Unisite and Multisite Catalysis in the ArsA ATPase*

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The ars operon of plasmid R773 encodes an As(III)/Sb(III) extrusion pump. The catalytic subunit, the ArsA ATPase, has two homologous halves, A1 and A2, each with a consensus nucleotide-binding sequence. ATP hydrolysis is slow in the absence of metalloid and is accelerated by metalloid binding. ArsA M446W has a single tryptophan adjacent to the A2 nucleotide-binding site. Tryptophan fluorescence increased upon addition of ATP, ADP, or a nonhydrolyzable ATP analogue. Mg2+ and Sb(III) produced rapid quenching of fluorescence with ADP, no quenching with a nonhydrolyzable analogue, and slow quenching with ATP. The results suggest that slow quenching with ATP reflects hydrolysis of ATP to ADP in the A2 nucleotide-binding site. In an A2 nucleotide-binding site mutant, nucleotides had no effect. In contrast, in an A1 nucleotide-binding mutant, nucleotides still increased fluorescence, but there was no quenching with Mg2+ and Sb(III). This suggests that the A2 site hydrolyzes ATP only when Sb(III) or As(III) is present and when the A1 nucleotide-binding domain is functional. These results support previous hypotheses in which only the A1 nucleotide-binding domain hydrolyzes ATP in the absence of activator (unisite catalysis), and both the A1 and A2 sites hydrolyze ATP when activated (multisite catalysis).

The ars operon of R-factor R773 encodes an arsenite extrusion system that confers resistance in Escherichia coli to the metalloids arsenite (As(III)) and antimonite (Sb(III)) (1). This efflux pump has a catalytic subunit, the ArsA ATPase, and a membrane subunit, the ArsB arsenite carrier (2, 3). ArsA has N-terminal (A1) and C-terminal (A2) halves that are homologous to each other, most likely as the result of ancestral gene duplication and fusion (4). The enzyme has two nucleotide-binding domains, NBD1 and NBD2, both of which are composed of residues from both the A1 and A2 halves (5). Both NBDs are required for metalloid resistance (6, 7).

Other pumps with multiple NBDs that exhibit multisite catalysis such as F-type ATPases and ATP-binding cassette ATPases have been proposed to have mechanisms that involve catalytic alternation between the NBDs (where only one is active at a time) and a high degree of cooperativity between the sites (8, 9). Kaur (10) has suggested that ArsA exhibits both unisite and multisite catalysis in which only NBD1 participates in unisite catalysis and that a functional A1 NBD is required for NBD2 to participate in multisite catalysis. We have considered a similar alternating site model for the function of ArsA, where the two NBDs alternate between open and closed conformations in a concerted and interactive manner, coupling the energy of ATP hydrolysis to the transfer of As(III) or Sb(III) at the metal site of ArsA to the ArsB carrier (11, 12).

In the absence of metalloid, ArsA catalyzes hydrolysis of ATP at a low basal rate (k = 0.001 s−1) (13). Under presteady state conditions the addition of Sb(III) produces two bursts of phosphate liberation, one of which is ~250-fold faster than the other (k = 49 and 0.2 s−1) (12). From these results, it appears that both NBDs hydrolyze ATP in the activated state. However, the two NBDs are not equivalent in either structure (5, 11) or catalytic rates (12), which raises the question of whether they are functionally equivalent or whether they play different roles in metalloid resistance.

To examine the role of the individual NBDs, we previously constructed two single tryptophan ArsAs (F141W and W159) that report nucleotide occupancy and hydrolysis in NBD1 (14, 15). In each half there is a 12-residue sequence (DTAPTGH) that is found in all ArsA homologues from bacteria to humans (14). In the ArsA structure these are seen as extended regions that connect the single regulatory metalloid-binding domain with the two NBDs and probably function in signal transduction between the two substrate sites and the regulatory site (5). Trp447 is located at the N-terminal end of the A1 sequence and Trp446 is near the C-terminal end. Asp142 is a Mg2+ ligand in NBD, and His148 is an Sb(III) ligand in the metalloid binding site (5, 16). Trp441 primarily reports binding of MgADP, but indirectly reports ATP hydrolysis by the filling NBD1 with the product ADP (14, 15). Trp159 reports conformational changes in the vicinity of NBD1 during hydrolysis of ATP (14, 15). The response of spectroscopic signals to nucleotide binding and hydrolysis at NBD1 has allowed a detailed analysis of the catalytic cycle of ArsA (12, 13, 17).

In this study we report the properties of M446W, a single tryptophan derivative of ArsA. Trp446 is adjacent to the A2 sequence and is in the vicinity of NBD2. Trp446 occupies the equivalent position in A2 that Trp441 does in A1. In the absence of metalloid (unisite conditions), both ADP and ATP produced an enhancement of M446W protein fluorescence that was stable with time, indicating binding but not hydrolysis of ATP in NBD2. When the enzyme was preincubated with Sb(III) and Mg2+ (multisite conditions), ADP produced a rapid quenching of fluorescence. Addition of nonhydrolyzable ATP analogues resulted in a stable enhancement of fluorescence. In contrast, ATP rapidly enhanced fluorescence followed by a slow quenching to the level with ADP, consistent with ATP hydrolysis under multisite conditions. Thus, although Trp441 and Trp446 occupy equivalent positions in the A1 and A2 halves of

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1 The abbreviations used are: NBD, nucleotide-binding domain; ATPγS, adenosine-5′-O-(thiotriphosphate); MOPS, 4-morpholinepropanesulfonic acid.
ArsA, respectively, their fluorescent responses to nucleotide binding and hydrolysis differ considerably, indicating non-equivalence of NBD1 and NBD2. These results support the hypothesis that both NBD1 and NBD2 hydrolyze ATP under multisite conditions but that only NBD1 and not NBD2 participates in unisite catalysis (10).

MATERIALS AND METHODS

Media and Growth Conditions—All of the E. coli strains and plasmids used in this study are listed in Table I. Cells were grown in Luria-Bertani medium (18) at 37°C. Ampicillin (125 μg/ml) and tetracycline (12.5 μg/ml) were added as required. ATP-S was purchased from Sigma.

DNA Manipulations—Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed by standard methods (19). Restriction enzymes and nucleic acid-modifying enzymes were purchased from Invitrogen. WizardTM plus miniprep DNA purification system and WizardTM DNA clean-up system (Promega) were used to prepare plasmid DNA for restriction enzyme digestion and to recover DNA fragments from low melting agarose gels, respectively.

Site-directed Mutagenesis—Mutations in the arsA gene were introduced by site-directed mutagenesis using the Altered SitesTM mutagenesis system (Promega) with plasmid pTZ6H6, which contains the arsA gene with all 4 tryptophan codons mutated to those of tyrosines and six histidine codons added to 3’-end, Tc’. 

| Strain/Plasmid | Genotype/description | Ref. |
|----------------|----------------------|------|
| E. coli strains | lacZ53 mutS201:Th5 thyA36 rha-5 metB1 docC IN[rvnD-rneE] recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F’ [traD36 proA’ B’ lacU’ lacZAM15] | Promega (19) |
| pTZ4H6 & pTZ4H6M446W, Tc’ | arsA gene with all 4 tryptophan codons mutated to those of tyrosines and six histidine codons added to 3’-end, Tc’ | (14) |
| pTZ4H6M446WG18R, Tc’ | Site-directed mutagenesis of codon 18 to Arg codon in arsA gene of pTZ4H6M446W, Tc’ | This study |
| pTZ4H6M446WG337R, Tc’ | Site-directed mutagenesis of codon 337 to Arg codon in arsA gene of pTZ4H6M446W, Tc’ | This study |
| pTZ4H6M446WG18RG337R, Tc’ | Site-directed mutagenesis of codons 18 and 337 to Arg codons in arsA gene of pTZ4H6M446W, Tc’ | This study |

Fluorescence Measurements—Fluorescence measurements were performed using an SLM-8000C spectrophotometer with a built-in magnetic stirrer. The bandwidths for emission and excitation monochromators were 4 nm. Tryptophan fluorescence was monitored with an excitation wavelength of 295 nm and an emission wavelength of 321 nm. The fluorescence of the assay buffer (50 mM MOPS-KOH, pH 7.5) alone was subtracted from each spectrum. ATP, ADP, ATP-S, MgCl2, and antimonite (in the form of potassium antimonyl tartrate) were added as indicated. The concentration of ArsA was 1.25 μM in all assays.

Under Multisite Conditions Trp446 Fluorescence Reports Nucleotide Binding and Sb(III)-dependent ATP Hydrolysis in the M446W ArsA Fluorescence Reports Multisite Catalysis

| Strain/Plasmid | Genotype/description | Ref. |
|----------------|----------------------|------|
| E. coli strains | lacZ53 mutS201:Th5 thyA36 rha-5 metB1 docC IN[rvnD-rneE] recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F’ [traD36 proA’ B’ lacU’ lacZAM15] | Promega (19) |
| pTZ4H6 | arsA gene with all 4 tryptophan codons mutated to those of tyrosines and six histidine codons added to 3’-end, Tc’ | (14) |
| pTZ4H6M446W & pTZ4H6M446WG18R | Site-directed mutagenesis of codon 18 to Arg codon in arsA gene of pTZ4H6M446W, Tc’ | This study |
| pTZ4H6M446WG337R | Site-directed mutagenesis of codon 337 to Arg codon in arsA gene of pTZ4H6M446W, Tc’ | This study |
| pTZ4H6M446WG18RG337R | Site-directed mutagenesis of codons 18 and 337 to Arg codons in arsA gene of pTZ4H6M446W, Tc’ | This study |

The intrinsic protein fluorescence of the M446W ArsA was analyzed (Fig. 1). M446W denatured with 8 M urea had a fluorescence emission spectrum indistinguishable from an equimolar amount of free tryptophan, with a λmax of 354 nm. Native M446W exhibited a substantial blue shift (λmax = 328 nm) and fluorescence enhancement. Addition of Sb(III) and saturating MgATP (1 mM) increased the fluorescence, and Sb(III) and 1 mM MgADP quenched the fluorescence. Sb(III) alone had no effect (data not shown). There was no shift in wavelength associated with the addition of those ligands, suggesting that the polarity of the environment of the indole side chain of Trp446 was not greatly altered.

To examine the structural basis of fluorescence changes in the ATP- and ADP-bound forms, the position of the tryptophan residue under both sets of conditions was modeled on the known crystal structures of the wild type enzyme with the same ligands (11). Because the structure of nucleotide-free ArsA has not been determined, the differences between the nucleotide-bound and nucleotide-free enzymes cannot be modeled. In the ADP-bound form (Fig. 2A) Trp446 is oriented toward the bound nucleotide with the ATP-bound form (Fig. 2B). This brings the indole ring closer to the phenyl ring of Phe342 (Fig. 2C). In the ATP-bound form, the imidazole–NH of Trp446 is −6 Å at its closest approach to the Phe342 phenyl ring. In the ADP-bound form the two aromatic rings are brought closer together (nearly perpendicular) so that the imidazole–NH comes within 3 Å of the phenyl ring. At this distance an indole amino proton can form a hydrogen bond with a phenyl ring that can quench tryptophan fluorescence (22). This suggests that the difference in fluorescence between the ATP- and ADP-bound forms is related to the relationship of the indole ring of Trp446 to the phenyl ring of Phe342.
The time dependence of the nucleotide responses under multisite conditions (in the presence of Sb(III)) was examined in more detail. The fluorescence quenching by MgADP observed in Fig. 1 was rapid; in the presence of Sb(III), addition of 50 μM MgADP to M446W produced a quick decrease in fluorescence intensity (Fig. 3, curve 5). In contrast, either 50 μM or 1 mM MgATP in the presence of Sb(III) produced a rapid rise in fluorescence followed by a slower quenching (Fig. 3, curves 3 and 4). With extended incubation time with 50 μM ATP, the fluorescence decreased to the same level as with MgADP, and the rate of quenching paralleled the rate of ATP hydrolysis (data not shown). Note that the concentration of ATP in curve 4 is only 5% of that in Fig. 1 so that ATP is depleted during the course of this assay but not in the duration of the assay shown in Fig. 1. The simplest interpretation of this result is that the decay of fluorescence represents the change in occupancy of the A2 NBD from the substrate ATP to the product ADP during the course of hydrolysis. Consistent with this idea, addition of the nonhydrolyzable ATP analog ATPγS also induced enhancement of fluorescence, but no decay was observed even with prolonged incubation (Fig. 3, curve 1). When added with MgATP, beryllium fluoride (BeF₂), which traps ATPases in a conformation resembling the transition state (23), also produced an enhancement of fluorescence with no subsequent decay of the fluorescence signal (Fig. 3, curve 2).

**Under Unisite Conditions Trp⁴⁴⁶ Reports Nucleotide Binding but Not Hydrolysis**—In the absence of antimonite ArsA hydrolyzes ATP at a low basal rate, which has been attributed to unisite hydrolysis in NBD1 (10). Consistent with this hypothesis, addition of either 1 mM or 50 μM MgATP produced a fluorescence enhancement of the M446W ArsA without subsequent decay of fluorescence even after extended times (Fig. 4, curves 1 and 2). This suggests that ATP is bound in NBD2 but not hydrolyzed under unisite conditions. Under these conditions MgADP produced a fluorescence enhancement rather than quenching, although this was to a lesser extent than ATP (Fig. 4, curve 3).

The transition from unisite to multisite conditions was examined by sequential addition of nucleotide, Mg₂⁺, and Sb(III) (Fig. 5). There was fluorescence enhancement following addition of ATP (Fig. 5, curve 1), ATPγS (Fig. 5, curve 2), or ADP (Fig. 5, curve 3). Subsequent addition of Mg₂⁺ partially reversed the fluorescence enhancement, which could result from a conformational change in the local environment of NBD2 when Mg₂⁺ is bound. Multisite conditions were initiated by the addition of Sb(III). As when the protein was preincubated with Sb(III), this resulted in a rapid quenching by ADP (Fig. 5, curve 3) and a slow quenching by ATP (Fig. 5, curve 1). The ATP quenching most likely reflects hydrolysis in NBD2, since there was no slow quench with ATPγS but rather a further enhance-
What is the requirement for two functional NBDs to produce these nucleotide-dependent effects on Trp 446 fluorescence? To examine this question, mutations in the P-loops of the two NBDs were combined with the M446W mutation individually and together. The G18R and G337R mutations have been shown to result in loss of resistance to arsenicals and loss of nucleotide binding in the A1 or A2 NBD, respectively (6, 7, 24). In M446W into which the G337R mutation was introduced there was no response of nucleotide under either unisite or multisite conditions (Fig. 6A). However, with M446W G18R, addition of ATP (Fig. 6B, curve 1), ATPγS (Fig. 6B, curve 2), or ADP (Fig. 6B, curve 3) under unisite conditions gave the same response as the wild type (Fig. 5). Addition of Sb(III) to initiate multisite conditions produced a small quench that may be caused by a conformational change upon Sb(III) binding, but there was no evidence of ATP hydrolysis by a slow quenching with ATP. This result suggests that NBD2 can bind nucleotides in the absence of a functional NBD1 but that it is locked into a conformation that prevents subsequent steps in the catalytic cycle.

**DISCUSSION**

Many types of transport ATPases are multisubunit complexes that contain two or more nucleotide-binding sites. The ways in which these sites interact with each other and participate in catalysis are still incompletely understood for even the best characterized proteins. For example, F-type ATPases have two classes of NBDs, both catalytic and noncatalytic (25). To explain the basis of the strong interactions between the catalytic NBDs in F1, Boyer and co-workers (26) proposed a binding change mechanism involving an alternation of catalytic sites, which took two decades of intensive study in many laboratories to be verified. Subsequently the ATP-binding cassette-type drug transporter P-glycoprotein has been suggested to utilize a similar alternating site mechanism (9, 27).

The ArsAB As(III)/Sb(III)-translocating ATPase is a resistance pump that catalyzes efflux of toxic metalloid salts (1). The
FIG. 5. Fluorescence of M446W during the transition from unisite to multisite conditions. Unisite conditions were initiated by addition of the indicated nucleotides (ANP) added followed by 0.5 mM MgCl₂. The transition to multisite catalysis was initiated by addition of 1 mM antimonite. Curve 1, 1 mM ATP; curve 2, 50 μM ATP-γ-S; curve 3, 50 μM ADP.

FIG. 6. Effect of mutations in NBD1 and NBD2 on fluorescence of M446W. Fluorescence of M446W ArsAs with mutations in NBD1 or NBD2. A, fluorescence responses of NBD2 mutant G337R and the double mutant G18R/G337R are superimposed. At the arrows 1 mM ATP, 0.5 mM MgCl₂, and 1 mM antimonite were added sequentially. B, fluorescence response of NBD1 mutant G18R. At the arrows, 1 mM ATP (curve 1), 1 mM ATP-γ-S (curve 2), or 1 mM ADP and 0.5 mM MgCl₂ (curve 3) were added. Multisite conditions were initiated by addition of 1 mM antimonite. ANP indicates the time of addition of adenine nucleotides.
catalytic subunit, ArsA, has two interacting NBDs (2). In the absence of Sb(III) or As(III) the enzyme hydrolyzes ATP at a slow basal rate, which seems to be catalyzed by only NBD1 (unisite catalysis), whereas in the presence of metalloid salts both sites are catalytic (10, 12). Under presteady state conditions in the presence of Sb(III) (multisite conditions), one NBD hydrolyzes ATP 250-fold faster than the other (12). From the rate of fluorescence quenching in the single tryptophan derivatives of ArsA that have tryptophan residues near NBD1 (Trp\(^{141}\) and Trp\(^{159}\)), the fast site can be identified as NBD1, which identifies the slow site as NBD2. The fluorescent properties of the F141W and W159 single tryptophan-containing ArsAs have been extremely informative about the catalytic properties of NBD1. In particular, under unisite conditions both Trp\(^{141}\) and Trp\(^{159}\) report slow hydrolysis (12), whereas under multisite conditions Trp\(^{159}\) reports rapid conformational changes associated with ATP hydrolysis (13). From these results it is clear that NBD1 participates in both unisite and multisite conditions.

From structure-based alignment of the A1 and A2 halves of ArsA, Met\(^{446}\) is identified as the residue in A2 that is equivalent to Phe\(^{141}\) in A1 (11). To examine the properties of NBD2, Met\(^{446}\) was changed to a tryptophan residue in a tryptophan-free background. The fluorescent responses of M446W ArsA are quite different from those of the F141W or W159 proteins. Both F141W and W159 ArsAs exhibit quenching of fluorescence with ATP that parallels the unisite rate of hydrolysis (14). In contrast, in the absence of Sb(III), ATP produces a stable enhancement of fluorescence of M446W, indicating that NBD2 does not hydrolyze ATP under unisite conditions. On the other hand, under multisite conditions W159 ArsA exhibits a quenching of fluorescence that is much more rapid than the steady state rate of hydrolysis. The interpretation of these results is that the highly fluorescent form of W159 under unisite conditions is because of buildup in the steady state of a conformational intermediate that is in slow equilibrium with the ground state of the enzyme (13). Under multisite conditions isomerization of the two forms is much faster such that a prior step in the catalytic cycle such as product release becomes rate-limiting. In contrast, the quenching rate of M446W fluorescence in the presence of Sb(III) is close to the steady state rate of hydrolysis, indicating that NBD2 is catalytic under multisite conditions.

From their evolutionary relationships and overall structural similarities, it is tempting to speculate that NBD1 and NBD2 have equivalent roles in ArsA function. In the crystal structure in which both are filled with MgADP, NBD1 is occluded while NBD2 is open (5). The occluded nature of NBD1 prevents exchange of ADP with other nucleotides, while the open NBD2 allows facile exchange with ATP or ATP analogues (11). Yet the occlusion in the crystal structure may result from capturing the enzyme in one particular conformation that represents one step in the catalytic cycle, and the two sites may be symmetric at other times during catalysis. These considerations have led to the alternating site models for ArsA (10–12). On the other hand, it is possible that the two sites have different roles in ArsA function. For example, under multisite conditions the role of NBD1 may be to activate NBD2, and perhaps hydrolysis only at NBD2 is coupled to transport. Although the results of this study do not distinguish between different models, they demonstrate that the two nucleotide-binding sites have intrinsic differences. This gives more weight to the possibility that the two NBDs have dissimilar functional roles in catalysis.

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