Phosphate Binding Proteins

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How Bacteria Choose Phosphate

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In 1987, the eminent physical organic chemist Frank Westheimer published a scholarly and highly respected article entitled "Why Nature chose Phosphates".[1] Amongst many aspects addressed in this publication, he considers other potential elements or moieties that might conceivably fulfill a similar function to phosphate, its esters, and anhydrides. One of these is arsenate, which is indeed superficially an attractive alternative owing to the similarity of its basic structure as a tribasic acid capable of forming esters and anhydrides, as well as the similar size of arsenate and phosphate ions (arsenate is only 4% larger than phosphate) and the almost identical pK_a values. However, he dismisses the possibility of arsenate being able to play a similar role to phosphate because of the extremely rapid hydrolysis of its esters in water. One of the hallmarks of phosphate chemistry is the enormous hydrolytic stability of phosphodiesters (half-life estimated to be ca. 30 million years at 25°[2]), which is an important basis for DNA stability and one of the reasons why it is possible to sequence DNA from Neanderthals that lived at least 30000 years ago. Chemists were therefore very surprised when a publication from 2011 presented what was considered to be evidence that a bacterium from the arsenic-rich Mono Lake in California, *Halomonas* strain GFAJ-1, could grow in the absence of phosphate and instead use arsenate.[3] In the meantime, it seems likely that this interpretation was incorrect, especially since the DNA of the bacteria grown under conditions of very high arsenate concentration and very low phosphate concentration was shown to contain phosphate but no arsenate.[4]

Despite the demonstration of the false conclusions of the earlier work, the observation that certain bacteria can grow at very high arsenate to phosphate ratios is of great interest and the mechanism of this effect is intriguing. This puzzle has essentially been solved in a recent contribution. Tawfik and co-workers[5] examined several proteins that are involved in phosphate uptake into bacteria at low phosphate concentrations, and are referred to as high-affinity periplasmic phosphate binding proteins (PBPs). These are components of the Pst (phosphate-specific transport) system, which also includes an ABC (ATP binding cassette) transporter protein that is able to pump against a phosphate gradient by using the free energy of ATP hydrolysis. In contrast to the low-affinity and low-specificity Pit (phosphate inorganic transport) system that operates at high phosphate concentration, PBPs are highly specific.

The Tawfik research group compared several PBPs, including two paralogues from GFAJ-1. They showed that all of these proteins discriminated strongly against arsenate, in several cases by a factor of approximately 500. Interestingly, in the case of the PBP-2 paralogue from GFAJ-1, this factor is approximately 4500, meaning that the discrimination against arsenate is almost a factor of 10 higher than in other PBPs. Together with the observation that PBP-2 expression is strongly upregulated at low phosphate concentrations, this explains how the bacteria are able to grow at a 4000-fold excess of arsenate over phosphate, with only a small excess of intracellular arsenate over phosphate being generated. It is presumably also the explanation of the original erroneous interpretation concerning the ability of the strain to use arsenate instead of phosphate.

The discrimination between phosphate and arsenate is a remarkable property considering the similarities between the two ions. To understand the basis for this specificity, Tawfik and colleagues crystallized *Pseudomonas fluorescens* PBP with either phosphate or arsenate bound and determined their structures by X-ray crystallography. Initial inspection of the structures indicated an identical mode of binding of the dibasic (i.e. singly protonated) form of the anions, with a large number of hydrogen bonding interactions to the phosphate/arsenate oxygen atoms. Fortunately, the structures were determined at resolutions of better than 1 Å, and this allowed identification of individual hydrogen atoms, which is not possible at lower resolution. Again, for most of the interactions no difference could be detected, but there was one detectable difference. In the case of the interaction of the singly protonated oxygen of phosphate or arsenate with the side chain of aspartate 62 of the PBP, a short hydrogen bond was seen (in both cases ca. 2.5 Å), in agreement with earlier work.[6] This is characterized as a low-barrier hydrogen bond, specifically a heteromolecular negative-charge-assisted hydrogen bond. In such bonds, the hydrogen atom is essentially shared between the two oxygen atoms. In the phosphate-bound structure, this appears to be a perfect example of this type of bond, with the hydrogen atom located almost equidistant between the oxygen atoms and with nearly ideal bond lengths and bond angles (Figure 1). In the arsenate structure, the longer As–O bond leads to a distortion of this structure, so that the angles are well removed from their

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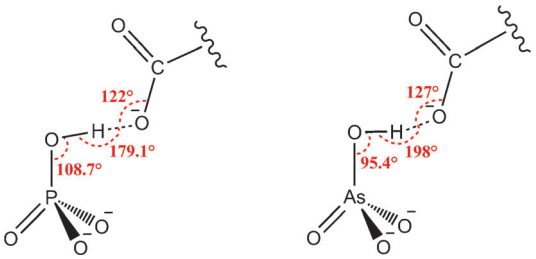

Figure 1. Comparison of the short hydrogen bond in the phosphate (left) and arsenate (right) complexes of *Pseudomonas fluorescens* PBP. Only one of the 12 hydrogen bonding interactions is shown.

canonical values, and the hydrogen atom is located closer to the arsenate than the carboxylate moiety. The bond appears to be forced into this energetically unfavorable geometry by the rigid nature of the active site, with the position of the phosphate or arsenate ions being dictated by the large number of other hydrogen bonding interactions (11 in addition to the short bond) and a steric effect mentioned below. This lack of flexibility is indicated by the low crystallographic B-factors around the active site. Hydrogen bonding to backbone NH groups in well-defined regions of the polypeptide chain and to stably fixed short side chains contributes to the strong fixation of the anions. The lack of flexibility in anion binding is augmented by unfavorable interactions of phosphate with the C-β atoms of alanine 7 and leucine 9, which become even less favorable with arsenate. Interestingly, mutation of either of these residues to glycine reduced the discrimination against arsenate by a factor of four, thus supporting the idea of the importance of a tightly confined active site to the discrimination mechanism.

The role of the short hydrogen bond to aspartate 62 was further investigated by mutation of the residue to asparagine. This results in a loss of discrimination against arsenate by about a factor of ten. Studies on the equivalent mutation in *E. coli* PBP show that there is no loss of affinity for phosphate.[6] The conclusion of the authors of the earlier contribution was that this means that the short hydrogen bond is not especially strong, and this is echoed in the views of the Tawfik group. Their results are rationalized in the statement "these structural features therefore support the notion that anion binding and anion selectivity are two independently evolved features". This is at present not easy to understand. Thus, if the short hydrogen bond does not contribute significantly to affinity (of phosphate), why should distortion of this bond (in the case of arsenate) decrease its affinity (in comparison to phosphate) dramatically? Could this be telling us that the short hydrogen bond in the arsenate case is contributing negatively to binding energy and is formed because it is the only solution to the steric problems imposed by the rigid binding site? Perhaps the as yet undetermined structures of phosphate and arsenate complexes with the even more specific GFAJ-1 PBP-2 will help to understand the mechanism more exactly.

Whatever the fine details of the mechanism of discrimination against arsenate, it is apparent that this has evolved by fine-tuning of active-site interactions in a tight binding site completely shielded from water. The pronounced spatial constraints have allowed selection on the basis of possibly the only exploitable difference between the two anions, namely the small difference in size. This is perhaps an even more remarkable feat than the discrimination of potassium channels against sodium ions, where the difference in ionic radii is approximately 40%, compared with the 4% difference between phosphate and arsenate. Interestingly, there are both parallels and differences between these two situations. Thus, potassium channels also have very rigid ionic binding sites, but they favor the interaction with the larger potassium ions because the rigid channel cannot shrink to allow favorable interactions of backbone carbonyls with the smaller sodium ions.[7] In the case of the PBPs, it appears that the very tight fit of the phosphate group to the rigid binding site does not allow easy expansion to the dimensions needed for the slightly larger arsenate. In both cases, the discrimination is an essential aspect of the mode of action of the respective proteins.

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