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Arsenate Replacing Phosphate: Alternative Life Chemistries and Ion Promiscuity[†]

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ABSTRACT: A newly identified bacterial strain that can grow in the presence of arsenate and possibly in the absence of phosphate, has raised much interest, but also fueled an active debate. Can arsenate substitute for phosphate in some or possibly in most of the absolutely essential phosphate-based biomolecules, including DNA? If so, then the possibility of alternative, arsenic-based life forms must be considered. The physicochemical similarity of these two oxyanions speaks in favor of this idea. However, arsenate-esters and arsenate-diesters in particular are extremely unstable in aqueous media. Here, we explore the potential of arsenate to be used as substrate by phosphate-utilizing enzymes. We review the existing literature on arsenate enzymology, that intriguingly, dates back to the 1930s. We address the issue of how and to what degree proteins can distinguish between arsenate and phosphate and what is known in general about oxyanion specificity. We also discuss how phosphate−arsenate promiscuity may affect evolutionary transitions between phosphate- and arsenate-based biochemistry. Finally, we highlight potential applications of arsenate as a structural and mechanistic probe of enzymes whose catalyzed reactions involve the making or breaking of phosphoester bonds.

A recently published article in *Science* describes a newly discovered bacterium isolated from the unique environment of California's Mono Lake. The authors provide data that indicate the presence of arsenate in nucleic acids and proteins. They thereby argue that arsenate can be a viable substitute for phosphate in the DNA of the *Halomonadaceae* (a family of halophilic protobacteria) GFAJ-1 strain (*1*). Following the four most abundant elements, carbon, hydrogen, nitrogen, and oxygen, phosphorus comprises one of the major elements of life. Foremost, phosphorus is a key element in the metabolic currencies of all known life forms. In addition, phosphate provides the connecting bridge between the nucleobases in RNA and DNA. That arsenic can potentially take over any of the roles of phosphorus is therefore a paradigm shift with far-fetched implications. Indeed, Wolfe-Simon et al. have raised the intriguing possibility that arsenate-based life forms may have existed at the early stages of evolution of life on this and potentially on other planets (*2*, *3*).

INTRIGUING YET CONTROVERSIAL FINDINGS

Why is arsenate an attractive option? Phosphate is not an uncommon compound in the earth's crust, but it is relatively scarce in oceans (where the first life forms may have emerged). Unlike the major elements (C, N, O, and H), there exists no common gas form of phosphorus that could have made it available to those niches where life emerged. Even for present life forms, phosphorus is a rate-limiting factor for growth. For example, some soil bacteria have evolved to exploit phosphate-containing pesticides (organophosphates), presumably to supplement shortages in inorganic phosphate (*4*, *5*). Arsenic and phosphorus share key chemical properties, including oxidative states. In its most common 5+ (V) oxides, arsenate ($HAsO_4^{-2}$) exhibits very similar pK_a values to those of phosphate (HPO_4^{-2}) and forms analogous esters. Arsenic rich environments are available on earth, and these locations may have been particularly relevant when life originated (*3*). Why then did nature choose phosphorus? The reasons for this preference are quite clear (*6*): The reduction of As(V) to As(III) is much easier than that for phosphorus, allowing phosphorus to be more stable in the preferred higher oxidation state. In particular, as further discussed below, arsenate esters are notoriously unstable in water, and rapid hydrolysis of the corresponding high-energy arsenate esters leads to wasteful "futile cycles" (*7*). This higher reactivity with water would also create serious problems for the long-term stability of the repository of genetic information for life forms containing arsenate-linked nucleic acids.

While Wolfe-Simon et al. raise an intriguing hypothesis about possible alternative life chemistries, their supporting data are rather slim. Several scientists have already criticized the published data (*8*, *9*), and our reaction upon reading the paper has been similar. The isolated strain grows faster on arsenate on its own than on phosphate on its own (Figure 1A in ref *1*). However, the growth rate under the condition which might be optimal for this strain, namely, high concentration of arsenate *plus* low phosphate concentration, has not been reported. DNA preparations from the newly isolated strain have been shown to contain arsenate, but these preparations also contain phosphate. Arsenate may be associated with the DNA, but no direct evidence has been provided to support the hypothesis that arsenate substitutes for phosphate in the DNA's base-linking diester bonds. In addition, whereas total nucleic acid preparation (DNA *plus* RNA samples analyzed for As and P content) from the cells grown on

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phosphate appear normal by gel-electrophoresis, the arsenate grown bacteria yielded only one band of discrete size (possibly chromosomal DNA) and no low molecular weight smear that usually corresponds to RNA (Figure 2A in ref *1*). It could therefore be that growth under arsenate induces a drastic change in the nucleic acid content and/or properties, as suggested by the authors. These nucleic acid samples extracted with standard methods, and examined in aqueous solution may therefore be irrelevant. Finally, the DNA from bacteria grown on phosphate only contains also arsenate, and vice versa. However, where the contaminating elements come from remains unclear.

HYDROLYTIC INSTABILITY OF ARSENATE ESTERS

Compared to the extensive literature on the hydrolysis of phosphate esters, data on the hydrolysis of arsenate esters is quite limited. It is known, however, that arsenate esters, including tri-, di-, and monoesters, and pyro-arsenate, hydrolyze many orders of magnitude faster than the equivalent phosphate esters. The mechanisms of hydrolysis also differ, with arsenate esters hydrolyzing via mechanisms with a more associative character (ref *10* and references therein). High reactivity and the use of a more associative mechanism result in a fundamental difference in the hydrolysis kinetics between phosphate and arsenate esters. Phospho-triesters are hydrolyzed $\sim 10^5$-fold faster than the corresponding diesters. The latter comprise the connecting bonds in DNA, and their remarkable stability is critical for genome integrity (*6*). Arsenate-triesters are highly labile, and the half-life of trimethyl-arsenate in water, at neutral pH and ambient temperature, is in the range of 0.02 s. However, unlike the analogous phosphate esters, the resulting dimethyl ester of arsenate hydrolyzes even more rapidly (extrapolating from the data of ref *10*). The 5′- and 3′-ribosyl groups of DNA bases exhibit about a 10-fold lower rate of hydrolysis than methyl groups (diethyl-arsenate hydrolyzes ~5-fold slower than dimethyl-arsenate) (*10*), but beyond that, no fundamental differences in reactivity between the dialkyl-arsenates used as a model and the diester bonds of DNA are expected. The corresponding arsenate derivatives of small metabolites such as NTPs and dNTPs (nucleotide and deoxy-nucleotide triphosphates) are expected to be even more labile and will not be sustained in aqueous environments.

Therefore, how would an arsenate-based DNA maintain its vital genetic information along generations? Wolfe-Simon et al. suggest that DNA and perhaps other key molecules might be stored in vacuole-like regions, and that these nonaqueous environments may prevent or at least slow down hydrolysis (*1*). However, arsenate esters are labile even when water is present at low concentrations: Baer et al. report for trimethyl-arsenate half-lives of < 0.1 s at pH ~9 with 0.25 M water in organic solvent, i.e., $< 0.5\%$ water (*10*). Another possibility is that "Arsenic-based life simply has a higher turnover for molecular disintegration and assembly than does conventional life", as stated by one of the authors (Paul Davies, Arizona State University) (*8*). This is not unprecedented; the extremophilic bacterium *Deinococcus radiodurans* that can resist extreme levels of ionizing radiation possesses a highly robust DNA repair system capable of reassembling its constantly fragmented genome. The reassembly of the fragmented DNA in this organism is based on diploidy and the annealing and elongation of overlapping fragments (*11*). Still, repairing frequent strand breaks and dealing with the rapid hydrolysis of nearly every base-connecting bond are two quite different scenarios.

Other complications for the incorporation of arsenate-based compounds in living organisms arise from the low reduction potential of arsenate (*6*). Free thiols can readily reduce arsenate to give As(III) trithiolates and oxidized dithiols (*12*). Although the arsenite-thiolates hydrolyze at pH > 7, this chemistry implies a highly oxidizing environment where reduced cysteines and other free thiols would be hard to maintain.

THE ARSENATE−PHOSPHATE ANALOGY

Whether or not *Halomonadaceae* GFAJ-1 uses arsenate instead of phosphate in its macromolecules (RNA, DNA, proteins) and/or small molecules remains controversial, but this work does provide preliminary indications for such a possibility and will thus inspire many to examine various aspects of arsenate biochemistry. Skepticism is an essential trait of every good scientist, and making scientists doubtful or even angry is a proven inspirational tool for many previous paradigm shifts (*13*).

From our own point of view, beyond the above-discussed issues, this work opens several interesting questions with respect to the oxyanion promiscuity of enzymes and specifically regarding the potential of arsenate to be used as substrate by phosphate utilizing enzymes. How readily can this replacement occur? What are the possible consequences of such replacements? We are obviously not the first to be interested in this problem. Some of the 20th century's most notable enzymologists, Otto Warburg, Frank Westheimer, and Henry Dixon, have each addressed this issue (refs (*6* and *7*), and references therein). However, these first raised interests in the enzymology of arsenate and other arsenic derivatives have not been sustained, and at present, the enzymology of arsenic derivates is rarely explored.

Here, we review the sparse existing literature on arsenate enzymology and discuss the relevance of these studies to the hypothesis of arsenate-based life forms. We address the issue of to what degree and how proteins can distinguish between arsenate and phosphate and what is known in general about oxyanion specificity. We also discuss how the lack of ion specificity (i.e., ion promiscuity) may affect evolutionary transitions between phosphate and arsenate-based biochemistry. Finally, we highlight potential applications of arsenate as a structural and mechanistic probe of enzymes whose catalyzed reactions involve the making or breaking of phosphoester bonds.

ENZYME PROMISCUITY WITH ARSENATE

Arsenic and phosphorus share many physicochemical properties. However, their atomic radii and electron shells differ, hence the below discussed different reactivities of their esters. The bond lengths and relative charges on the arsenic and oxygens of arsenate also vary from the corresponding phosphate: P−O bond lengths are 1.52−1.54 Å versus 1.68−1.71 for As−O (*7*, *14*). The partial negative charge on the oxygen atoms is −0.952 for phosphate versus −0.892 for arsenate (*14*). This difference, although relatively small, also affects the nucleophilicity of these ions. The overall sizes of these ions are, however, quite similar: the thermochemical radius for phosphate is 2.38 Å, and that of arsenate is 2.48 Å (*15*).

Could enzymes that utilize phosphate or phospho-esters bind the equivalent arsenates, and if so, would they utilize them as substrates? The main hurdle for exploring this question is stability. Arsenate ($HAsO_4^{-2}$) can be used as a substitute for inorganic phosphate, but because most phosphate liberating processes are effectively irreversible (e.g., ATP → ADP + Pi),

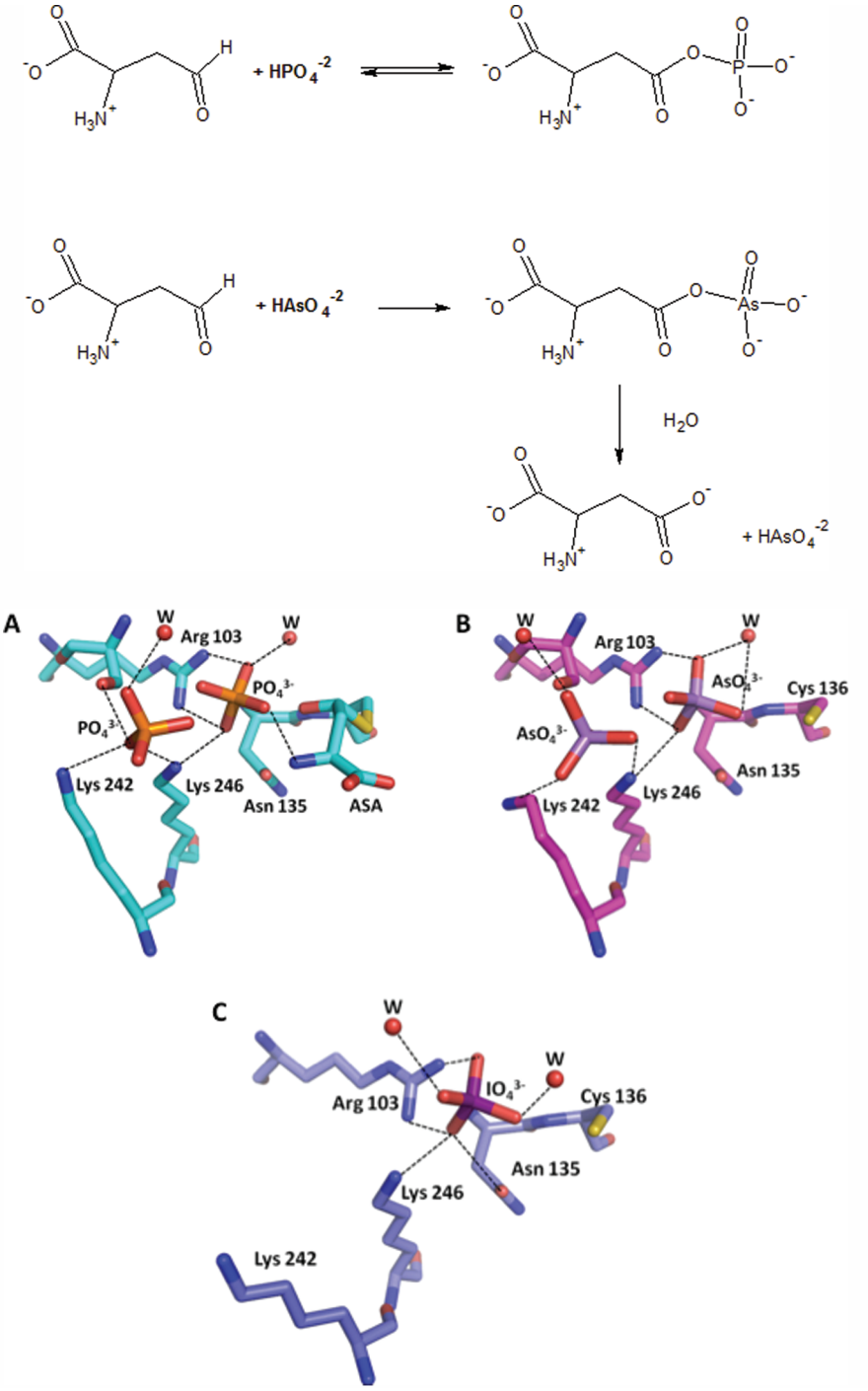

FIGURE 1: Reactions catalyzed by L-aspartate-β-semialdehyde dehydrogenase with phosphate and arsenate (top panel) and structures of the enzyme complexes with different oxyanions (bottom panel; PDB codes 1NX6, 1TA4, and 1TB4; adopted from ref *18*). (a) The phosphate-bound structure with the substrate, aspartate-β-semialdehyde (ASA), as a covalent adduct with active-site Cys136. (b) The arsenate-bound structure. The positions of the bound arsenate ions and the interacting protein ligands are identical for phosphate and arsenate. (c) The periodate-bound structure. Unlike the binding of the oxyanion substrates phosphate and arsenate, periodate is an inhibitor of this enzyme. Only one molecule of periodate is bound, causing conformational changes that alter the alignment of the active site functional groups into a non-catalytic configuration (Ref. 18).

the number of enzymes that use inorganic phosphate as substrate is limited (for example, only about a dozen such enzymes could be identified in the *E. coli* genome using EcoCyC). Nevertheless, several key phosphate utilizing enzymes have been explored with arsenate (*7*, *14*, *16*, *17*), and two detailed examples are discussed below.

The protein data bank (PDB) contains two enzyme structures with a bound arsenate that replaces phosphate. One structure is L-aspartate-β-semialdehyde dehydrogenase (*H. influenzae* ASADH), an enzyme that reversibly catalyzes the reductive dephosphorylation of β-aspartyl phosphate to give L-aspartate-β-semialdehyde. The position of the bound arsenate, and those of its protein ligands, are identical for both phosphate and arsenate. Critical hydrogen bonds with catalytic residues and with a water molecule ligand are also maintained (*18*) (Figure 1). Accordingly, arsenate comprises a surprisingly good substrate for this enzyme, both in terms of K_M (1.6 mM, versus 2.9 mM for phosphate) and k_{cat} (510 and 710 min^{-1}) (*14*). The primary difference between the

phosphate versus the arsenate reaction is the product: whereas phosphate yields β-aspartyl-phosphate, a somewhat unstable acyl phosphate that can nevertheless be easily isolated, the corresponding arsenate product rapidly hydrolyses to yield aspartic acid and free arsenate. In fact, ASADH was examined with a variety of potential oxyanions. Several ions were found to be effective inhibitors of this enzyme, including tungstate and iodate. The latter has been shown to bind in ASADH's active-site in an altered manner that explains the inhibitory effect. Of those oxyanions examined, only vanadate and arsenate comprised alternative substrates, with arsenate being as good a substrate as phosphate, while vanadate showed a 2-fold lower k_{cat} but a 20-fold lower K_M and hence ~10-fold higher k_{cat}/K_M than phosphate (*14*).

Arsenate and vanadate, as well as molybdate, comprise alternative substrates for another well-characterized dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This enzyme had been explored with arsenate by Otto Warburg, as early as 1939. As is the case with ASADH, the acyl-arsenate product rapidly decomposes (estimated half-life is < 2.5 s), resulting in a futile cycle and an irreversible reaction (ref (*7*) and references therein).

Another case of an arsenate-bound structure is purine nucleoside phosphorylase (PNP). This enzyme catalyzes the reversible phosphorolysis of various nucleosides including inosine and guanosine, to yield the free bases and D-ribose-1-phosphate. PNP also catalyzes the arsenolysis of inosine and guanosine to yield the corresponding arsenic-containing product. However, in opposition to D-ribose-1-phosphate, ribose 1-arsenate is unstable and rapidly hydrolyzes to ribose and arsenate, thus rendering the reaction irreversible (*17*). As in other cases of arsenate-ester products, the hydrolysis of ribose 1-arsenate is not catalyzed by the enzyme but occurs spontaneously (*17*). The K_M values for PNP (human erythrocyte) are similar for arsenate and phosphate (1.8 mM and 0.8 mM, respectively) (*19*). While we could not identify a reference for the k_{cat} values, the microscopic parameters measured with arsenate as a substrate indicate that the rate-limiting step is dissociation of the enzyme-free-base complex, suggesting that the rate of chemistry with arsenate is not significantly slower than that with phosphate. The structure of *Plasmodium falciparum* PNP was solved with inosine and arsenate, and was found to contain a mixture of products and reactants: hypoxanthine, ribose, and arsenate (*20*). The arsenate is ligated in a prototypic phosphate binding site, through bidentate interactions to 2−3 arginyl residues along with an additional side chain and backbone donor atoms, and is seen to be in an attacking position with respect to C1 of the ribose (Figure 2).

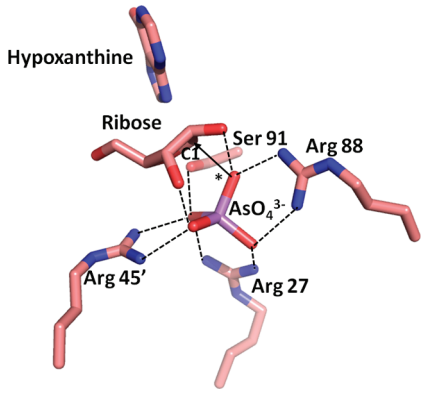

FIGURE 2: *Plasmodium falciparum* PNP's active site with bound hypoxanthine (the free base, in cyan), ribose (cyan), and arsenate (purple) (PDP code: 3ENZ). The arsenate position corresponds to the nucleophilic attack by one of the oxygens (marked as O*) on the CI of ribose (O*-C1 distance is 2.3 Å). The arsenate ion is bound via hydrogen bonds (dashed lines) to three arginine side chains (Arg 88, Arg 27, and Arg 45′ of the neighboring subunit). Other hydrogen donors to the bound arsenate include the hydroxyl group of Ser 91 and its backbone amide nitrogen.

ENZYME PROMISCUITY WITH ARSENATE ESTERS

In contrast to arsenate, the repertoire of arsenate analogues of phosphate esters that can be explored is limited because arsenate esters are unstable in aqueous solution. Nonetheless, several enzymes that act on phospho-monoesters have been examined via the *in situ* enzymatic synthesis of the arsenate-monoester, and found to readily accept the corresponding R-O-AsO_3^{-2} esters (e.g., glucose-6-arsenate; see ref *7* and references therein). In addition, alkylarsonic acids (R-CH_2-AsO_3^{-2}) are stable and can be used, similarly to phosphonates (R-CH_2-PO_3^{-2}), as nonhydrolizable analogues of phospho-monoesters (ref (*7*) and references therein). However, the replacement of oxygen by methylene generally renders these analogues inactive since enzyme pockets appear to be highly specific toward oxygen atoms at the reaction's center. Thus, for example, the arsonomethyl analogue of ADP is a very poor substrate for enzymes for which ADP reacts via the β-phosphate group (*7*).

That being said, an interesting precedent for accepting an R-CH_2-AsO_3^{-2} instead of R-O-PO_3^{-2} relates directly to the issue of whether nucleic acids may include arsenate. Dixon et al. examined the reverse reaction of *E. coli* RNA polymerase, i.e., the reaction of RNA and pyrophosphate to give dNTPs. Pyrophosphate was replaced with arsonomethyl-phosphonic acid ($^{-2}O_3$P-CH_2-AsO_3^{-2}), and the reaction did occur, indicating that the polymerase accepted the CH_2-AsO_3^{-2} replacement of phosphate. However, because the corresponding arsenic analogue of dNTPs rapidly hydrolyses, the isolated products were dNMPs (deoxynucleotide mono phosphates) (*21*). The polymerase therefore acted as a nuclease with these alternative substrates (*7*). As discussed above, such futile cycles potentially underlie all enzyme reactions with arsenate esters as the substrate instead of phosphate esters.

PROMISCUITY TOWARD OTHER ARSENIC-BASED SUBSTRATES

Replacements of -O-P- with CH_2-As- are not strictly isosteric and are rarely of potential metabolic relevance. Nevertheless, the RNA polymerase and other cases provide clear indications that enzymes tend to mistake arsenate for phosphate, not just in binding but in some cases also for catalysis. The relatively few cases where C-P- bonds are relevant do provide interesting insights with respect to ion selectivity. In cases where C-PO_3^{-2} (a phosphonate group) comprises part of the substrate, but the C−P bond is not transformed, the analogous arsonate substrate (C-AsO_3^{-2}) seems to be well accepted. For example, a transaminase, that convert 2-aminoethyl-phosphonate to the 2-oxoethyl-phosphonate while releasing ammonia enables certain bacteria to utilize the former as a sole nitrogen source. The enzyme readily accepts 2-aminoethyl-arsonate as substrate, thus enabling growth on this compound as a nitrogen source (Figure 3). However, the subsequent reaction that involves breakage of the C-P bond (converting 2-oxoethylphosphonate into acetaldehyde and phosphate by phosphonoacetaldehyde hydrolase) does not occur in the case of the arsonate analogue (*22*).

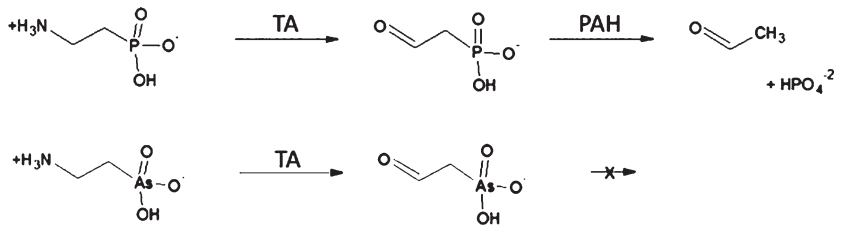

FIGURE 3: Reactions catalyzed by *P. araginusa* transaminase (TA) and phosphonoacetaldehyde hydrolase (PAH) on 2-aminoethyl-phosphate and on its arsenic analogue 2-aminoethyl-phosphonate.

This lack of distinction between arsonate and phosphonate is reflected not only in the promiscuity of this enzyme but also in the growth of bacteria on 2-aminoethylarsonate instead of 2-aminoethylphosphate (*22*). The latter indicates the promiscuity of transporters and of regulatory proteins (transcription factors, etc.). Indeed, it is generally assumed that arsenate penetrates cells via phosphate transporters that exhibit similar affinities to both ions, although certain mammalian transporters have been shown to exhibit 10−40-fold lower affinities to arsenate than to phosphate (*23*).

Another enzyme that transforms a C−P bond, phosphoenolpyruvate mutase, has been examined with the analogous C−As substrate. The K_M value was similar with this alternative substrate, suggesting that recognition was not adversely affected. However, k_{cat} for the synthesis of the 3-arsonopyruvate analogue was reduced by 6000-fold (*24*).

The above examples indicate that in contrast to the oxygens of arsenate and phosphate (and of arsonate and phosphonate), which are poorly distinguished when carbon−phosphorus bonds are directly involved in the enzymatic reaction, enzymes can readily distinguish between phosphorus and arsenic (as is probably the case for the distinction between other similar elements such as sulfur and selenium).

STRUCTURAL ASPECTS OF OXYANION PROMISCUITY

What about the structural protein features that underline arsenate−phosphate binding and the above-discussed lack of discrimination? There are very few examples where arsenate has been specifically added to form enzyme complexes for structural studies. However, cacodylate (dimethylarsenic acid), a common buffer that has been incorporated into many crystallization screening kits, is seen in numerous crystal structures within specific binding sites. Examining the binding interactions seen with cacodylate may provide some insights into the nature of arsenate and other oxyanion recognition. Because of its two C−As bonds, cacodylate is not a precise mimic of natural phosphate containing compounds, yet the fact that it can selectively bind to enzyme binding sites further supports the hypothesis that proteins hardly discriminate between the oxides of these closely related elements.

Our analysis of nearly 90 structures in the PDB in which the nature of the interactions to cacodylate has been described, indicates that almost half are monodentate interactions, either to a wide range of amino acid side chains (with histidine, arginine, and carboxylate groups being most prominent) or to a bound metal ion (mostly Zn^{2+}). The next most prominent examples are bidentate interactions with a metal ion and an amino acid side chain, followed by bidentate interactions to different amino acid side chain or backbone amide groups. It is likely that the high concentrations of cacodylate used (typically 100 mM) leads to nonspecific binding to sites with only one or two well-defined protein ligands. However, there are several examples of potentially more selective oxyanion binding sites in which cacodylate is interacting through its oxygen atoms to three or more amino acid side chains or backbone amide groups.

An even more common oxyanion used in protein crystallization is sulfate, which is present in high concentrations as part of the use of ammonium sulfate for ionic precipitation of proteins. Over 8000 structures in the PDB list sulfate as a bound ligand, clearly showing the high likelihood of nonspecific binding of oxyanions to proteins sites that do not normally bind oxyanions or to sites that normally bind other oxyanions such as phosphate.

ARSENATE: A HIGHLY USEFUL MECHANISTIC PROBE

In addition to the discussion of alternative life chemistries and oxyanion promiscuity, we also wish to highlight the utility of arsenate as a structural and mechanistic probe for phosphate-utilizing enzymes. The irreversibility of the arsenate reaction catalyzed by PNP (purine nucleoside phosphorylase) was used to help decipher the catalytic mechanism and the microscopic rates for various steps of the enzymatic reaction (*17*). In an earlier example of the application of this approach, Fitting and Doudoroff used arsenate to distinguish between the mechanism of action of sucrose phosphorylase (which acts via a glucosyl-enzyme intermediate) and maltose phosphorylase that acts via direct attack of phosphate on maltose (*16*). Similar mechanistic approaches are still being used with glycosyltransferases that use phospho-sugars as substrates (*25*, *26*). Arsenate-based structures can provide a glimpse of the nature of reaction intermediates, as demonstrated with PNP (*20*) (Figure 2), and can also shed some light on the ion binding mode in phosphate-utilizing enzymes such as ASADH (*18*) (Figure 1).

Arsenate has also been applied in a reconstituted glycolytic system based on purified yeast enzymes. By effectively acting as an ATPase, arsenate uncoupled ATP synthesis from glycolysis. Uncoupling was driven by the extremely rapid hydrolysis (millisecond half-life) of the intermediate 1-arseno-3-phosphoglycerate. The latter was formed by GAPDH promiscuously using arsenate instead of phosphate (*35*).

Also of interest as potential mechanistic probes are alkylaronates, not only as nonhydrolizable analogues of phospho-esters but also as substrate analogues for enzymes acting on phosphonate substrates. The latter are becoming increasingly interesting, as phosphonates have been shown to comprise a key component of the dissolved marine organic phosphorus pool (*27*). For example, phosphonates are a major component of total cellular phosphorus in the marine cyanobacterium *Trichodesmium erythraeum* (*28*). The enzymology of marine phosphonates is still substantially unexplored, but alkylaronates are likely to

be useful as inhibitors in the isolation and characterization of the relevant enzymes involved in phosphonate metabolism.

ION SELECTIVITY AND PROMISCUITY

Judging by the above examples and others that have not been discussed here, it appears that enzymes and others proteins show very low phosphate−arsenate binding selectivity. As is the case with other protein traits, this lack of discrimination can be due to two unrelated reasons: (i) physicochemical constrains may limit the ability of proteins to differentiate between these two highly similar ions with significant (orders-of-magnitude) differences in affinity; (ii) the absence of evolutionary pressure to prevent arsenate competition at phosphate sites results in little discrimination when arsenate is introduced.

How physicochemically feasible is a strict distinction between arsenate and phosphate? What, in general, dictates the specificity of binding of small ions? Proteins can easily distinguish between small charged atoms such as H^+, Na^+, and K^+, or F^-, Cl^-, and Br^-, primarily by virtue of considerable differences in size, charge density, and hydration energy. For example, ion channels show high specificity for the closely related Na^+ and K^+. However, these ions differ considerably in their radii (> 20%) and dramatically in their hardness. The latter is reflected in 10^6-fold differences of the solubility of Na^+ and K^+ in certain hydrophobic organic molecules (*15*). Relatively high selectivity is also observed with larger metal ions, for example, with metalloenzymes that may accept a range of transition metals, yet with affinities that differ by orders of magnitude and different catalytic activities (for example, refs (*29* and *30*)). Here, ligand donor atom type and coordination geometry preferences offer additional selectivity criteria.

For larger oxyanions such as arsenate and phosphate, size differences are relatively small (the thermochemical radius for phosphate is 2.38 Å, arsenate is 2.48 Å, and sulfate is 2.30 Å) (*15*). Binding selectivity is therefore unlikely to be based on size differences, and differences in geometry and charge distribution are more likely to be important (*14*). For example, at neutral pH, carbonate, which is abundant in living media, has the same net charge as phosphate (−2) and essentially the same partial charge on the charged oxygens (−0.964 versus −0.952 for phosphate). However, its size is much smaller (1.85 Å), and in particular, its geometry is planar as opposed to tetrahedral for phosphate. A study of the oxyanion interactions with ASADH indicates that ions that are tetrahedral and exhibit high negative charge densities on their oxygens are either good substrates or effective inhibitors for the enzyme. For example, sulfate is neither a substrate nor an inhibitor; while it is tetrahedral, the partial negative charge on its oxygens (− 0.861) is significantly lower than that of phosphate (−0.952). The only exception observed for ASADH is periodate, which is tetrahedral but exhibits very low negative charge (−0.719). Interestingly, periodate is the most tightly bound ion, but it disrupts an important hydrogen bond with Glu243, thus rendering the enzyme inactive (Figure 1) (*14*, *18*). It appears, therefore, that the relatively small differences in size between phosphate and arsenate (~4%), and in the partial negative charges of the oxygens (−0.952 for phosphate and −0.892 for arsenate) suggest that a strict distinction between arsenate and phosphate might be rather challenging.

Can very high oxyanion binding selectivity be achieved, and, if so, then which structural features may mediate it? The recently described ultrahigh resolution (0.88−0.98 Å) structure of a phosphate binding protein from *Pseudomonas fluorescens* affords an interesting insight by virtue of the electronic density that is observed for certain hydrogen atoms. This structure suggests that oxyanion discrimination may involve some unique interactions, such as low-barrier hydrogen bonds (LBHBs) to selected donor atoms. The bound HPO_4^{-2} ion in this protein seems to accept as many as 11 hydrogen atoms in binding interactions. But it only donates a single hydrogen atom, to an aspartate side chain, while forming a LBHB to this functional group. This LBHB is likely to mediate the dibasic phosphate binding specificity, allowing the protein to discriminate phosphate from sulfate by a factor of 10^5 (*31*). Thus, beyond the type and geometry of the donor atoms in an oxyanion binding site, the specific polarity of the hydrogen-bonding pattern and the incorporation of special binding interactions may also provide a possible basis for ion discrimination.

As for evolutionary pressures, arsenic and arsenate are toxic for most organisms. The toxicity of arsenic(III) oxide, the favorite murder weapon of Italian Renaissance aristocrats and murder mystery novelists, relates to the As(III) state. But other toxic effects may stem from its oxidation to arsenate, with the latter occupying essential phosphate sites. This may result in inhibiting essential protein functions or yielding enzymatic products that are rapidly hydrolyzed. Arsenate itself is also toxic to most organisms. By virtue of its similarity to phosphate, it uncouples organismal metabolism by impairing ATP synthesis (*32*). Since mechanisms for As(III) and arsenate detoxification have been identified in various organisms, evolutionary pressures to minimize arsenate binding to phosphate sites may exist. However, the relatively few enzymes that have been analyzed with arsenate come from a very narrow range of organisms, and these enzymes are not likely to have been under selection for high phosphate−arsenate selectivity. It appears, therefore, that the lack of discrimination in these enzymes stems from both factors, namely, from the physicochemical similarity of these ions and from the absence of evolutionary selection pressure.

EVOLUTION OF ARSENATE-BASED BIOCHEMISTRY

Promiscuity generally provides the starting points for the evolution of new protein functions (*33*). Arsenate−phosphate cross-reactivities may therefore serve as the basis for evolutionary transitions from a phosphate-based organism into an arsenate-based one or vice versa. However, the extreme sensitivity of arsenate esters to hydrolysis suggests that many metabolites and macromolecules must remain phosphate-based. Thus, in an organism that does utilize arsenate, enzymes which need to work with phosphate-based substrates and products must evolve to incorporate high phosphate selectivity. Thus, a bacterium that dwells in environments containing high concentrations of arsenate and low concentrations of phosphate faces a paramount challenge, even if arsenate does not get incorporated into its metabolites and/or macromolecules. Selective transport to maximize phosphate uptake and minimize entry of arsenate, and/or active transport to export arsenate, and storage in specialized compartments, including insoluble precipitates, are essential for an organism that dwells in an arsenic rich environment to protect itself from toxic effects. But these mechanisms alone cannot eliminate arsenate altogether. Numerous proteins, including transporters and enzymes, must evolve high selectivity toward phosphate. The DNA repair system in such organisms might also be unique in having to deal with strand breakages that may occur

due to the accidental (or nonaccidental) incorporation of arsenate instead of phosphate.

Alternatively, we need not exclude the possibility that arsenic-based life forms do not involve a simple replacement of phosphate-esters by arsenate-esters. Alternative chemistries, such as stable C−As bonds (see the methylene-arsonates discussed above), could replace O−P bonds or even a completely different chemistry that has no precedents in known life forms. Needless to say, however, these chemistries present their own difficulties (how, for example, DNA polymerization and editing can readily occur when C−As bonds must be frequently made and broken).

CONCLUDING REMARKS

Whatever the ultimate case may be regarding the role of arsenic, an in-depth study of the *Halomonadaceae* strain described by Wolfe-Simon et al. should yield intriguing insights, as well as the motivation to study other bacterial strains that can grown under high arsenate levels (e.g., see ref *34*). The notion of life without phosphate might be proven wrong, but life with arsenate presents extreme challenges as well as intriguing research opportunities. Specifically, as discussed here, the distinction between arsenate and phosphate ions represents a challenging problem in molecular recognition. The study of the structural, functional, and evolutionary aspects of arsenate−phosphate discrimination and of the mechanisms for the discrimination of similar ions may therefore yield key insights into the possibilities of alternative life chemistries.

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