDVH    | 355–482                    | (6) |

control (lane 1) or the ATP (lane 2) sample. To determine the origin of this fragment, Western blot analysis was carried out. Data in Fig. 4B show that the 25-kDa band cross-reacts with the anti-N18 antibody (lane 3) but not with the anti-C30 antibody (lane 6), thus implying that this fragment is the result of further breakdown of the otherwise compact N32 fragment (residues 1–290). This fragment is designated N25. N-terminal amino acid sequence analysis showed that this fragment contains the sequence VTDPFKGVL, thus this fragment also results from cleavage at Arg-88 in A1 (Table 1). Based on its size of 25 kDa, this fragment is expected to lie between Arg-88 and Arg-290. Effect of FSBA binding to the point mutants, G20S and K340E, was also studied. The data in Fig. 4A show that the N25 fragment is produced when G20S is treated with FSBA (lane 6). Interestingly, however, trypsin proteolysis of K340E results in the N25 fragment irrespective of the presence or absence of FSBA (lanes 7–9).

**Fig. 2.** Trypsin analysis of the AS conformation of ArsA. Wild type ArsA (10 μM) incubated with 5 mM ATP and 0.5 mM antimonite for 10 min at 37 °C was subjected to trypsin digestion at a trypsin/protein ratio of 1:100 or 1:50 for 60 min. The samples were analyzed by 12% SDS-PAGE. A, Coomassie Blue staining. Lane 1, undigested control; lanes 2 and 4, 5 mM ATP; lanes 3 and 5, 5 mM ATP and 0.5 mM antimonite. B, samples digested with 1:100 trypsin were analyzed by Western blot using anti-N18 and anti-C30 serum. Lanes 1 and 2, anti-N-18; lanes 3 and 4, anti-C30; lanes 1 and 3, 5 mM ATP; lanes 2 and 4, 5 mM ATP and 0.5 mM antimonite.
N-terminal 32-kDa and a C-terminal 27-kDa fragment (Fig. 9A, lanes 3 and 4). We find that in a buffer containing 10% glycerol (as used in this experiment), the C27 fragment is stable and is not digested further, thus allowing us to determine the relative labeling of N32 versus C27 with γ-35S-ATP.

![Fig. 3. A linear depiction of the wild type ArsA](image)

Fig. 3. A linear depiction of the wild type ArsA. The two halves of ArsA, A1 and A2, and the intervening linker region (hatched lines) are shown. Trypsin cleavage sites, Arg-88 in A1 and the Arg-290 in the linker, are marked. Various peptides of ArsA resulting from cleavage at Arg-290 (N32 and C27) or the alternate site Arg-88 (N25 and NC40) are shown below the linear map of ArsA. The binding sites for the ATP analogs, FSBA (355–482, stippled) and γ-35S-ATP (291–442, vertical lines), are also marked.

The autoradiogram in Fig. 9B shows that the C-terminal 27-kDa fragment is the predominant labeling site in wild type ArsA and in G20S (lanes 3 and 4). A smaller fragment of about 18 kDa is also labeled (lanes 3 and 4). The N-terminal amino acid sequence of the 18-kDa fragment was determined and it corresponds to291LXSXQPVA, indicating that it is the breakdown product of C27 (Table I and Fig. 3).

The data from the experiments outlined above suggest that ATPγS, like FSBA, binds to the A2 domain of ArsA. If so, does binding of ATPγS to A2 also produce a conformational change in A1? Trypsin analysis showed that binding of ATPγS, in the presence of magnesium, releases a 40-kDa fragment (Fig. 10, lane 5). N-terminal sequence analysis of the 40-kDa fragment showed that it also results from cleavage at Arg-88 and contains the sequence89IVDPIKGVL (Table I). This fragment is thus similar to the NC40 fragment produced by trypsin cleavage of ArsA in the AS conformation and it, once again, suggests a conformational change in A1 upon binding of nucleotide to A2.

![Fig. 4. Effect of FSBA binding on the conformation of the A1 domain of ArsA](image)

Fig. 4. Effect of FSBA binding on the conformation of the A1 domain of ArsA. A, wild type or mutant (G20S and K340E) ArsA proteins incubated with 5 mM ATP or 1 mM FSBA for 10 min at 37 °C were subjected to trypsin proteolysis at 1:1000 for 60 min. The samples were analyzed by 12% SDS-PAGE and stained with Coomassie Blue. Lanes 1–3, wild type ArsA; lanes 4–6, G20S; lanes 7–9, K340E. Lanes 1, 4, and 7, no substrate; lanes 2, 5, and 8, 5 mM ATP; lanes 3, 6, and 9, 1 mM FSBA. B, wild type ArsA samples from A were analyzed by Western blotting using anti-N18 or anti-C30 antibodies. Lanes 1–3, anti-N18; lanes 4–6, anti-C30; lanes 1 and 4, no substrate; lanes 2 and 5, 5 mM ATP; lanes 3 and 6, 1 mM FSBA.

![Fig. 5. Dose-dependent inhibition of the ATPase activity of ArsA by ATPγS](image)

Fig. 5. Dose-dependent inhibition of the ATPase activity of ArsA by ATPγS. 15 μg of ArsA was incubated with different concentrations of ATPγS in 1 ml of the coupled ATPase assay reaction mixture in 50 mM MOPS-KOH (pH 7.5), 0.25 mM EDTA. The reaction mixture also contained 1 mM ATP and 0.5 mM antimonite. After incubation for 10 min at 37 °C, the reactions were initiated by the addition of MgCl₂. A decrease in absorbance at 340 nm was monitored. The percentage ATPase activity was plotted as a function of the ATPγS concentration. The solid line represents a non-linear regression of the data and IC₅₀ of about 20 μM.
Fig. 6. Kinetic analysis of ATP hydrolysis by ArsA in the presence of ATPγS. The experimental conditions were same as in Fig. 5. Effect of different concentrations (squares, 0 μM; triangles, 10 μM; inverted triangles, 20 μM; diamonds, 40 μM) of ATPγS on hydrolysis of a range of ATP concentrations by ArsA was determined. The data are presented in the form of a double-reciprocal plot. A Vmax = 946.9 ± 114.3 nmol/min/mg and Kcat = 131.2 μM were obtained in the absence of the inhibitor. The Ks values obtained in the presence of ATPγS were 1.48 mM (10 μM ATPγS), 4.38 mM (20 μM ATPγS), and 4.54 mM (40 μM ATPγS).

Fig. 7. Effect of ATPγS on unisite and multisite catalytic activity of ArsA. 32P, release assay was used to determine the effect of ATPγS on the initial rate of hydrolysis by ArsA either in the absence (squares) or presence (triangles) of 0.5 mM antimonite. The samples were withdrawn at different time points (20, 40, 60, 80, and 100 s in the presence of antimonite and 1, 2, 3, 4, and 5 min in the absence of antimonite) and analyzed by TLC.

**DISCUSSION**

This article describes the long range conformational changes that occur in the A1 domain of ArsA on nucleotide binding to the A2 domain and elucidates the role of the linker in transducing these changes. It is previously known that ArsA, in the presence of ATP and antimonite, acquires a conformation strikingly different from the conformation produced in the presence of either by itself (6). We have proposed earlier that the A conformation of ArsA results from ATP binding to the A1 site, whereas the AS conformation results from binding of ATP to A1 and A2 in the presence of antimonite, followed by interaction between the domains (6). The significance of the AS conformation of ArsA is evident from the fact that point mutants in either NBD of ArsA do not produce the AS conformation (Fig. 1). This suggests that AS conformation is indeed the result of interaction between two functional NBDs with ATP bound to one or both. AS conformation is the trypsin-resistant conformation so that Arg-290 in the linker is not accessible to trypsin. However, we find that at a higher concentration of trypsin, an alternate site, Arg-88, becomes accessible in AS. Cleavage at Arg-88 results in the NC40 fragment. These data imply a conformational change in A1 resulting from its interaction with A2 in the presence of antimonite. Do these data also indicate that ATP binding to A2 in the presence of antimonite results in a conformational change in A1? To answer that question, further experimental evidence was collected.

Since FSBA, an ATP analog, binds preferentially to the A2 domain of ArsA (6), we asked if binding of FSBA to the A2 domain produces a conformational change in A1. In this study, we find that FSBA binding to A2 protects C27 from further cleavage by trypsin (as shown before in Ref. 6), and, at the same time, it produces a conformational change in the A1 domain making it accessible to trypsin (Fig. 4). This results in a new fragment N25, which originates from Arg-88 in A1 (Figs. 3 and 4). Thus FSBA binding studies demonstrate very clearly that nucleotide binding to A2 results in a conformational change in A1. Interestingly, our results also show that a mutation in the A2 domain (e.g. K340E) results in the N25 fragment both in the presence or absence of FSBA, suggesting that the A1 domain in K340E is locked in an open conformation, thus providing further evidence that the conformational change in A1 is induced by events at A2.

Furthermore, we find that binding of another ATP analog, ATPγS, to the A2 domain of ArsA also results in a conformational change in A1. A 40-kDa fragment, originating at Arg-88, is produced in the presence of ATPγS and magnesium (Fig. 10). This 40-kDa band is similar to the NC40 fragment produced on trypsin cleavage of the AS conformation (Fig. 1). Thus, cleavage at Arg-88 on nucleotide binding to A2 results in either the N25 (with FSBA) or the NC40 (with ATP or ATPγS) fragment depending, most likely, on the binding characteristics of the nucleotide in question (for binding sites, please see Fig. 3).

The two NBDs of ArsA are connected by a linker sequence, which has been shown to play a crucial role in the function of the protein (3). Several mutations in the linker region were reported earlier (3). One mutation, D303G, was found to be particularly interesting (3). In addition to resulting in high arsenite sensitivity, this mutation was shown to produce a conformational change in the A1 domain resulting in a new fragment N25 (3). In light of the studies reported here, this observation takes on a new significance. We decided to determine its N-terminal amino acid sequence and found that it too...
results from cleavage at Arg-88 (Table I). Thus a mutation in the linker (D303G) has the same effect on the conformation of A1 as a mutation in A2 (K340E), or binding of an ATP analog to A2 (FSBA). It implies, as suggested earlier (3), that the interaction between A1 and A2 involves the linker. That these three components (A1, A2, linker) interact closely is further evidenced by the fact that a mutation in any of these three components prevents the formation of the AS conformation (Fig. 1 and Ref. 3). It suggests that A1 communicates with A2 via the linker, and this flow of information is disrupted in the D303G mutation (no AS is seen). Thus, the linker functions as a transducer of conformational changes from A1 to A2 and vice versa.

In summary, the point mutants in the two binding sites and the linker allow us to look at the conformational changes that would normally occur in the wild type ArsA. Whereas wild type ArsA switches from A to AS on addition of antimonite, the point mutants in any of the three domains do not make this transition. The A2 mutation and the D303G linker mutation, however, have the effect of locking the A1 domain in a particular trypsin-sensitive conformation, irrespective of whether nucleotide is bound to A2 or not. Thus there is evidence for two separate effects resulting from events at A2: a long range conformational change in A1 (reflected in trypsin accessibility of Arg-88), and a movement of the A1 and A2 domains (reflected in the AS conformation and inaccessibility of Arg-290). In the wild type ArsA the long range conformational change in A1 is accompanied by the formation of the AS, whereas in the mutants it is not.

There is no doubt that these conformational changes reflect interaction between A1 and A2, most likely via the linker; however, this raises the question: what is the significance of these changes in the mechanism of catalysis by ArsA? The answer may be linked to the asymmetric nature of the two sites in ArsA. We have proposed that the two sites in ArsA are non-equivalent and that only the A1 NBD is competent to bind ATP in the absence of antimonite, whereas ATP binding to A2 is "switched on" in the presence of antimonite (6). Even though clear evidence for this proposal is still lacking, this could very well be the basis for sequential participation of A1 and A2 in catalysis (7). We have shown that A1 participates in catalysis first in the absence of the ligand (unisite catalysis), whereas A2 comes into play later, in the presence of the ligand and a functional A1 (multisite catalysis). This has recently been verified in independent studies carried out by Zhou et al. (8).

Thus the conformational changes observed in this study may play a role in the cycle of ATP binding and ADP release from A1 and A2. The following sequence of steps might be envisioned: ATP binding and hydrolysis occurs in A1 first in the absence of antimonite, which produces a change in A2 so that A2 is now competent to bind ATP. If antimonite is available, ATP binding to A2 (and perhaps hydrolysis by A2) (in turn) produces a conformational change in A1, causing release of tightly bound ADP from A1 and allowing binding of another molecule of ATP to A1. Whether antimonite indeed acts as a switch for ATP binding to A2 could be answered by structural studies, however, crystals of ArsA in the absence of antimonite have not been obtained (11). Nevertheless, the crystal structure studies have confirmed that the two sites in ArsA are non-equivalent and that the exchange with nucleotides occurs only at the A2 site (11).

Further, these studies have also shown that the nature of the nucleotide bound at A2 is reflected in the conformation of the A1 domain (12). Simultaneous changes in the conformation of the linker region of ArsA have also been reported (12). Thus, our biochemical studies are, to a large extent, supported by the crystal structure data.

Finally, a comparison of ArsA to CFTR and P-glycoprotein, two other proteins that contain two NBDs each (13, 14) suggests that ArsA is more similar to CFTR in its mode of function than it is to P-glycoprotein. The two NBDs in CFTR and ArsA seem to be non-equivalent and participate sequentially in the catalytic cycle;
whereas in Pgp, the two sites seem to be equivalent and even though they alternate in catalysis, there is no sequence to the steps. Each cycle of hydrolysis and transport in Pgp involves hydrolysis of only one ATP at either site. However, in both CFTR and ArsA, the N-terminal NBD is the first site to come into play; for one complete cycle, both NBDs seem to be involved. For example, in CFTR, a hydrolytic event at the N-terminal site opens the channel and another event at the C-terminal site closes the channel. In ArsA, binding of ATP to the N-terminal site and its hydrolysis occurs first and it is essential for participation of the C-terminal NBD and for multisite catalysis.

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