

## Minireview

# Biochemistry of arsenic detoxification

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**Abstract** All living organisms have systems for arsenic detoxification. The common themes are (a) uptake of As(V) in the form of arsenate by phosphate transporters, (b) uptake of As(III) in the form of arsenite by aquaglyceroporins, (c) reduction of As(V) to As(III) by arsenate reductases, and (d) extrusion or sequestration of As(III). While the overall schemes for arsenic resistance are similar in prokaryotes and eukaryotes, some of the specific proteins are the products of separate evolutionary pathways. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Arsenic; Transport ATPase; Resistance pump; Arsenate reductase

### 1. Introduction

This review will focus on selected mechanisms of arsenic detoxification in prokaryotes and eukaryotes. Arsenic is a semi-metal or metalloid with two biologically important oxidation states, As(V) and As(III), as the oxyacids arsenic acid ( $\text{H}_3\text{AsO}_4$ ) or arsenous acid, also called arsenic trioxide ( $\text{As}_2\text{O}_3$ ). In solution at neutral pH, arsenic acid exists as the arsenate oxyanion. The  $\text{pK}_a$  of arsenous acid is 9.2, so that, at neutral pH, it would be primarily present in solution as neutral  $\text{As}(\text{OH})_3$ . As described below, this difference in  $\text{pK}_a$  is relevant for the type of transport system that catalyzes uptake of the pentavalent and trivalent forms of arsenic. The other aspect of arsenic chemistry relevant to biological activity is reactivity of As(III) as a soft metal ion, forming strong bonds with functional groups such as the thiolates of cysteine residues and the imidazolium nitrogens of histidine residues.

Arsenic enters the biosphere primarily by leaching from geological formations [1]. For example, in Michigan, arsenic leaches into water from the Marshall Sandstone and Coldwater Shale. Anthropomorphic sources include arsenical-containing fungicides, pesticides and herbicides. The health effects of environmental arsenic can be devastating, and, over a period of decades, can progress to various forms of cancer. Its ubiquity in the environment has led to the evolution of arsenic defense mechanisms in every organism studied, from *Escherichia coli* to man [2] (Fig. 1). As described below, organisms take up As(V) via phosphate transporters [3,4] and As(III) by aquaglyceroporins [5–7]. As(V) is reduced to As(III) [8,9],

which is either extruded from cells or sequestered in intracellular compartments, either as free arsenite or as conjugates with GSH or other thiols [10,11]. In addition, arsenic can be methylated [12], although this process may increase arsenic toxicity rather than contributing toward detoxification [13].

### 2. Arsenic uptake systems

In the prokaryote *E. coli* there are two phosphate transporters, Pit and Pst [4]. Both catalyze arsenate uptake, but the Pit system appears to be the predominant system for arsenate [14]. Similarly, in the eukaryote *Saccharomyces cerevisiae* several phosphate transporters participate in arsenate uptake [3,15]. It can be assumed that arsenate is taken up similarly in mammals, although this has not been demonstrated.

Pathways for arsenite uptake into cells have only recently been discovered. We first identified a trivalent metalloid transporter as GlpF, the glycerol facilitator of *E. coli* [5]. A screen of a random mutagenesis library turned up a mutant resistant to Sb(III). The chemical properties of Sb(III) and As(III) are very close, which makes it likely that GlpF is also an As(III) transporter even though the cells did not exhibit increased arsenite resistance. GlpF is an aquaglyceroporin, a member of the aquaporin superfamily (Fig. 2A). Aquaglyceroporins are multifunctional channels that transport neutral organic solutes such as glycerol and urea [16]. Fps1p, the yeast homolog of GlpF, was recently shown to be the route of uptake of arsenite in *S. cerevisiae* [6]. In that study the *FPS1* gene was targeted for deletion specifically to examine its role in arsenite resistance.

More recently we have shown that mammalian aquaglyceroporins catalyze uptake of trivalent metalloids [7]. We had previously constructed a strain of *S. cerevisiae* with deletions in the genes for in the two arsenite extrusion transporters, Acr3p and Ycf1p [10], as described below. A strain with disruptions of both ACR3 and YCF1 was hypersensitive to arsenite. We next constructed the triple deletion of ACR3, YCF1 and FPS1. This strain was unable to take up arsenite, which resulted in arsenite resistance. To examine the arsenite transport properties of mammalian aquaglyceroporins, we cloned the genes for the rat AQP9 and mouse AQP7 into a yeast vector and tested their ability to complement the arsenite-resistant phenotype of the triple yeast mutant. AQP9 was expressed well and restored arsenite sensitivity. Cells expressing AQP9 transported both  $^{73}\text{As}(\text{III})$  and  $^{125}\text{Sb}(\text{III})$ , thus demonstrating that this protein can transport metalloids. AQP7 was not expressed in yeast. However, when either

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AQP7 or AQP9 cRNA was microinjected into *Xenopus laevis* oocytes, increased transport of  $^{73}\text{As(III)}$  was observed [7]. We have recently expressed human AQP3, AQP9 and AQP10 in yeast and found that only hAQP9 complemented the arsenite phenotype, suggesting that only some aquaglyceroporins transport As(III) (Z. Liu and B.R., unpublished). What chemical species of As(III) do the aquaglyceroporins recognize? With a  $\text{pK}_a$  of 9.2,  $\text{As(OH)}_3$  would predominate in solution at neutral pH. Since AQP7 and AQP9 conduct transmembrane movement of neutral species,  $\text{As(OH)}_3$ , which might be considered an inorganic equivalent of glycerol, is the likely substrate. This finding may have relevance to human health and disease. On the one hand, considerable individual variability in sensitivity to arsenic in drinking water has been observed in countries such as India and Bangladesh [17]. It is possible that this variability is due in part to different levels of expression of aquaglyceroporins in those individuals. On the other hand, arsenite is an effective cancer chemotherapeutic agent. Its anhydrous form, arsenic trioxide (Trisenox) has been approved as a chemotherapeutic agent for the treatment of acute promyelocytic leukemia [18,19]. Differences in expression of AQP7 or AQP9 could lead to variability in response to drug therapy in different patients.

### 3. Arsenate reductases

When the pentavalent oxyanion arsenate is taken up, it is reduced to As(III) prior to extrusion or sequestration. Three independently evolved families of arsenate reductase enzymes have been recognized [20] (Fig. 2B). We reported the first sequence of an arsenate reductase as the product of the last gene of the *ars* operon of *E. coli* plasmid R773 [21]. Homologues of this *arsC* gene are found in many bacteria, both on plasmids and in chromosomes [1]. The crystal structure of the 16 kDa R773 ArsC has been reported with bound substrate (arsenate) and product (arsenite) [22]. In the reaction cycle, arsenate first binds to the anion site that consists of three basic residues, Arg60, Arg94 and Arg107. Phosphate and sulfate are competitive for binding at this site. In the next step arsenate (but not phosphate or sulfate) forms a covalent arsenate thioester intermediate with the active site Cys12. It is then reduced in two steps by glutaredoxin and glutathione, producing the Cys12-S-As(III) intermediate, which hydrolyzes to release arsenite. *E. coli* has three glutaredoxins, Grx1, Grx2 and Grx3, any one of which will serve as source of reducing potential for arsenate reduction, although Grx2 is preferred [23].

A second family of arsenate reductases also widely distributed in bacteria is typified by the *arsC* gene product of *Staphylococcus aureus* plasmid pI258 [8]. It is unfortunate that this enzyme is also called ArsC considering that it is unrelated to the first family of arsenate reductases. The pI258 enzyme uses thioredoxin as the source of reducing potential [24] and has two intramolecular cysteine residues that participate in the catalytic cycle [25]. The crystal structures of the pI258 ArsC and its homolog from *Bacillus subtilis* have recently been reported [26,27]. Interestingly, this enzyme is related to low-molecular-weight protein tyrosine phosphate phosphatases and exhibits low-level phosphatase activity [26].

The third family of arsenate reductases is also related to protein tyrosine phosphate phosphatases, although to a different family of phosphatases than the pI258 ArsC. The only

member of the family to be characterized to date is Acr2p from *S. cerevisiae* [28,29]. It is related to the superfamily of protein phosphatases that includes CDC25a, a cell cycle phosphatase [30] and uses a similar HisCys(X)<sub>5</sub>Arg motif in its active site [31].

Like the R773 ArsC, Acr2p has a single active site cysteine residue and uses glutaredoxin and glutathione as reductants [32]. Even though it is not related to either of the bacterial ArsC arsenate reductases, it can be heterologously expressed in *E. coli* and complements an *arsC* deletion. It does not exhibit phosphatase activity. However, we have constructed an Acr2p mutant that has gained phosphatase activity and lost arsenate reductase activity (R. Mukhopadhyay and B.R., unpublished). The ease by which a reductase can be changed into a phosphatase has led us to propose that, under the selective pressure of ubiquitous arsenate in the environment, arsenate reductases evolved from phosphatases.

So far no mammalian members of any of the three families of arsenate reductases has been identified. It should be pointed out, however, that the relatedness of Acr2p to the widespread protein tyrosine phosphatase family makes it difficult to identify homologs with arsenate reductase activity through a genomics approach. Arsenate reductase activity has been observed in vitro in extracts from human liver [33]. Recently the activity has been attributed to the enzyme purine nucleotide phosphorylase [34]. Whether this enzyme functions in vivo in arsenic detoxification has not yet been examined.

Cytosolic arsenite, whether as the product of arsenate reductase or from uptake via an aquaglyceroporin, is detoxified by removal from the cytosol [1,35]. It is rather counterintuitive that arsenate, which is not very toxic, should be converted to the much more toxic arsenite prior to transport out of the cytosol. Why are there no arsenate-specific efflux systems? We speculate that this is an accident of evolution. Since the primordial atmosphere was not oxidizing, most arsenic would have been in the form of As(III), and early organisms would have evolved detoxification mechanisms to cope with As(III), not As(V). Once the atmosphere became oxidizing, arsenite in oceans and other surface waters would have been oxidized to arsenate. Thus mechanisms to cope with As(V) evolved to use existing arsenite extrusion systems. Since the conversion of a phosphatase to a reductase is relatively easy to accomplish in the laboratory, its evolution during the formation of an oxygenic world would have been rapid.

### 4. Prokaryotic arsenic extrusion systems

In bacteria there are two basic mechanisms of arsenite extrusion. One is carrier-mediated efflux via an arsenite carrier protein, where energy is supplied by the membrane potential of the cell, and the other by an arsenite-translocating ATPase [36]. Two unrelated families of arsenite carriers have been identified (Fig. 2C). An arsenite-resistance membrane protein was identified in the SKIN element of *B. subtilis*, and is found in some other bacteria, archaea and fungi [37]. However, the majority of bacteria use ArsB, which is found in most *ars* operons, to extrude arsenite. When ArsA is co-expressed with ArsB, an ArsAB complex is formed that is obligatorily coupled to ATP. Some bacteria have three-gene *arsRBC* operons and extrude arsenite by ArsB alone, while others have five-gene *arsRDABC* operons and use the ArsAB pump [1]

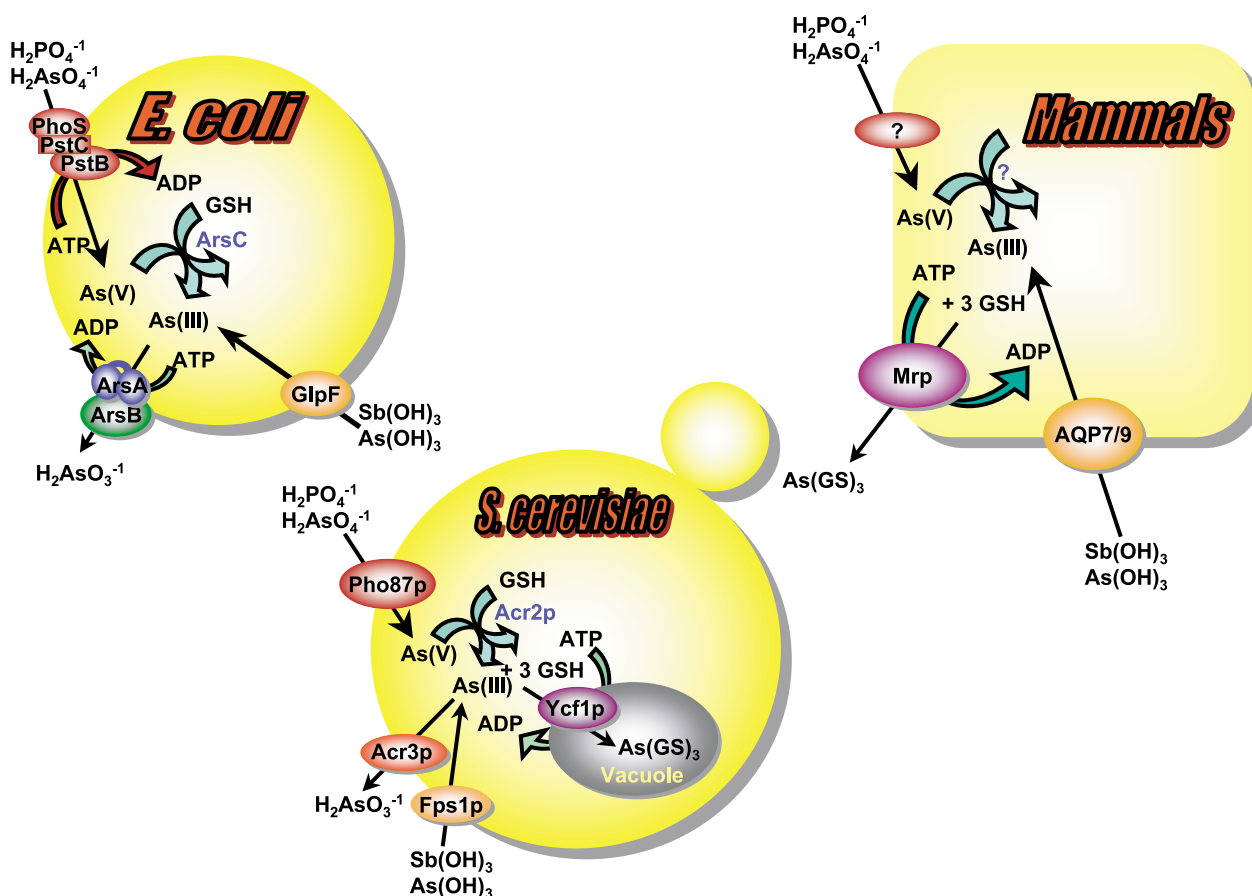


Fig. 1. Arsenical detoxification in prokaryotes and eukaryotes. Arsenate ( $\text{As(V)}$ ) is taken up by phosphate transporters, and  $\text{As(III)}$  is taken up by aquaglyceroporins (GlpF in *E. coli*, Fps1p in yeast and Aqp9 in mammals). In both *E. coli* and *S. cerevisiae*, arsenate is reduced to arsenite by the bacterial ArsC or yeast Acr2p enzymes. In both organisms, glutathione and glutaredoxin serve as the source of reducing potential [23,32]. The proteins responsible for arsenate uptake and reduction in mammals have not yet been identified. In *E. coli*, arsenite is extruded from the cells by ArsB alone or by the ArsAB ATPase [36]. In yeast Acr3p is a plasma membrane arsenite efflux protein, and Ycf1p, which is a member of the MRP family of the ABC superfamily of drug-resistance pumps, transports  $\text{As(GS)}_3$  into the vacuole. In mammals Mrp isoforms pump  $\text{As(GS)}_3$  out of cells. For example, Mrp2 extrudes  $\text{As(III)}$  into bile [11].

(ArsR and ArsD, which are  $\text{As(III)}$ -responsive repressors of the *ars* operons, are not discussed here; for a review, see [38]). We have speculated that the five-gene operons arose by insertion of the *arsDA* genes into a three-gene operon [1].

ArsB is a 429-residue integral membrane protein with 12 membrane-spanning segments [39]. It appears to be a uniporter that uses the membrane potential, positive exterior, to extrude arsenite. Even though the  $\text{pK}_a$  of 9.2 favors  $\text{As(OH)}_3$  over  $\text{As(OH)}_2\text{O}^-$ , the equilibrium ensures that some anion is always available to ArsB. *E. coli* has a chromosomal *arsRBC* operon that confers moderate resistance to arsenite. However, when ArsA is synthesized in *E. coli* from the plasmid R773 *arsRDABC* operon, cells are more resistant to arsenite because the ArsAB ATPase is much more efficient at arsenite extrusion than ArsB alone [36].

The 583-residue ArsA ATPase is a member of a family of ATPases that probably arose from GTPases [40]. It is normally bound to ArsB [41], but, in the absence of ArsB, ArsA is found in the cytosol and can be purified as a soluble protein. ArsA has two halves, A1 and A2, that are connected by a 25-residue linker [42]. A1 and A2 are homologous to each other, and each has a consensus nucleotide-binding sequence (NBS) [43]. Both sequences are required for activity, and considerable effort has been expended to elucidate the role of the

individual sites in arsenite transport (see [44] for a recent summary). The crystal structure of the enzyme has been determined [45]. Three types of domains can be resolved. First, there are two nucleotide-binding domains (NBDs). These domains are more than NBSs: the NBDs are folded structures that both contain residues from both A1 and A2. Second, there is a single metalloid-binding domain (MBD). This is an allosteric site at the opposite end of the protein from the NBDs. The MBD consists of a number of residues including Cys113, Cys172 and Cys422 [46,47] and His148 and His453 [48]. The MBD binds three  $\text{Sb(III)}$  or  $\text{As(III)}$ , and ArsA ATPase activity is activated by metalloid. Our working hypothesis is that the contacts between A1 and A2 are loose in the absence of metalloid, and the NBDs are not fully functional (Fig. 3). Binding of metalloid at the MBD acts as a 'molecular glue' that holds the A1 and A2 halves of ArsA in tight contact with each other. In this form the two NBDs are completed by contact with residues from both A1 and A2 and are thus fully activated.

Connecting the single MBD to the two NBDs are signal transduction domains, one in each half of the protein that can be recognized by a 12-residue signature sequence (D<sub>142</sub>TAPTGHITIRLL and D<sub>447</sub>TAPTGHITIRLL) [49]. ArsA homologs have been found in every sequenced genome of eubacte-

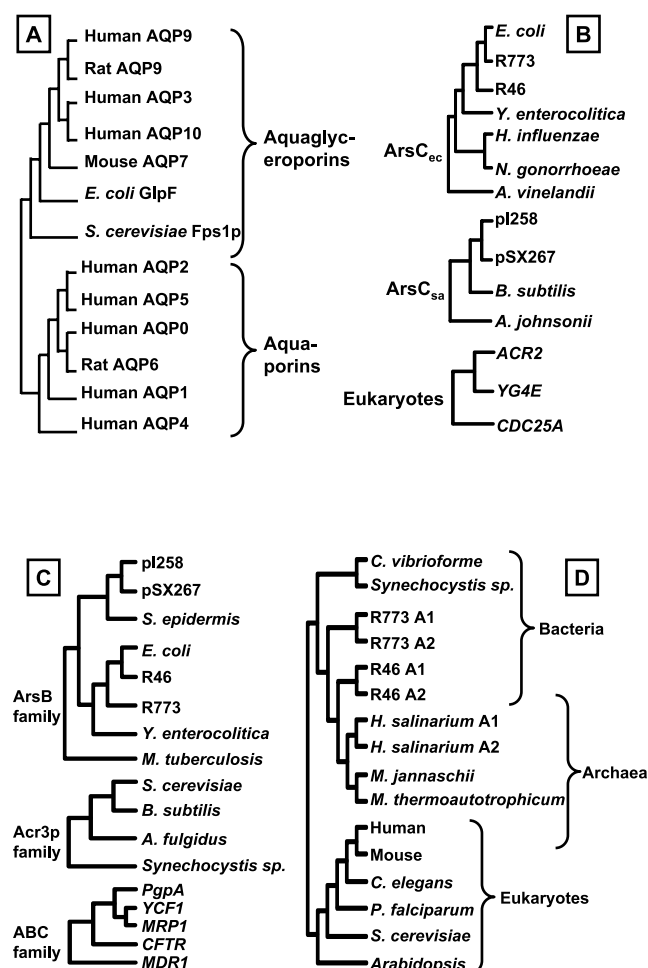


Fig. 2. Families of arsenic detoxification proteins. These families are growing rapidly, so only representative ones are shown to illustrate the nature of the lineages. The dendrograms were made using the CLUSTAL4 algorithm [67]. A: The aquaporin superfamily. There are two subfamilies of aquaporins [16]. The true aquaporins such as AQP1 allow only water through the channel. The aquaglyceroporins have larger pores and allow uncharged solutes such as glycerol and urea through. As(OH)<sub>3</sub> enters cells by members of this subfamily. B: Three families of arsenate reductases. Reductases involved in arsenate detoxification have apparently evolved independently at least three times [20]. One family is typified by the *E. coli* plasmid R773 ArsC that utilizes Grx and GSH as reductants. A second family represented by the *S. aureus* plasmid pI258 ArsC utilizes thioredoxin as a reductant and is related to a family of low-molecular-weight protein phosphotyrosine phosphatases. Acr2p belongs to the superfamily of PTPases that includes the Cdc25a cell cycle phosphatase. C: Three families of arsenite transporters. The ArsB family of arsenite uniporters has been so far identified only in bacteria [1]. The Acr3p family was first identified in the *B. subtilis* SKIN element, but members were subsequently found in yeast and archaea. The ABC superfamily of solute-translocating ATPases includes the Mrp family. Members of this family that have been shown to transport As(GS)<sub>3</sub> include the human Mrp1 and Mrp2, yeast Yfc1p and *Leishmania* PgpA. D: The ArsA family. The ArsA family is defined by the signature sequence DTAPTGHTRILL and is found in members of every kingdom [49]. Those involved in arsenic transport have two ATPase modules, with a duplicated A1–A2 structure [48]. All eukaryotic members thus far identified have only one ATPase module, and their physiological functions are not known [50].

ria, archeobacteria, fungi, plants and animals (Fig. 2D). However, the physiological roles of eukaryotic homologs are not known, although probably unrelated to arsenic resistance [50]. In the ArsA structure these sequence are seen as extended stretches of residues physically linking the NBDs through Asp142 and Asp447 at the N-terminal end to the MBD through His148 and His453. This physical connection between the domains allows ATP hydrolysis at the NBDs to alter the affinity for As(III), and, reciprocally, allows metalloid binding at the MBD to alter the affinity for and rate of hydrolysis of ATP.

This is most easily observed by the introduction of specific spectroscopic probes into strategic locations in ArsA (Fig. 4). The probes that we have used are single tryptophan residues whose fluorescent properties change in response to nucleotide binding, hydrolysis and/or conformational changes in domains [49,51–53]. In the crystal structure the two NBDs appear to be hidden within caverns formed by the interface of the A1 and A2 halves of ArsA [45]. The tryptophans serve as lamps that light up the caverns that hide the nucleotides from our view. The intrinsic fluorescence of Trp148 has allowed real time monitoring of the conformation of ArsA during the individual steps of the catalytic cycle and has allowed modeling of the reaction cycle [54,55]. The results suggest that the rate-limiting step in the overall reaction in the absence of metalloid activation is the isomerization of a long-lived conformation of the enzyme, and that binding of metalloid overcomes this rate-limiting step, increasing the rate of hydrolysis. This is not unlike the E1 to E2 transition of P-type ATPases, where a conformational change from one state to the other similarly distinguishes the reaction cycle.

In the crystal structure NBD1 is occluded and has ADP bound [45]. NBD2 is more open, and ATP can be exchanged into the site. This gives the appearance of an alternation of sites, and alternating site mechanisms are found in other transport ATPases [56,57]. Yet a single crystal structure is like a single frame of a movie. Whether the two NBDs look like two cylinders of a reciprocating engine cannot be determined from viewing one frame. As an alternative, the two sites could be performing different functions. There is good evidence to suggest non-equivalence of the two sites. NBD1 hydrolyzes ATP in the absence of activation while NBD2 does not [58]. For this reason, basal hydrolysis has been termed unisite catalysis. In the presence of metalloid, both sites hydrolyze ATP and participate in multisite catalysis. We have used the fluorescence of two single tryptophan residues, Trp141 and Trp446, to light up either NBD1 or NBD2. Trp141 is adjacent to NBD1 and to the N-terminal Asp142 of the A1 signature sequences. Trp446 is in the equivalent position in NBD2, next to Asp447 of the A2 signature sequence. The fluorescent properties of these two mutants allow measurement of the rate of hydrolysis in each site singly. The fluorescent properties of Trp141 clearly show that NBD1 hydrolyzes ATP during unisite catalysis [49]. In contrast, Trp447 fluorescence indicates that NBD2 is catalytically inactive until metalloid is bound, that is, only under multisite conditions [51]. Although the two NBDs are not equivalent in all respects, the existing data cannot yet distinguish between different functions for the two or alternating sites catalysis. The use of more such site-directed spectroscopic probes will greatly enhance our ability to understand the catalytic cycle of this ion-translocating ATPase.



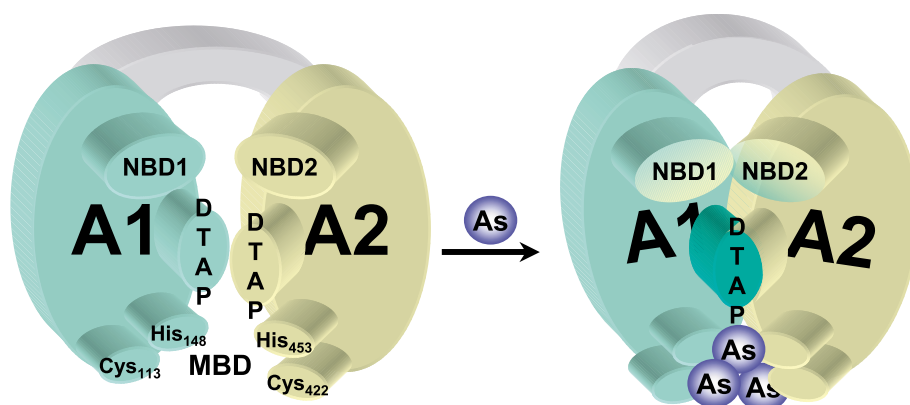


Fig. 3. Model for allosteric activation of ArsA by As(III). In the absence of As(III) activator, the A1 and A2 halves of ArsA are loosely interacting, with only the A1 NBD exhibiting a basal rate of ATP hydrolysis (unisite catalysis) [51,58]. Binding of Sb(III) or As(III) to the MBD 'glues' the two halves of ArsA together, completing the NBDs and accelerating catalysis [49,68].

### 5. Eukaryotic arsenic extrusion systems

Arsenite resistance is conferred by members of the MRP (multidrug resistance-associated protein) group of the ABC superfamily of transport ATPases [59] (Fig. 2C), which physiologically catalyze export of GS-conjugates such as leukotriene C<sub>4</sub> (LTC<sub>4</sub>) [60]. MRP1-catalyzed export of glutathione from cells was increased by arsenite, suggesting that MRP1 functions as a As(GS)<sub>3</sub> carrier [61]. In the liver MRP2 ex-

trudes arsenic–glutathione complexes into bile and may be a major route of arsenic detoxification in humans [11].

MRP homologs have been shown to confer arsenic resistance in eukaryotic microbes. Metalloid-containing drugs are still the first line therapy for trypanosomiasis and leishmaniasis, and clinical resistance is a serious problem in treatment. In arsenite-resistant strains selected in vitro there is increased expression of *pgpA*, which encodes an MRP homolog [62]. We have recently demonstrated that PgpA transports As(GS)<sub>3</sub>

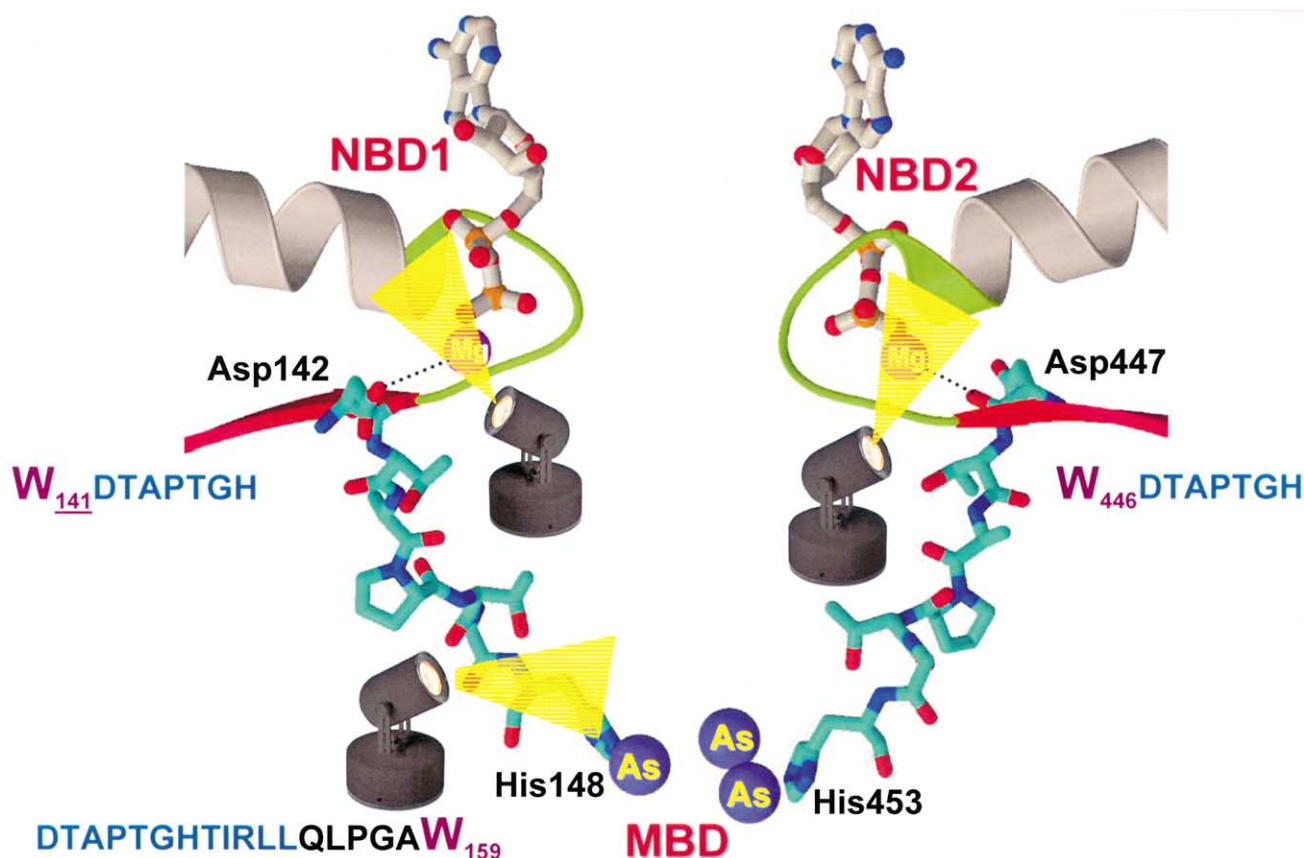


Fig. 4. Lamps into the caverns of the nucleotides. Single tryptophans introduced into specific locations of ArsA by site-directed mutagenesis serve as spectroscopic probes of the two NBDs. Three single tryptophan ArsA mutants were constructed containing Trp159 [49,52], Trp141 [49] or Trp446 [51]. Trp141 'shines' directly into NBD1 and shows that NBD1 is active in both unisite and multisite catalysis. The equivalent residue in A2, Trp446, 'lights up' NBD2 and shows that it is not active during unisite catalysis but is during multisite catalysis. Trp159 'illuminates' the MBD and allows the transition from unisite to multisite catalysis to be quantified [54].

[63]. In *S. cerevisiae* an MRP homolog, Ycf1p, has been shown to confer Cd(II) resistance by pumping Cd(GS)<sub>2</sub> into the vacuole [64,65]. We have demonstrated that Ycf1p also transports As(GS)<sub>3</sub> into the vacuole and confers arsenite resistance in yeast [10]. In addition to Ycf1p, *S. cerevisiae* has a gene cluster of three ACR genes that also confer arsenic resistance [28]. Acr1p is a transcription factor, and Acr2p is the yeast arsenate reductase described above. ACR3 encodes a membrane protein that is homologous to the *B. subtilis* SKIN element arsenite carrier protein and confers arsenite resistance [10,66]. While Ycf1p is located in the vacuolar membrane and catalyzes sequestration of As(GS)<sub>3</sub> in the vacuole, Acr3p is a plasma membrane carrier protein that catalyzes extrusion of arsenite from cytosol. Thus these two systems are independent of each other and provide parallel pathways for arsenic detoxification in yeast. Acr3p homologs have not yet been found in mammals.

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