Nonequivalence of the Nucleotide Binding Domains of the ArsA ATPase*

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The arsRDABC operon of Escherichia coli plasmid R773 encodes the ArsAB pump that catalyzes extrusion of the metalloids As(III) and Sb(III), conferring metalloid resistance. The catalytic subunit, ArsA, is an ATPase with two homologous halves, A1 and A2, connected by a short linker. Each half contains a nucleotide binding domain. The overall rate of ATP hydrolysis is slow in the absence of metalloid and is accelerated by metalloid binding. The results of photolabeling of ArsA with the ATP analogue 8-azidoadenosine 5′-[α-32P]-triphosphate at 4 °C indicate that metalloid stimulation correlates with a >10-fold increase in affinity for nucleotide. To investigate the relative contributions of the two nucleotide binding domains to catalysis, a thrombin site was introduced in the linker. This allowed discrimination between incorporation of labeled nucleotides into the two halves of ArsA. The results indicate that both the A1 and A2 nucleotide binding domains bind and hydrolyze trinucleotide, even in the absence of metalloid. Sb(III) increases the affinity of the A1 nucleotide binding domain to a greater extent than the A2 nucleotide binding domain. The ATP analogue labeled with 32P at the γ position was used to measure hydrolysis of trinucleotide at 37 °C. Under these catalytic conditions, both nucleotide binding domains hydrolyze ATP, but hydrolysis in A1 is stimulated to a greater degree by Sb(III) than A2. These results suggest that the two homologous halves of the ArsA may be functionally nonequivalent.

In Escherichia coli the ArsAB pump encoded by the ars operon of plasmid R773 confers resistance to arsenicals and antimonials (1). ArsA is the catalytic subunit of the pump that hydrolyzes ATP in the presence of the trivalent forms of the two metalloids. ArsA ATPase is coupled to extrusion of As(III) or Sb(III) through ArsB, which serves both as a membrane anchor for ArsA and as the substrate-conducting pathway. Upon overexpression, ArsA exists primarily as a soluble protein in the cytosol. Soluble ArsA has been purified and characterized and has been shown to be an As(III)/Sb(III)-stimulated ATPase (2).

ArsA is composed of homologous N-terminal (A1) and C-terminal (A2) halves that are connected by a short linker (3). Each contains a nucleotide binding domain (NBD)3 (4) (Fig. 1), and both NBDs are required for ATPase activity and metalloid transport (5, 6). A novel metalloid binding domain located at the A1-A2 interface diametrically opposite to the NBDs modulates ATPase activity (4, 7, 8). Two stretches of residues, D142TAPTGH148 in A1 and D447TAPTGH453 in A2, physically connect the metalloid binding domain to the A1 and A2 NBDs, respectively. The metalloid binding domain is composed of three metalloid atoms, each of which is coordinated by two protein ligands, one of which is donated by A1 and the other by A2. Thus the metalloid atoms serve as the “molecular glue” that brings the two halves of the protein together, activating catalysis. The basal rate of catalysis has been thought to reflect the activity of only NBD1 and thus was termed unisite catalysis (9, 10). The metalloid-activated rate, termed multisite catalysis, has been proposed to be the result of stimulation of the activity of both NBD1 and NBD2.

In this study, that hypothesis was tested with the use of the ATP analogue 8-azidoATP, which has been used to examine the catalytic mechanism of other transport ATPases such as the F1 ATPase (11) and the P-glycoprotein (12, 13). Incorporation of the label from either 8-azido[α-32P]ATP or 8-azido[γ-32P]ATP at low temperature allows measurement of nucleotide binding. The apparent Kd value for trinucleotide of wild type ArsA was decreased more than 10-fold by Sb(III). Comparison of incorporation of 8-azido[α-32P]ATP and 8-azido[γ-32P]ATP under catalytic conditions indicates whether hydrolysis of the trinucleotide has occurred. As expected, nucleotide hydrolysis was stimulated by Sb(III). To distinguish between events at NBD1 and NBD2, a thrombin site was introduced into the linker that connects A1 and A2. Following labeling and thrombin digestion, A1 and A2 migrated with different mobilities on SDS-PAGE. Under noncatalytic conditions, both NBD1 and NBD2 bound 8-azido[α-32P]ATP and 8-azido[γ-32P]ATP. Under catalytic conditions, both NBD1 and NBD2 hydrolyzed the trinucleotide in the presence or absence of Sb(III). In both cases, hydrolysis was dominated by NBD1 activity. Thus the two NBDs have different properties, suggesting that they may have different functions.

**EXPERIMENTAL PROCEDURES**

Chemicals—8-Azido[α-32P]ATP (15–20 Ci/mmol) and 8-azido[γ-32P]-ATP (15–20 Ci/mmol) were purchased from Affinity Labeling Technologies (Lexington, KY). Restriction grade thrombin was purchased from

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† The abbreviations used are: NBD, nucleotide binding domain; 8-azidoATP, 8-azidoadenosine ATP; 8-azido[α-32P]ATP, 8-azido-adenosine 5′-[α-32P]ATP; 8-azido[γ-32P]ATP, 8-azidoadenosine 5′-[γ-32P]ATP; MOPS, 4-morpholinopropanesulfonic acid.
Bertani medium to an Bio-Max MR film (Eastman Kodak Co.) at SDS-PAGE on 12% acrylamide gels (16). Gels were dried and exposed to ThArsA (0.5 mg/ml) in buffer A was incubated in the dark at 4 °C or ageQuant software. The data were analyzed using SigmaPlot 2000. The Storm 860 PhosphorImager system (Amersham Biosciences) and Immunolabeling incorporated into the ArsA band was quantified using a 8-azido[32P]ATP incorporation into the ArsA bands was quantified on a PhosphorImager and expressed as arbitrary units. The error bars represent the average of three experiments calculated with SigmaPlot 2000. For clarity, incorporation was normalized to the value of 8-azido[32P]ATP incorporation at 4 °C.

thrombin. Following proteolysis, the two halves of ArsA were separated by SDS-PAGE on 12% acrylamide gels, and the radioactivity incorporated into the ArsA bands was quantified as described above. The data were analyzed using SigmaPlot 2000.

**RESULTS**

**Sb(III) Increases the Affinity of ArsA for Trinucleotide**—To determine the effect of metalloid on ArsA activity, purified wild type enzyme was incubated individually with 8-azido[α-32P]ATP or 8-azido[γ-32P]ATP at either 4 °C or 37 °C (Fig. 2). At 4 °C the amount of [32P] incorporated was similar whether the label was in the α or γ position, showing that the assay reflects trinucleotide binding under noncatalytic conditions. The addition of Sb(III) increased the amount of label incorporated with the label in either position. Under catalytic conditions (37 °C), the amount of label incorporated with 8-azido[α-32P]ATP remained the same as at 4 °C. The amount of label incorporated from 8-azido[γ-32P]ATP decreased substantially with higher temperature, both in the presence and absence of Sb(III), reflecting release of the γ-phosphate.

The effect of Sb(III) on the affinity of ArsA for 8-azido[α-32P]ATP was determined (Fig. 3). The apparent affinity for trinucleotide was −15 μM in the presence of Sb(III). In the absence of metalloid the affinity was difficult to measure accurately but appeared to be at least 10-fold less than in the presence of Sb(III). Thus, a major effect of metalloid binding was to increase the affinity of ArsA for trinucleotide.

**Introduction of a Thrombin Enzyme Cleavage Site in the Linker Sequence of ArsA**—Binding of nucleotide to the individual NBDs of P-glycoprotein can be measured because the two sites can be differentiated following brief trypsin digestion (17). However, limited trypsin treatment of ArsA results in near complete digestion of the A2 half of ArsA (18). To overcome this limitation, the sequence for a thrombin enzyme cleavage site was introduced into the linker that connects the two halves of ArsA. Within limits, neither the length nor the sequence of the linker is critical for ArsA function (3). The site was created by the addition of an alanine residue following Asp-311 and an arginine residue following Pro-313. Thrombin cleavage would be predicted to produce an A1 fragment of 315 residues (33,746 Da) and an A2 fragment of 276 residues (30,507 Da).

**Fig. 1. Catalytic sites in ArsA.** The structure shows the relative position of the two Mg-ADP filled NBDs. The two signal transduction domains (STD1 and STD2) in each half of the protein are shown to connect the NBDs with the Ars(III) filled metalloid binding domain. The model was derived from the 2.3 Å crystal structure of ArsA (4).

**Fig. 2. Binding and hydrolysis of 8-azidoATP by wild type ArsA.** Wild type ArsA (25 μg) was incubated for 10 min at 4 °C or 37 °C with either 8-azido[α-32P]ATP or 8-azido[γ-32P]ATP in the presence or absence of 0.1 mM Sb(III). Samples were photocross-linked and electrophoresed as described under "Experimental Procedures." The amount of 8-azido[α-32P]ATP or 8-azido[γ-32P]ATP incorporation into the ArsA bands at 4 °C (filled bars) or 37 °C (open bars) was quantified on a PhosphorImager and expressed as arbitrary units. The error bars represent the average of three experiments calculated with SigmaPlot 2000. For clarity, incorporation was normalized to the value of 8-azido[α-32P]ATP incorporation at 4 °C.
There was little difference between the arsenite resistance conferred by the wild type \(\text{arsA}\) gene and the \(\text{tharsA}\) gene containing the sequence for the thrombin site (Fig. 4), demonstrating that the gene product is functional \textit{in vivo}. To compare their properties \textit{in vitro}, wild type \(\text{ArsA}\) and \(\text{ThArsA}\) were purified. In an average of three separate preparations, wild type \(\text{ArsA}\) had a specific activity of 70 ± 17 nmol/min/mg of protein in the absence of Sb(III) and 779 ± 68 nmol/min/mg of protein in the presence of 0.1 mM Sb(III). The corresponding activities for the \(\text{ThArsA}\) were 74 ± 46 and 404 ± 49 nmol/min/mg of protein, respectively. Thus, although lower than wild type, \(\text{ThArsA}\) exhibits substantial stimulation by Sb(III).

ATP hydrolysis elicited similar conformational changes in the wild type enzyme and the thrombin site-containing derivative, as demonstrated by ligand-dependent changes in intrinsic protein fluorescence (Fig. 5). During ATP hydrolysis, \(\text{ArsA}\) attains...
a conformation in which the fluorescence of Trp-159 is enhanced (19). In the absence of Sb(III), the decay of this fluorescent conformer is a rate-limiting step in the catalytic cycle. Binding of Sb(III) accelerates the decay of this intermediate and consequently stimulates the rate of hydrolysis (20). Wild type and ThArsA exhibited similar enhancements of protein fluorescence during ATP hydrolysis and similar rates of quenching following addition of Sb(III). To measure binding of trinucleotide to ThArsA, the protein was labeled with 8-azido-[γ-32P]ATP in the presence or absence of 0.1 mM Sb(III) at 4 °C (Fig. 6). ThArsA exhibited binding of trinucleotide that was stimulated by Sb(III). The apparent Kd value in the presence of Sb(III) was approximately 30 μM within a factor of two of that of the wild type. The Sb-stimulated catalytic properties of the ThArsA derivative warrant further analysis.

**Separation of the A1 and A2 Halves of ArsA and Labeling of NBD1 and NBD2**—To examine the activity of the NBD1 and NBD2 individually, both wild type ArsA and ThArsA were digested with thrombin, and the products were separated by SDS-PAGE. From Coomassie Blue staining, cleavage of wild type ArsA by thrombin with time was observed from the 63-kDa intact protein to a slightly smaller form (ArsA') (Fig. 7A) even though ArsA does not contain an authentic thrombin site. From immunoblotting results, the cleavage site could be localized to the C-terminal end of the protein. Digestion at the C terminus was observed with every commercial batch of thrombin, so it is unlikely the result of a contaminating protease. Both forms were observed with a polyclonal antibody directed against entire ArsA (Fig. 7B) and against the N-terminal 18 kDa designated N18 (Fig. 7C). Only the full-length protein was observed when an antibody to the C-terminal six-histidine tag was used (Fig. 7D), indicating that the tag had been lost in the shorter form. Treatment of ThArsA with thrombin resulted in time-dependent formation of two fragments. The larger fragment corresponded to the A1 half of ArsA because it reacted with the N18 antibody but not the His-tag antibody. With time the lower band was converted to a slightly smaller form that differed in mass by about 5 kDa, similar to the difference between ArsA and ArsA' (Fig. 7B). Neither reacted with anti-N18 (Fig. 7C), showing that they are derived from the A2 half of the protein. The larger of two reacted with the His-tag antibody but the smaller did not (Fig. 7D). Thus the larger is converted to the smaller by cleavage of about 5 kDa from the C terminus of ArsA.

**Binding and Hydrolysis of 8-AzidoATP by NBD1 and NBD2**—ThArsA was labeled with either 8-azido[α-32P]ATP or 8-azido[γ-32P]ATP in the presence or absence of 0.1 mM Sb(III) at either 4 °C or 37 °C. This allowed measurement of both

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**FIG. 7.** Thrombin digestion of wild type and ThArsA. Either wild type or ThArsA (0.5 mg/ml) was digested with thrombin (1 unit) at room temperature in a buffer comprised of 20 mM Tris-HCl, pH 8.4, 150 mM NaCl, and 2.5 mM CaCl2. At the indicated times, samples were withdrawn, and the reaction was terminated by the addition of 2 mM phenylmethylsulfonylfluoride. The samples were analyzed by SDS-PAGE on 12% polyacrylamide gels, followed by Coomassie Blue staining (A) or Western blot analysis using anti-ArsA (B), anti-N18 (C), and anti-His tag (D) antibodies. The experiment shown in this figure was repeated numerous times, and the data shown are representative of the results.

**FIG. 8.** Nucleotide hydrolysis mediated by NBD1 and NBD2. A, ThArsA (25 μg) was incubated with 10 μM of either 8-azido[α-32P]ATP or 8-azido[γ-32P]ATP at 4 °C or 37 °C, photocross-linked, and digested with thrombin as described under “Experimental Procedures.” Experimental conditions are depicted on the autoradiogram. B, radioactivity incorporated into the NBD1 and NBD2 bands in A was quantified using a PhosphorImager. The experiment shown in this figure was repeated twice, and the data shown are representative of the results.
binding and hydrolysis of nucleotide. The eight samples were digested with thrombin and separated by SDS-PAGE (Fig. 8A), and the autoradiographs were quantified by densitometry (Fig. 8B). Under noncatalytic conditions (4 °C), both NBDs were labeled with either 8-azido[\(\alpha^{32}P\)]ATP or 8-azido[\(\gamma^{32}P\)]ATP. The amount of label incorporated into NBD1 was always greater than into NBD2. Although this assay does not permit one to obtain absolute \(V_{\text{max}}\) values, the data indicate that there is an increase in \(V_{\text{max}}\) for both NBD1 and NBD2. We conclude from the data in Fig. 8 that both NBD1 and NBD2 bind nucleotide, but the two are nonequivalent.

Notably, binding to both NBD1 and NBD2 was increased by Sb(III). Binding to the two NBDs was measured as a function of trinucleotide concentration (Fig. 9). In the absence of Sb(III), the apparent affinity at either site was too low to quantify. In the presence of Sb(III), binding was increased in both sites. Binding to NBD1 was saturable with an apparent \(K_d\) of \(-35 \mu M\), which is similar to that of the intact ThArsA, but the affinity for NBD2 was greater than \(70 \mu M\).

Under catalytic conditions (37 °C), the amount of label derived from 8-azido[\(\gamma^{32}P\)]ATP was considerably less than under noncatalytic conditions, presumably from liberation of the \(\gamma\)-phosphate (Fig. 8B). Loss of label from 8-azido[\(\gamma^{32}P\)]ATP was observed in both NBD1 and NBD2. Thus, we can conclude that both nucleotide binding domains hydrolyze trinucleotide in the absence of Sb(III) and that ArsA does not conduct unisite hydrolysis. Hydrolysis in both NBDs was stimulated by Sb(III).

Hydrolysis was measured as a function of trinucleotide concentration (Fig. 10). Because there was somewhat less binding of 8-azido[\(\gamma^{32}P\)]ATP at 37 °C compared with 4 °C, hydrolysis was determined from difference between incorporation of label from 8-azido[\(\alpha^{32}P\)]ATP and 8-azido[\(\gamma^{32}P\)]ATP at 37 °C. The data shown in Fig. 10 are the average of three separate experiments, and the \(K_m\) values are the best fit to the Michaelis-Menten equation using SigmaPlot 2000. The apparent \(K_m\) for 8-azidoATP in NBD1 in the absence of Sb(III) was \(>70 \mu M\). The apparent \(K_m\) for 8-azidoATP in NBD1 in the presence of Sb(III) was \(-18 \mu M\). The apparent \(K_m\) for 8-azidoATP in NBD2 in the absence of Sb(III) was \(>70 \mu M\). The apparent \(K_m\) for 8-azidoATP in NBD2 in the presence of Sb(III) was \(35 \mu M\).

Thus, in NBD1, Sb(III) produced an \(-4\)-fold increase in affinity for trinucleotide, whereas in NBD2 the Sb(III)-dependent increase in affinity was somewhat less. Although there was lower incorporation in both, there was a clear and reproducible difference between NBD1 and NBD2 in the amount of trinucleotide hydrolyzed.

**DISCUSSION**

The ArsAB pump provides resistance to trivalent metalloids in *E. coli*. The ArsA subunit is an As(III)/Sb(III)-stimulated ATPase (21), and the ArsB subunit is an antiporter that catalyzes As(III)/H\(^+\) exchange (22). The 583-residue ArsA ATPase...
is composed of two homologous A1 and A2 halves that are connected by a short linker (3). Each of the two halves contains a single NBD (4), both of which are required for ATPase activity and resistance (5, 6). To gain insight into the contributions of the individual NBDs in catalysis, we used the ATP analogue, 8-azidoATP, which has been used to examine the catalytic mechanism of other ATPases. Incorporation of label from either 8-azido[α-32P]ATP or 8-azido[γ-32P]ATP at low temperature allows measurement of nucleotide binding. Similarly, comparison of incorporation of 8-azido[α-32P]ATP and 8-azido[γ-32P]ATP under catalytic conditions indicates whether hydrolysis of the trinucleotide has occurred. Labeling studies on wild type ArsA with 8-azido[α-32P]ATP showed that the apparent Kₘ was decreased ~10-fold in presence of Sb(III). Similarly, the Kₘ for nucleotide hydrolysis was decreased by Sb(III) (Fig. 10).

The two ATP sites of several ABC transporters such as P-glycoprotein, MRP1, and CFTR can be distinguished by SDS-PAGE following brief trypsin digestion. Monitoring incorporation of 8-azido[α-32P]ATP, 8-azido[γ-32P]ATP, and 8-azido[α-32P]ADP into the two NBDs has provided mechanistic insights into the role of each NBD (for reviews, see Refs. 23–25). However, limited trypsin treatment of ArsA results in nearly complete digestion of A2 (18). To overcome this limitation, the sequence for a thrombin enzyme cleavage site was introduced into the linker that connects the two halves of ArsA. E. coli cells bearing wild type copy of arsB along with the gene for the thrombin site containing ArsA (TharsA) were resistant to As(III) and Sb(III) (Fig. 4), and purified TharsA catalyzed metalloid-stimulated ATP hydrolysis. Following labeling and thrombin digestion, A1 and A2 fragments migrated with different mobilities on SDS-PAGE. Under noncatalytic conditions (4 °C), both NBD1 and NBD2 bound 8-azido[γ-32P]ATP, and although the relative incorporation of nucleotide at NBD1 was 4-fold more than in NBD2. Addition of Sb(III) showed an ~4-fold increase in the affinity for 8-azidoATP in NBD1 and a lower increase in NBD2. Under catalytic conditions, both NBD1 and NBD2 hydrolyzed trinucleotide in the presence or absence of Sb(III), but in both cases the extent of hydrolysis appeared to be ~4-fold greater in NBD1 than NBD2. It should be emphasized that this photolabeling assay does not lend itself to rate determinations, and a number of factors could influence the extent of labeling. The importance of these results is that they clearly show that 1) both NBDs are catalytic and hydrolyze ATP in the presence and absence of Sb(III), 2) Sb(III) increases the affinity for ATP in both NBD1 and NBD2, and 3) NBD1 always has higher affinity for ATP than NBD2.

Both we (10) and Kaur (9) proposed that only NBD1 hydrolyzes ATP in the absence of metalloid (unisite catalysis), whereas both NBDs hydrolyze ATP in the presence of As(III) or Sb(III) (multisite catalysis). In this study we show for the first time that both NBD1 and NBD2 are catalytic in both the absence and presence of metalloid, so it is incorrect to label the basal rate of hydrolysis as unisite. It is more likely that NBD2 has a sufficiently low rate of hydrolysis such that most of the basal rate is contributed by NBD1. This conclusion raises the question as to the function of NBD2. There are a number of possibilities. First, it could be vestigial and not involved in transport. These seems unlikely because mutagenesis of NBD2 resulted in loss of both As(III) resistance and ATPase activity (6). Second, one NBD could play a regulatory role with the energy for metalloid transport supplied by hydrolysis at the other NBD. This is similar to the roles proposed for the two NBDs of MRP1 and CFTR (26, 27). Third, the two NBDs could play equivalent roles in the intact ArsAB complex, and apparent differences could be caused by analysis of ArsA in the absence of ArsB. Future studies will be designed to examine each of these possibilities.

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