Unraveling the inner workings of respiratory arsenate reductase

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It began back in 1994 with a short note in the journal *Nature*, about a curious bacterium from the Aberjona watershed, strain MIT-13, that could grow on arsenic (1). Arsenic resistance had been well established, as it had been found in many clinical species like *Staphylococcus aureus* and *Escherichia coli*. Arsenite oxidation linked to resistance was also known. However, this was different—the organism, later to be named *Sulfurospirillum arsenophilum* (2), could couple the oxidation of lactate to the reduction of arsenate [As(V)] for growth. More significantly, incubation experiments revealed dissolution and reduction of arsenic from the sediments. This suggested that such organisms could be responsible for mobilizing arsenic in aquifers, resulting in the poisoning of millions of people worldwide. As more researchers began investigating microbial arsenic metabolism, it became apparent that not only was there a robust biogeochemical cycle, but arsenic played a role in the evolution of life on Earth (3). Thus began the journey to decipher how these organisms were capable of harnessing the energy from arsenic oxyanions. Now, Glasser et al. (4) have found the holy grail by solving the crystal structure of the respiratory arsenate reductase, Arr, from *Shewanella* sp. ANA-3. In doing so, they answer several questions, including the type of iron sulfur clusters in both ArrA and ArrB, the coordinating ligand to the molybdenum (cysteine, as had been predicted), the nature of the catalytic pocket, the binding of arsenic to the reaction site, and the mechanism of substrate transformation. Furthermore, they propose a reasonable solution to the preferred electron flow in these bidirectional enzymes.

The respiratory arsenate reductase, Arr, was established both biochemically and through gene knockout studies (5–7). Once key conserved regions in the protein sequence were recognized, more examples were found in both Bacteria and Archaea. Although the operon structure varied, with different anchoring subunits (e.g., ArrC, CymA), chaperone proteins (ArrD), and regulatory elements, the core enzyme, consisting of the large catalytic subunit ArrA and the smaller iron–sulfur cluster subunit ArrB, was highly conserved (8). Interestingly, the need for both subunits for enzyme activity was found regardless of the organism (5–7). So one of the first mysteries solved in the current work was the significance of the fourth iron–sulfur cluster [4Fe4S] in ArrB (the binding site for the electron transfer partner, in this case, methyl viologen).

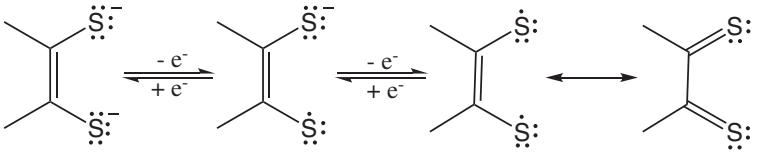

Fig. 1. Redox noninnocence of dithiolene unit in the pyranopterin cofactor.

Another important feature was in the large subunit. Like other members of the DMSO reductase family of enzymes, the catalytic Mo center of ArrA is coordinated by two equivalents of the pyranopterin cofactor (present as the guanine dinucleotide). In addition to the four sulfur atoms from the pyranopterin cofactor, the Mo center is coordinated by a cysteine sulfur (C193) similar to DMSO reductase with its serinato oxygen coordination (9). In addition, a long Mo–O bond has been detected that is presumably due to the reduction of a Mo=O unit in the synchrotron radiation. This completes six coordination sites about the Mo center with a distorted octahedral overall geometry as opposed to trigonal prismatic structures found in DMSO reductase and polysulfide reductase (PsrA). The composition of the first coordination sphere is very similar to that of polysulfide reductase (10) and the initial structure of periplasmic nitrate reductase (NapA) (11) that has subsequently been revised (12). Thus, compositionally the Mo center is similar to other members of the DMSO reductase family.

A related enzyme arsenite oxidase, Aio, catalyzes the oxidation of arsenite. The crystal structures of Aio from two different organisms, *Rhizobium* sp. NT-26 and *Alcaligenes faecalis*, have shown no coordination of a protein-based ligand to the Mo center (13, 14). It

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has long been suggested that different amino acid coordination sets the substrate specificity. Reactivity toward other substrates has been examined for a select few enzymes in this class. For example, NapA from *Paracoccus denitrificans* cannot reduce arsenate and ArrA from *Bacillus selenitisreducens* MLS 10 or *Shewanella* strain ANA 3 cannot reduce nitrate, even though their immediate coordination environments are very similar. Even more striking is that the related bidirectional enzyme system, the ArxAB complex, whose coordination environment should be very similar to ArrAB, can carry out the reverse of the physiological reaction, oxidation of arsenite to arsenate, while AioAB whose physiological function to oxidize arsenite cannot reduce arsenate (15). These differences in reactivity cannot be completely understood considering the nature of the first coordination sphere alone. The current paper (4) discusses the presence of a second binding site that may be used for phosphate binding. The presence of a second binding site provides an excellent opportunity in understanding the substrate transformation.

One of the intriguing aspects of the crystallographic studies is that the conformation of the pyranopterin cofactor changes upon binding of the substrate, arsenate. The S–C–C–S fragment exhibits a small but definable deviation from planarity upon substrate binding that has been interpreted as due to a reduction of the dithiolene fragment. Dithiolene ligands are well known for their redox noninnocent behavior. In the fully reduced state, they can undergo two one-electron oxidations yielding the fully oxidized dithione form; during the process, the S–C–C–S torsion angle can change (Fig. 1). The conformational changes reported in ArrAB structures underscore the involvement of pyranopterin cofactor in the reaction process.

The dithiolene is not the only redox active unit in the pyranopterin cofactor, as the pterin itself can exhibit a variety of redox states from the fully reduced tetrahydro to the partially reduced dihydro to the fully oxidized state (16). The conformation of the pyranopterin cofactor provides a hint to the redox state with the tetrahydro state being more distorted than the dihydro. The conformational analyses of the cofactor in crystallographically characterized enzymes have provided a basis for establishing the redox states in those enzymes (17, 18). In this case, the conformation change indicates the role of the pyranopterin cofactor, at least the P pterin (the pterin distal from the partner [4Fe4S] prosthetic group) in electron transfer.

The substrate transformation of Arr occurs at the Mo center, and the proposed mechanism involves a direct oxygen atom transfer reaction from the substrate to the reduced Mo(IV) center (4). The reaction scheme is consistent with that proposed for DMSO reductase (9). The catalytic center is regenerated by two one-electron transfer reactions from the ArrB subunit. The crystal structure of the ArrAB complex provides a roadmap for the electron transfer pathway in the enzyme. The smaller ArrB subunit shows the arrangement of four [4Fe4S] clusters that are electron transfer partners of the Mo center.

To date, no EPR signal attributable to a Mo(V) species has been reported for Aio complexes. The electrochemical behavior of this system examined by voltammetry indicates that the thermodynamically uphill redox step at the Mo center is an obligate two-electron process. In contrast, the ArrAB complex reported here exhibits EPR signals due to Mo(V) species by reducing the fully oxidized enzyme with a mixture of dithionite, arsenate, and methyl viologen. The Mo(V) species exhibit near axial EPR features. A small hyperfine structure was detectable in ENDOR and pulsed EPR experiments conducted in the Q-band. The small hyperfine was interpreted as due to the β-proton present in coordinating cysteine, not to a Mo–OH species. This suggests that the Mo–OH intermediate is not accumulating in an appreciable quantity during the experiment. With a higher concentration of methyl viologen, a Mo–arsenite species was observed in the EPR experiments. The EPR signals due to the iron–sulfur clusters were observed as expected. The observation of the EPR signals strongly supports the notion that mechanistically Aio and Arr behave differently.

The current work by Glasser et al. marks a milestone in the structure/function of arsenic enzymes. It has established a clear mechanistic difference between the classic arsenite oxidase (Aio) and respiratory reductase (Arr) and provided a model from which to investigate Arx.

Microbial activity can have a significant impact on the toxicity and mobility of arsenic (3). Although arsenate respiring bacteria are typically grown in media containing millimolar amounts of As(V), in the environments in which they live the concentration is often micromolar or the arsenic is bound in the sediment. Interestingly, all three enzymes that have been linked to respiration (e.g., Aio, Arr, and Arx) are topologically located on the outer side of cytoplasmic membrane, facing into the periplasmic space. Arr has both high affinity (K_m of 44.6 μM) and fast kinetics (k_{cat} of ~10,000 s^{-1}), more than compensating for the less than optimal orientation for the generation of a proton gradient. Glasser et al. surmise that the rate-limiting step is either other components of the electron transfer chain (e.g., CymA, ArrC), or the diffusion rate of solid-phase arsenic (4). Interestingly, AioAB from *Rhizobium* sp. NT-26 also functions near the diffusion control limit ($k_{cat}/K_m \sim 10^8$), while Aio from *A. faecalis* is less efficient ($k_{cat}/K_m \sim 10^6$) (9). This suggests that the respiratory enzymes function at a higher efficiency than the resistance enzymes. It will be interesting to see whether the arsenite oxidases involved in light-dependent arsenite oxidation (e.g., Aio in *Chloroflexus aurantiacus*, Arx in *Ectothiorhodospira* spp.) behave similarly.

It has indeed been a remarkable journey over the past two and a half decades, one in which our understanding of arsenic in the environment and in microbiology has changed dramatically. The current work by Glasser et al. marks a milestone in the structure/function of arsenic enzymes. It has established a clear mechanistic difference between the classic arsenite oxidase (Aio) and respiratory reductase (Arr) and provided a model from which to investigate Arx. Furthermore, their advancement in overexpression of native proteins should prove extremely useful in the investigation of complex, multisubunit, metalloenzymes.

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