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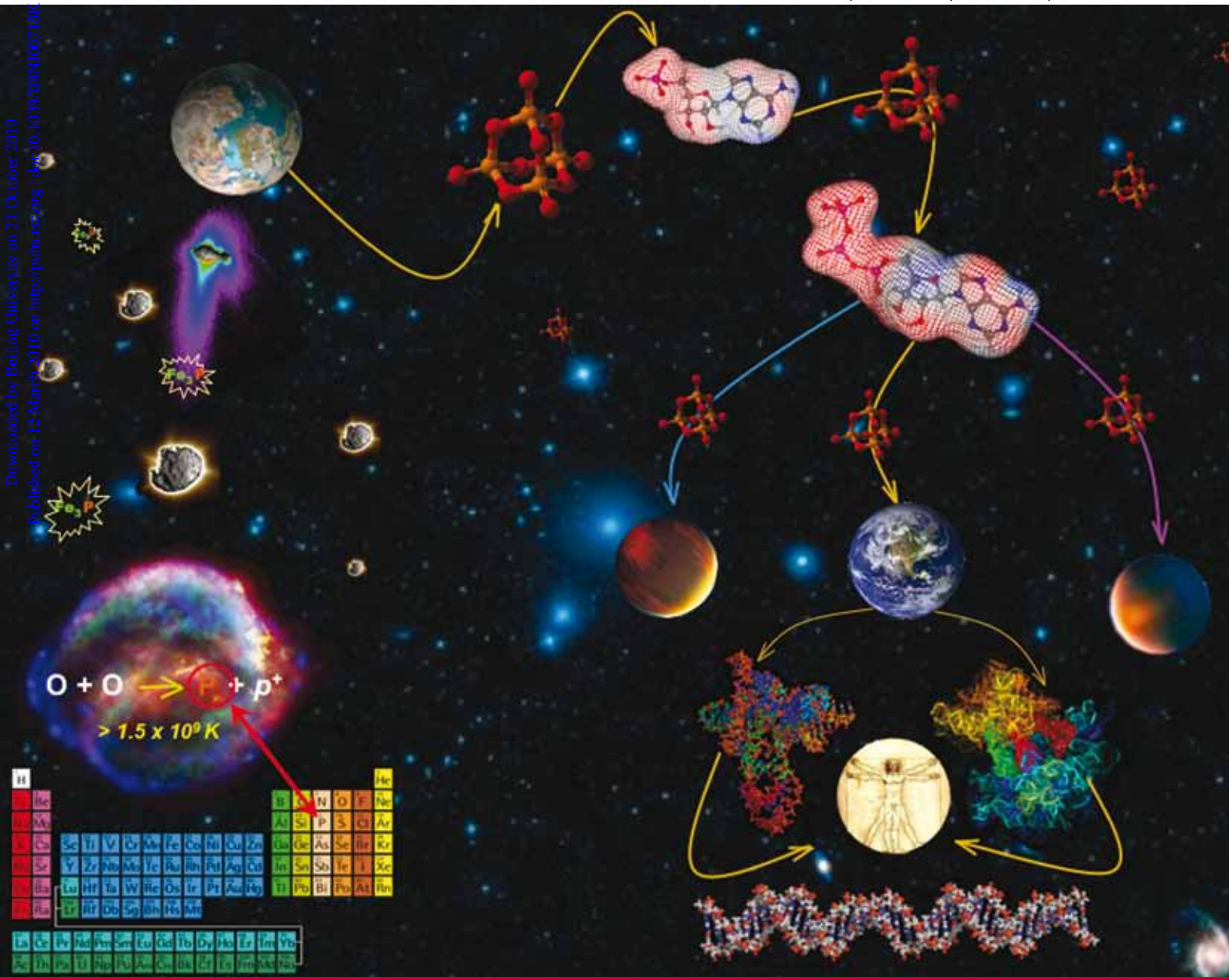

Themed Issue: Biophosphates

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PERSPECTIVE
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Why did Nature select phosphate for its dominant roles in biology?

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Why did Nature select phosphate for its dominant roles in biology?†‡

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Evolution has placed phosphate mono- and diesters at the heart of biology. The enormous diversity of their roles has called for the evolution of enzyme catalysts for phosphoryl transfer that are among the most proficient known. A combination of high-resolution X-ray structure analysis and ^{19}F NMR definition of metal fluoride complexes of such enzymes, that are mimics of the transition state for the reactions catalysed, has delivered atomic detail of the nature of such catalysis for a range of phosphoryl transfer processes. The catalytic simplicity thus revealed largely explains the paradox of the contrast between the extreme stability of structural phosphate esters and the lability of phosphates in regulation and signalling processes. A brief survey of the properties of oxyacids and their esters for other candidate elements for these vital roles fails to identify a suitable alternative to phosphorus, thereby underpinning Todd's Hypothesis "Where there's life there's phosphorus" as a statement of truly universal validity.

Outline

One of the most remarkable features of the chemistry of living organisms is the evolutionary development of phosphate esters, on the one hand to provide the extremely stable backbone for the biopolymers that encode the genetic information, RNA then DNA, on the other hand for the temporal protein regulation that is largely under the control of kinases and phosphatases, and yet again for the generation, distribution, and application of free energy throughout the cell by the manipulation of anhydrides of phosphoric acid and its esters, notably adenosine triphosphate. The solution to the paradox between the remarkable chemical stability of phosphate mono- and diesters and their facile manipulation lies in the catalytic power of enzymes to make and break P–O–C and P–O–P bonds rapidly, giving some of the largest enzymatic rate accelerations yet identified. The chemical basis for that catalysis lies at the heart of the natural selection of phosphate esters and anhydrides for ubiquitous central roles in biology.

Introduction

The early bioorganic chemistry of phosphate esters was dominated in the mid-20th century by the work of Alexander Todd and his colleagues in Cambridge (Fig. 1). They delivered the first chemical syntheses of adenosine 5′-triphosphate[1] (ATP) in 1948 and the of first dinucleoside phosphate[2] (dTpT)

Fig. 1 Alexander Todd 1907–1997. Nobel Prize 1958.

in 1955. This work advanced with Gobind Khorana into the production of the pyrophosphate linkage in vitamins and coenzymes,[3] and thence into the chemical synthesis first of DNA then of RNA. Todd's engagement with phosphate esters and anhydrides engendered a fascination with mechanisms of phosphorylation, both chemical and biological, which led to a strong focus on the concept of monomeric metaphosphate esters as transient, neutral, trigonal species ($R–O–PO_2$) in phosphoryl transfer processes.[4] His broader view of the dominant role of phosphates in biology was more securely founded and cogently expressed in a magisterial lecture in Osaka in 1981, as identified in the following quotation.[5]

"Let us try to summarise what nature needs to facilitate all she has to do with carbon-based building bricks.

(1) She needs a strong acid capable of forming anhydrides which can be used for energy storage and transport. Presumably other acids than phosphoric would do for that but phosphoric can fulfil many other purposes in addition.

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‡ Dedicated to Professor Wojciech J. Stec on the occasion of his 70th birthday.

(2) The acid must be tribasic so that it may act as a link between two molecules of groups and still have one free acidic hydroxyl for further reaction.

(3) The strength of the acid is important since it permits use in carbon–carbon bond synthesis.

(4) As a tribasic acid both it and its monoesters should be capable of forming internal anhydrides which can be powerful acylating agents.

"When one looks at the periodic table there is only one element which can give such an acid—phosphorus. The only other element of comparable character when it comes to forming oxy-acids is sulfur but it is quite useless for most of the above purposes because it gives only dibasic acids. It is my belief that the requirements listed above are so vital to the development of organised life that I would guess that if life exists anywhere else in the universe it will do so only on a planet on which phosphorus is readily available."

This statement is based on the general mid-20th century knowledge of phosphates, while now, fifty years later, we can see some of its limitations. Not only do phosphoric anhydride bonds yield high free energy on hydrolysis, some phosphate diesters also do so.[6] The use of the third phosphate hydroxyl remains significant only for *chemical* transformations of diesters, with no apparent natural biological counterpart. As most phosphate leaving groups in biology are dianions, their pK_{a2} is around 7.0, so acid strength is not of primary significance. However, the challenge is couched most strongly in Todd's inspired guess. Are phosphate esters truly universal components of life? It is the purpose of this essay to show that the case for this hypothesis has grown much stronger in the 30 years since its first articulation.

Vital roles for phosphates

In the 1970s, the structural roles for phosphate diesters in genomic macromolecules and for monoesters in lipids were well understood. But the explosion of protein phosphorylation and the dimensions of the kinome had yet to be discovered. Now, thirty years on, the number of major biological roles for phosphates has grown tremendously. The following list is by no means exhaustive.

- Genome stability—DNA structure demands ultra-stable phosphate diesters—and rapid repair processes on demand for survival.
- The RNA World—life likely started with RNA preceding DNA and proteins—for both genome, catalysts (ribozymes), and regulation (riboswitches).
- Lipids—cell membranes utilise phospholipids as essential stable components.
- Skeletal structure—bones, teeth, *etc.* use calcium phosphate (apatite) as a key mineral component.
- Compartmentalisation—small metabolites as ionic phosphate esters are unable to cross membranes and can be trafficked.
- Organelle membrane recognition—phosphoinositols.
- Energetics—"Energy-rich phosphates" store and distribute free energy for biological processes.
- Regulation and signalling—many key proteins become phosphorylated in order to modulate their activity, and second messengers such as cAMP and cGMP are vital for cell signalling. The time course of phosphoproteomics is a burgeoning field in post-translational modification of proteins.[7]

The remarkable paradox of this list is that it ranges from extreme stability (the half life of DNA to spontaneous water hydrolysis is 31 million years)[8] to millisecond manipulation (*Yersinia* phosphotyrosine phosphatase, YopH, has k_{cat} = 1000 s^{-1}).[9] It is this feature of phosphate chemistry that makes it unique in its biological functions and, we will argue, cannot be delivered by the esters of oxyacids of any other element in the Periodic Table.

The origin of phosphate ester stability

Frank Westheimer (Fig. 2) and coworkers initiated a physical organic chemistry analysis of the chemical and biological mechanisms of phosphate ester transfer in Harvard with the investigation of the unusual reactivity of ethylene phosphate, a diester with a five-membered ring, the potential role of a vicinal hydroxyl group, and the probable "in-line" geometry for the transition state of phosphate ester hydrolyses.[10,11] Notwithstanding a predilection[12] for metaphosphate akin to that of Todd, Westheimer made a major advance in the rationale for the evolutionary selection of phosphates by identifying the stabilising role of the negative charge on phosphate mono- and diesters as a deterrent to nucleophilic attack.[13]

"Phosphoric acid is specially adapted for its role in nucleic acids because it can link two nucleotides and still ionize; the resulting negative charge serves both to stabilise the diesters against hydrolysis and to retain the molecules within a lipid membrane. A similar explanation for stability and retention also holds for phosphates that are intermediary metabolites and for phosphates that serve as energy sources. Phosphates with multiple negative charges can react by way of the monomeric metaphosphate ion PO_3^- as an intermediate. No other residue appears to fulfil the multiple roles of phosphate in biochemistry."

There is ample evidence for such anionic stabilisation. Recent support comes from a comparison of the rates of hydrolysis of phosphate di- and tri-esters. The relative rates of reaction of a methyl triester and the corresponding diester

Fig. 2 Frank Westheimer 1912–2007. Reproduced with permission from the Harvard University Gazette.

with hydroxide shows the effect of neutralizing the phosphate ester negative charge is to accelerate the reaction by 10^9-fold.[8] Even more impressive is the retardation of the reaction of water with trimethyl phosphate by the two negative charges on methyl phosphate, a deceleration of at least 10^{12} (allowing for P–O *vs.* C–O cleavage). An equally remarkable stabilising effect is shown for a dineopentyl phosphate, where the suppression of C–O cleavage reveals that the spontaneous hydrolysis of the monoanion has a rate constant[8] of 7×10^{-16} s^{-1}, corresponding to a half life for the 3′,5′-diester linkage in DNA of 31 million years!

By comparison, the accelerations achieved by enzyme hydrolysis are staggering. *Staphylococcus* diesterase achieves a rate enhancement of 6×10^{14} for the dimethyl phosphate anion, while the *Yersinia* monoesterase accelerates the neutral hydrolysis of the phenyl phosphate dianion by 2×10^{16}. Such data have been extrapolated[8,15] to deduce that phosphate-cleaving enzymes such as Type 1 protein phosphatase, fructose bisphosphatase, and inositol phosphatase each catalyze direct hydrolysis of their phosphate monoalkyl ester substrate with a rate of water attack on the dianion enhanced by a factor of $\sim 10^{21}$. That is equivalent to binding the reacting substrate in the transition state with a formal dissociation constant[15] of $\sim 10^{-26}$ M.

Molecular mechanics can be used to illustrate this anionic nucleophile repulsion. The calculated electronic charge distribution over the Gaussian surface of a series of mono-, di-, and tri-esters of phosphates and sulfates reveals the localised negative charge (red) that is associated with tight water solvation and strong anion repulsion for the mono- and di-anions (Fig. 3). The reduced polarities for sulfate esters, having one charge less, is manifestly apparent. The situation is even more acute for phosphoryl transfer from anhydrides such as ATP, as manifest by the charge repulsion barrier to be overcome in order to equilibrate ATP + AMP with 2 ADPs (Fig. 4), two negative charges opposing four negative charges! Yet the malarial enzyme adenylate kinase achieves[20] this important reaction with a turnover of $k_{cat} = 35$ s^{-1}.

By contrast, the anionic character of phosphate esters enables proteins to bind them in precise positions inside

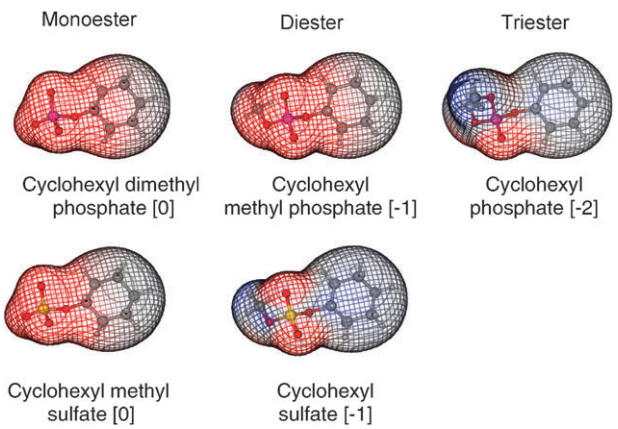


Fig. 3 Electrostatic surfaces calculated for mono-, di-, and tri-esters of phosphates and sulfates using MOE.[19] (Negative polarity—red; positive polarity—blue; net charge shown in parentheses).

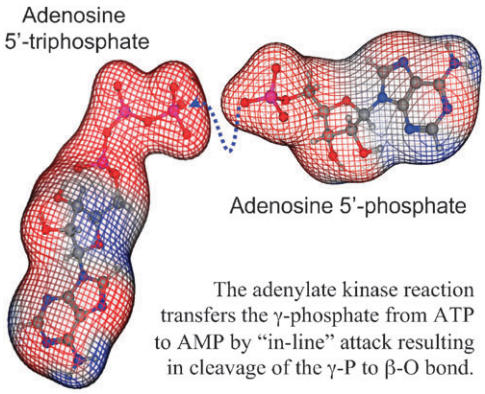


Fig. 4 Electrostatic surfaces calculated for the ATP tetra-anion (left) and the ADP dianion (right) using MOE.[19] (Negative polarity—red; positive polarity—blue).

enzyme active sites and signalling proteins. For example, trypanosome phosphoglycerate kinase, PGK, binds the substrate 3-phospho-D-glyceric acid inside its active site by means of a "phosphate clamp", using three arginines and a lysine to form six hydrogen bonds to the stable 3-phosphate ester dianion of D-glyceric acid. This locates its carboxyl group exactly to receive a γ-phosphate from ATP by "in-line" transfer (Fig. 5).

Here then, is the central paradox. Evolution has made full use of the kinetic stability of phosphate diesters for the polymeric backbone of the genomic materials and of monoesters for stable metabolic species. Alongside that it has developed modes of catalysis that outperform any chemist's hydrolytic reaction of phosphate esters (usually boiling in 4 N HCl). How has this been accomplished? It is clear that charge neutralisation is at the heart of such catalysis.

Enzyme catalysis of phosphoryl group transfer

There are several recent reviews of the mechanisms of enzyme catalysis of phosphoryl group transfer.[21] In general, these have approached the subject from a basis of physical organic chemistry allied to enzyme biochemistry, and with a strong focus on the associative/dissociative nature of the reactions.

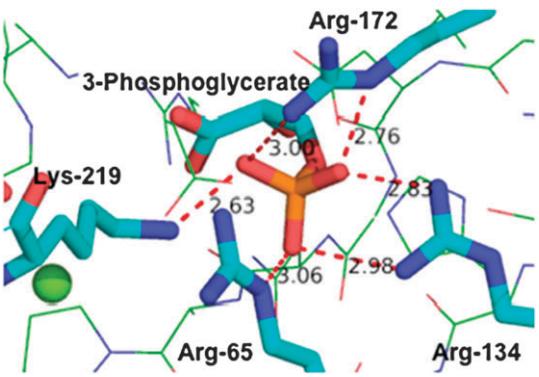


Fig. 5 The PGK phosphate clamp. 3-Phospho-D-glyceric acid in the binding site of *T. brucei* phosphoglycerate kinase showing hydrogen bonds (red), with bond lengths (black) from phosphate to amino acid residues.

However, in the last period of time, structural studies, especially juxtaposing X-ray analysis with NMR spectroscopy, have made examination of the transition states for enzyme phosphoryl group transfer accessible from a new direction. It is now opportune to compare transition state stabilisation for three classes of phosphoryl group transfer, with a focus on charge balance:

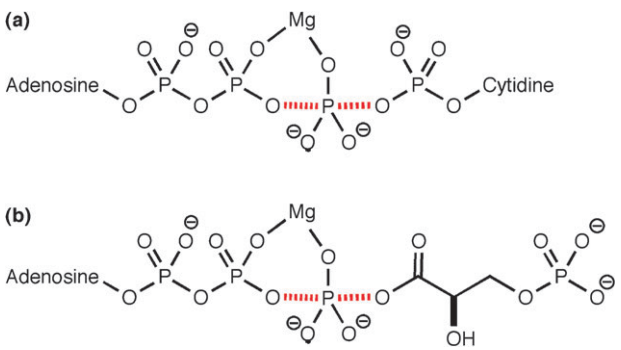
- Anionic nucleophile with anionic leaving group
- Anionic nucleophile with neutral leaving group
- Neutral nucleophile with neutral leaving group.

This is one way of describing the three classes: the alternative is to say that the phosphoryl group transfers between two anionic ligands, between an anionic and a neutral ligand, or between two neutral ligands. Examples of each class will now be explored and conclusions drawn about how enzymes have solved the problem of phosphate ester charge repulsion of the attacking nucleophile.

Anionic nucleophile and anionic leaving group

The two dominant types of enzyme in this group involve phosphoryl transfer between two anionic phosphate residues, characteristic of the nucleotide kinases, and transfer between an anionic phosphate residue and a carboxylate anion, typically represented by phosphoglycerate kinase, PGK (Scheme 1). Metal fluoride complexes have been known for UMP/CMP kinase and adenylate kinase as well as for other phosphoryl transfer enzymes for many years. While examples of octahedral complexes are conventionally assigned to AlF_4^- as anions that mimic the transferring phosphoryl group with sacrifice of geometrical accuracy, trigonal bipyramidal (tbp) complexes have been described as AlF_3^0 structures, albeit without adequate metal characterisation.[22] As more recent NMR studies have shown for the case of phosphoserine phosphatase (*v.i.*),[23] at least some of these complexes may more accurately be described as trifluoromagnesate (MgF_3^-) complexes that, because they are anionic, are isoelectronic as well as being isosteric with a tbp phosphoryl transition state. At least some of these switches from aluminium to magnesium fluorides arise because fluoride is displaced from aluminium-III by hydroxide as the pH rises, eventually giving (insoluble) aluminium hydroxide.[24]

The metal fluoride transition state complex for UMP/CMP kinase with ADP and CMP bound shows remarkable charge



Scheme 1 Transition states for (a) the UMP/CMP kinase reaction and (b) the phosphoglycerate kinase reaction. (Forming and breaking bonds in red).

balance.[25] The six negative charges of the two nucleotides (*cf.* Fig. 4, RCSB PDB accession 3UKD) are exactly neutralised by 8 positive charges (from the catalytic Mg^{2+}, 5 arginines and a lysine) diminished by two negative charges (2 aspartates) (Fig. 6). The three fluorides share a rich variety of hydrogen bonds to proximate donors and to the catalytic magnesium that hold them in the equatorial positions of a trigonal bipyramid. The separation of the two apical oxygens is 4.2 Å, which is significantly less than required for a monomeric metaphosphate intermediate (~6.5 Å) and rather towards the associative reaction distance (≥3.6 Å).

A closely similar situation applies to the reaction between ATP and 3-phospho-D-glyceric acid catalysed by PGK. The transition state complex has been identified by X-ray structure determination[26] of a ternary complex of human PGK with ADP, 3PGA, and MgF_3^- that identifies the 5 positive charges required to balance the 4 negative charges of ATP plus 1 negative charge of the phosphoglycerate carboxylate (Scheme 2). Two positive charges are required to clamp the 3PGA-phosphate group in position (*cf.* Fig. 4) and these are provided by Arg-85, Arg-122, and Arg-170 balanced by Glutamate-128. Analysis of this structure shows that the net charge balances to zero out to a radius of 10 Å from the central γ-phosphorus atom. The tbp structure has two apical oxygens at 4.27 Å separation and the three equatorial fluorides are located by 5 hydrogen bonds and the catalytic magnesium.

While phosphatases using sulfur as the nucleophile generally employ an anionic cysteine residue,[27] a crystal structure of bovine PTPase shows vanadate ion in covalent linkage with the nucleophile Cys-12 at the active site in trigonal bipyramidal geometry, corresponding to water attack in hydrolysis of the phosphocysteine. However, the state of protonation of the four vanadate oxygens is indeterminate, so charge neutralisation is uncertain, even though a proximate arginine-18 hydrogen-bonds to two equatorial vanadate oxygens.[28]

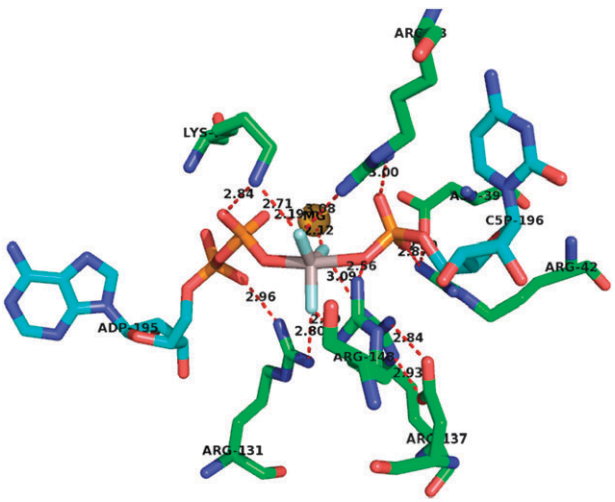
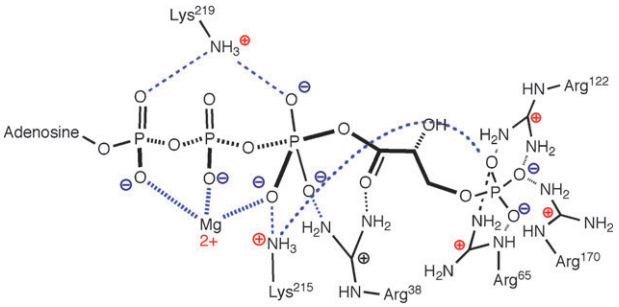


Fig. 6 Transition state for UMP/CMP kinase. The central, transferring phosphoryl group is mimicked by the metal trifluoride in a near-symmetrical tbp structure. Each phosphate is hydrogen bonded to a cationic residue, with the transferring phosphoryl group bridged to the ADP β-phosphate and to the CMP α-phosphate [RCSB PDB accession 3UKD].[22] There is overall zero charge within this complex out to 15 Å from the central metal atom.



Scheme 2 The transition state structure for human phosphoglycerate kinase based on the MgF_3^- TSA.[26] The 5 negative charges of the two substrates, ADP (left) and 1,3-bisphospho-glycerate (right) are exactly balanced by 5 positive charges (Mg^{2+}, Arg65, Lys215 and Lys219). The net charge in the "phosphate clamp" is made zero by Glutamate-128 (not shown). The transferring γ-phosphoryl group is bridged to ADP by magnesium and K-219, and to the 3PG carboxylate by K-215 and Arg-28 – – – –.

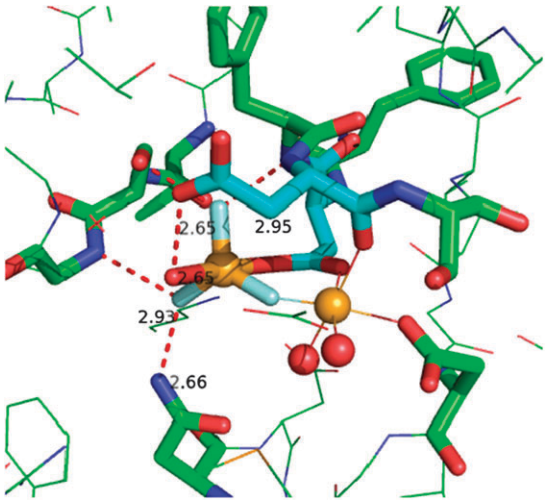


Fig. 8 Transition state complex for the hydrolysis of phosphorylated phosphoserine phosphatase. Trifluoromagnesate, MgF_3^-, occupies the site of the transferring phosphoryl group from Asp-11 to water. General base catalysis is provided by Asp-13. The catalytic magnesium (gold) coordinates Asp-11 and one of the three fluoride ions, and also forms a six-membered ring by ligating the nucleophilic oxygen of Asp-11.

Neutral nucleophile and anionic leaving group

Phosphoryl transfer mechanisms of this class have recently come into re-examination in the cases of β-phosphoglucomutase (βPGM) and phosphoserine phosphatase (PSP) (Figs. 7 and 8). Both of these enzymes, and many others, use an essential aspartic acid carboxyl function as a nucleophile to form a transient aspartyl phosphate intermediate that is either hydrolysed or donates its phosphoryl group to a second substrate hydroxyl group. [Such covalent activity by a neighbouring carboxyl group was first recognised in model studies of carboxylate mega-activation of phosphonate[29] and phosphate[30] ester hydrolyses in the late 1960s]. In both of these enzymes, our studies have established transition state structures for binary complexes using magnesium trifluoride anion as an isosteric and isoelectronic mimic of the transferring phosphoryl group. The use of ^{19}F NMR has shown that for β-phosphoglucomutase from *Lactococcus lactis*, the observed pH-switch in fluoride coordination from an octahedral tetrafluoro-complex at low pH to a trigonal bipyramid trifluoro-complex at high pH does not derive from an AlF_4^- moiety converting into AlF_3^0. Instead, AlF_4^- is progressively replaced by MgF_3^- as the pH increases.[23,31] Hence the enzyme prioritizes anionic charge at the expense of sacrificing native trigonal geometry to octahedral AlF_4^- over the pH range <6–8. We have observed similar behaviour for phosphoserine phosphatase from *Methanococcus jannaschii*. The two chemical reactions in question are shown above (Scheme 3).[23]

In the high resolution X-ray density structure of the trifluoromagnesate complex of βPGM with glucose 6-phosphate (Fig. 7, PDB accession 2WF5), the trigonal bipyramid

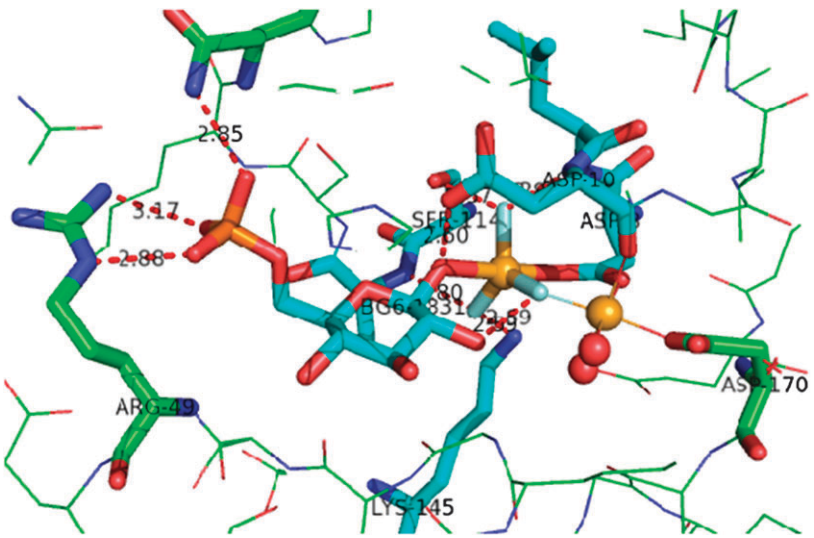
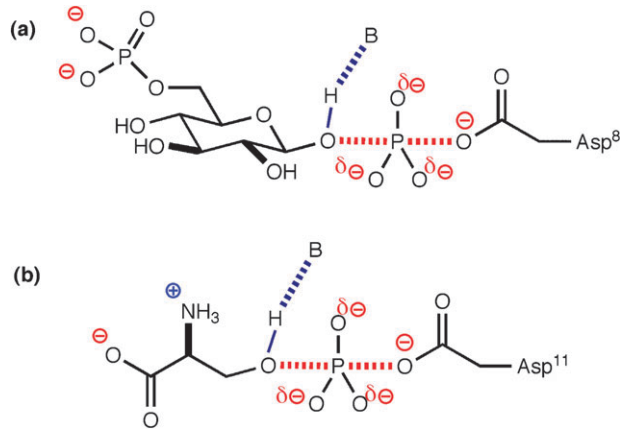


Fig. 7 Transition state complex for β-phosphoglucomutase, βPGM. Trifluoromagnesate, MgF_3^-, occupies the site of the transferring phosphoryl group from Asp-8 to the glucopyranose 1β-hydroxyl. General base catalysis is provided by Asp-10. The catalytic magnesium (gold) coordinates Asp-8 and one of the three fluoride ions, and also forms a six-membered ring by ligating the nucleophilic oxygen of Asp-8.



Scheme 3 Schematic transition states for the first step in the phosphoryl transfer reactions catalysed by **(a)** β-phosphoglucomutase and **(b)** phosphoserine phosphatase. In both cases, an intermediate aspartyl phosphate (right) is formed in the first step, that is hydrolysed by water in a second step to give phosphate, and there is general acid–base catalysis from a neighbouring aspartate residue to activate the neutral hydroxyl group (blue dashes).

geometry is clearly defined and can be interpreted for an "in-line", associative mechanism for βPGM. The carboxylate of aspartate-8 and the 1-β-hydroxyl group of D-glucopyranose 6-phosphate are the two ligands for the transferring phosphoryl group with a separation of 4.27 Å. Two of the three equatorial fluorides share 5 hydrogen bonds to proximate amino acids, identified by ^{19}F NOE analysis, and the third fluoride coordinates both to the catalytic magnesium and to the glucose 2-OH group.[32] The anionic charge is −2 from the glucose 1-phosphate and −1 from the catalytic aspartate, and these are balanced by 2 positive charges from the catalytic magnesium and 1 from lysine-145, giving nearly exact charge neutralisation out to 14 Å from the central phosphorus (Fig. 9). The carboxyl group of aspartic acid-10 makes a 2.5 Å hydrogen bond to the glucose 1-oxygen, showing that general acid–base catalysis operates on the "neutral" nucleophile/leaving group in the phosphoryl group transfer, confirmed by the catalytic inactivity of mutant D10N. It is noteworthy that the distant hexose phosphate is bound only by arginine-49 with no "clamp" feature. However, this enzyme has to recognise both D-glucose 6-phosphate and 1-β-phosphate in this site while it also forms a transition state complex with galactose 1-α-phosphate.[33] Thus strong binding is sacrificed for substrate tolerance.

The second example of phosphoryl transfer between a carboxylate anion and an alcohol is phosphoserine phosphatase from *Methanococcus jannaschii*. ^{19}F NMR examination of the tbp metal fluoride transition state complex has established that it is a trifluoromagnesate rather than an aluminium trifluoride complex (Fig. 8).[23,31] The transition state is remarkably similar to that for βPGM (above). Trigonal bipyramid geometry is clearly defined and identifies an "in-line", associative mechanism for PSP with a 4.24 Å separation of apical ligands. The fluorides are located in equatorial positions by coordination to the catalytic magnesium and hydrogen bonds to adjacent residues. The carboxylate of aspartate-11 and nucleophilic water are the two ligands for the transferring phosphoryl

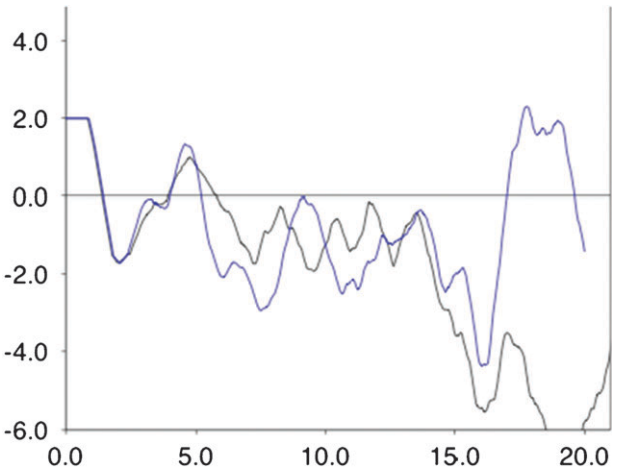


Fig. 9 Net integral charge (vertical axis) for MgF_3 transition state complexes calculated for spherical volume of defined radius (horizontal axis, in Å) from the Mg atom: β-phosphoglucomutase (blue) and phosphoserine phosphatase (black).

group. The anionic charge is −2 from the aspartyl-phosphate and this is balanced by 2 positive charges from the catalytic magnesium. As a result, there is net zero charge in the active site sphere out to 12 Å (Fig. 9, PDB accession 1L7N). The carboxyl group of aspartic acid-13 makes a 2.65 Å hydrogen bond to the nucleophilic water, showing general acid–base catalysis for its nucleophilic attack on the phosphate, endorsed by the catalytic inactivity of the mutant D13N (Scheme 3).

Protein kinase A provides a third example of this class. This enzyme catalyses the transfer of a γ-phosphoryl group from ATP to the serine of a substrate protein. A ternary complex of a substrate analogue peptide TTYADFIASGRTGRRASIHD, ADP, and aluminium fluoride shows a trigonal bipyramidal transition state with a 4.52 Å separation of the apical oxygens in which three fluorides share three hydrogen bonds to neighbouring residues, and two of them coordinate to the two catalytic magnesiums. The six negative charges of the reaction (4 from ATP and 2 from the catalytically active aspartates-166 and -184) are balanced by six positive charges (4 from two catalytic magnesiums and 2 from lysines-72 and -168). In line with the previous two examples, the serine-21 hydroxyl group is activated as a nucleophile by general base catalysis from Asp-166 (Fig. 10, PDB accession 1L3R).[34]

Neutral nucleophile and neutral leaving group

Two examples from this third category of phosphoryl transfer enzymes complete this mini-survey. *Escherichia coli* alkaline phosphatase operates by using serine-102 as a nucleophile to form a covalent phosphate ester which is hydrolysed by water in a second step. A covalent vanadate complex has been refined to 1.9 Å resolution in which vanadium assumes trigonal bipyramidal geometry and is covalently bound to the active site serine-102 nucleophile and to the attacking water. The equatorial oxygen atoms of the vanadate are stabilized by interactions with both Arg-166 and the two zinc ions in the active site, while the diaxial oxygens are 3.63 Å apart (Fig. 11, PDB accession 1B8J).[35] This is

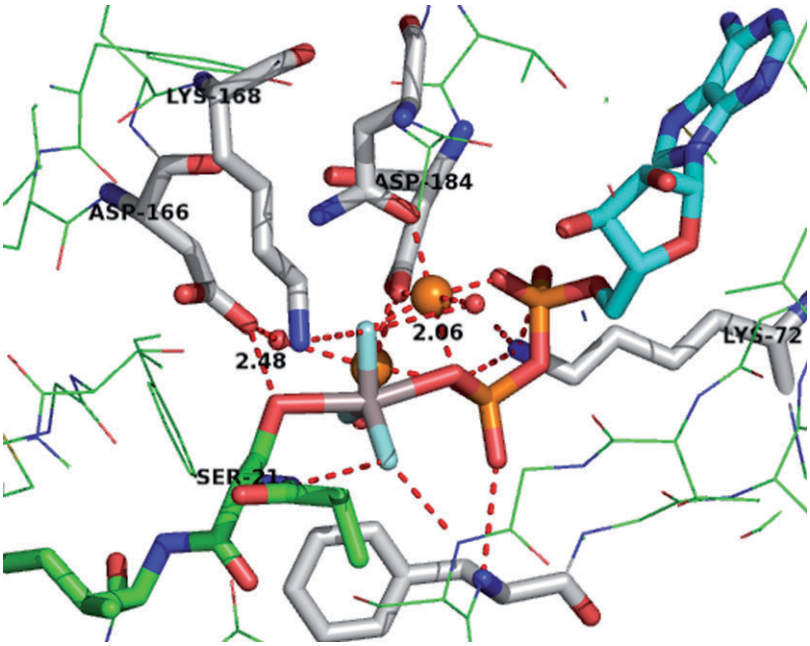


Fig. 10 Transition state for protein kinase A modelled with AlF_3, ADP, and substrate peptide.[34] 2 Magnesiums and 2 lysines provide 6 positive charges to balance the six negative charges (4 from ATP and 2 from aspartates 166 and 184). General acid–base catalysis for the serine-21 hydroxyl is provided by Asp-166 with a hydrogen bond length of 2.48 Å.

considerably shorter than the 4.36 Å separation seen in a vanadate.ADP.Mg complex of the myosin motor domain,[36] and is close to the axial separation for a covalent penta-oxyphosphorane. Therefore, these bond lengths should be interpreted with caution as mimics of the transition state: while the overall vanadate geometry clearly supports an "in-line" mechanism, it does not necessarily define a stable covalent phosphorane intermediate. Equally difficult is the interpretation of charge neutralization. Aspartates-51, -101, and -327 contribute 3 negative charges and the transferring phosphoryl anion makes a fourth. If both zincs have the state RO–Zn^+, they, together with the cations Arg-166 and Lys-328, give a total of 4 positive charges. Alternatively, the divalent magnesium cation can be deemed to counterbalance the negative charges from an ionized Ser-102 and Glu-322. More simply, the two zinc atoms provide Lewis acid catalysis to facilitate ionisation of the two neutral axial groups and enhance their affinity for the transferring phosphoryl group.

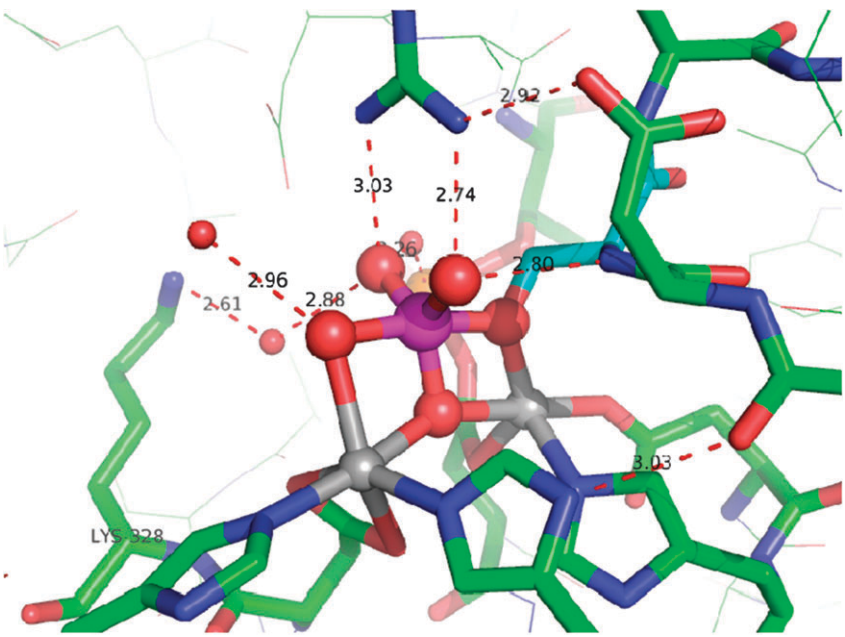


Fig. 11 Vanadate complex of *E. coli* alkaline phosphatase.[35] Vanadium (magenta) bonded to five oxygens: water (left), Ser-102 (right, cyan residue) and three equatorial oxygens in a tbp arrangement. Two zinc ions (grey) coordinate the two axial and the bottom equatorial oxygen. Arg-166 hydrogen bonds to the two upper oxygens. A magnesium ion (gold) at the rear is in the second coordination shell as is Lys-328 (left).

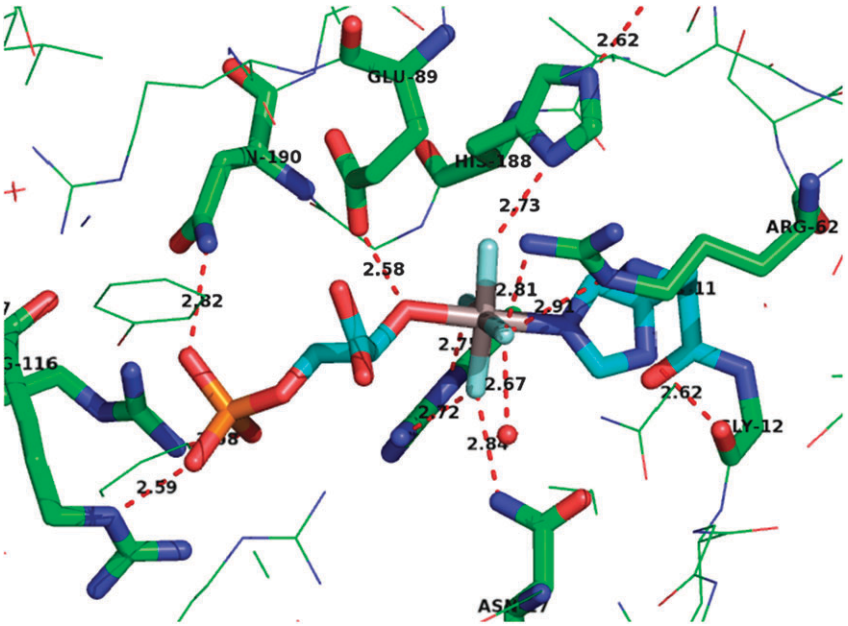


Fig. 12 Aluminium tetrafluoride complex for 3-phosphoglycerate mutase.[37] The octahedral AlF_4^- complex has 4 fluorines in the vertical plane mimicking the substrate 2,3-bisphosphoglycerate (left, blue) being attacked by the nucleophilic histidine-11 (right, blue). General base catalysis is provide by glutamate-89 (top) and charge balance by arginines-10 and -62. The 3-phosphoglycerate ester is clamped by arginines-116 and -117 (left).

Finally, 2,3-bisphosphoglycerate mutase provides a clear-cut example of a neutral nitrogen and neutral oxygen phosphoryl transfer reaction. The structure of an aluminium tetrafluoride complex has been solved to 2.0 Å resolution and gives good detail of the transition state (Fig. 12, PDB accession 2F90).[37] In this reaction, 2,3-bisphosphoglycerate binds as a primer. Its 3-phosphate is first transferred to neutral histidine-11 releasing 2-phosphoglycerate. 3-Phosphoglycerate then binds to the phospho-enzyme and the His-11 phosphate is transferred to hydroxyl-2. Rebinding with the two phosphates of the 2,3-bisphosphoglycerate interchanged now allows transfer of phosphate-3 to the enzyme and release of 2-phosphoglycerate. The repetition of this cycle results in the net conversion of 3-phosphoglyceric acid into 2-phosphoglyceric acid.

While interpretation of the aluminium tetrafluoride structure in transition state terms is impeded by the presence of the sixth ligand on the aluminium, a broad interpretation is simple. The complex shows clear "in-line" geometry with a 4.05 Å separation of the nucleophilic oxygen and nitrogen atoms. The "equatorial" fluorine ligands share 6 hydrogen bonds to proximate residues and one water. The two negative charges on the phosphate ester are nicely neutralised by the two positive charges on arginines-10 and -62. The "passive" 3-phosphate is clamped by arginines-116 and -117. The neutral histidine-188 coordinates to an oxygen in the tbp transition state. Finally, glutamate-89 provides general acid catalysis for cleavage of the P–O bond to the glycerate oxygen.

Conclusions on catalysis

All of the eight enzyme reactions described show a remarkable convergence in the primary features of catalysis as deduced by structural interpretation. First, the transition state geometry is uniformly "in line". The minor, second-order deviations from this seen in some structures may well be the result of the limitations of the transition state analogues as mimics of the true phosphoryl tbp, or of experimental inaccuracies in interpretation of the electron density maps, or may actually represent the true transition state geometry. Secondly, where it is possible to assess the net charge in the transition state complex, it tends to zero in the first co-ordination shell of the transferring phosphoryl group and usually into the second shell. For some kinases, it holds up to 15 Å from the core phosphorus atom.[26] This has to be one of the major factors in the successful evolution of rapid phosphoryl transfer reactions. Thirdly, where the nucleophile is an alkyl hydroxyl group, there is general base catalysis for its deprotonation in the transition state, equivalent to general acid catalysis in the transition state for P–O bond breaking. Next, in the six trigonal bipyramidal transition states examined, the equatorial oxygen ligands are well-coordinated to locate them in a stretched triangle through which the transferring phosphorus atom can shuttle between the tetrahedral structures of reactant and product phosphates. Lastly, although not emphasized in these analyses, with but a few exceptions there is general exclusion of water from the transition state inner complex except where waters are ligands for catalytic metals. It is also notable that for several enzymes here the lability of the phosphoryl group undergoing catalytic transfer contrasts dramatically with the passivity of structural phosphate(s) only a few Ångstroms from the reaction centre. It is also noteworthy that the covalent activity of the carboxyl group towards phosphate esters and anhydrides, not unrelated to the role of carboxylic-phosphoric anhydrides in protein biosynthesis, shows a proficiency that merits further investigation in the context of prebiotic chemical evolution of phosphoryl transfer processes.

Clearly, then, evolution has brought together a relatively simple set of chemical devices to accomplish catalytic rate

accelerations for phosphate monoesters that are among the largest in our biosphere. The comparable situation for phosphate diesters is as yet not so well investigated, but we can anticipate a similar overall outturn. This, then, brings us to the central Todd conjecture.[5] If evolution has employed phosphates across all forms of life on planet Earth, with roles stretching from extreme structural stability to sub-second, labile manipulation, must phosphates necessarily serve the same core functions for life on *any* planet in our Universe?

Are there alternatives to phosphate?

There are two parts to this question. The first part is: "Are there viable biotic alternatives to phosphorus in the Periodic Table?" We know that the Laws of Physics are universal.[38] While the Periodic Table of the Elements is also universal, the distribution of the chemical elements across the Universe is variable.[39] So the second part of the question is: "Are there other planets in the Universe with sufficient availability of phosphorus to support prebiotic evolution?"

The foregoing analysis has demonstrated the essentiality of anionic charge on oxyacid diesters to give them the requisite structural stability for their biopolymer roles[13] (Table 1). A brief survey of the characteristics of vanadate and arsenate esters (Group V elements) shows that although they have the desired anionic character, they do not have the required stability (Table 2). Sulfate diesters also fail the stability test, but their monoesters do have useful stability, although not comparable to that of phosphate monoesters.

Could not terrestrial evolution have developed a mixed economy, with phosphate diesters for biopolymers and sulfate monoesters for all other purposes? This question is not quite imponderable. The arguments against such a duality include the following considerations: polyphosphates as putative prebiotic phosphorylating species[40] have no polysulfate counterpart; the evolutionary simplicity of adenosine 5′-triphosphate as the phosphorylating agent contrasts with the complexity of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as sulfating agent (its structural complexity is needed for segregation of activities from ATP); coulombic forces and hydrogen bonding are much diminished for sulfate monoester monoanions relative to phosphate dianions; moreover, while tyrosine sulfation is the major post-translational modification of tyrosine residues, it first appeared consistently only in multicellular eukaryotic organisms—and is usually non-reversible.[41] It may also be relevant that the dominant, though not universal, role of magnesium ions in the catalysis of phosphoryl transfer relates to a dissociation constant for magnesium phosphate (8.5 mM)[42] compared to that of magnesium sulfate (2.2 μM).[43]

It thus appears that, in chemical evolution, the prebiotic selection of phosphate esters underpins the evolution of their biological dominance in the multifunctional roles described at the beginning of this review. What then of the universal availability of phosphate?

The cosmic formation of 31-phosphorus arises in the inner zone of stars massive enough (≥15 solar masses) to undergo hydrostatic C-burning, leading to explosive Ne-burning[44] at a temperature around 1.5×10^9 K, that primarily generates 16-oxygen and 24-magnesium but secondarily makes ^{31}P (2.5% yield). This is followed by with oxygen burning in thermonuclear processes that generate silicon, sulfur, and (some) phosphorus. Such reactions have resulted in a distribution of the elements now determined by analysis of galactic cosmic rays[46] and shows that the availability of phosphorus across the universe is rather higher than within our solar system (Fig. 13). These data may also challenge the opinion that there had to be a meteoric local accretion of phosphorus for prebiotic chemistry to begin. They clearly enhance the possibility of life linked to water, carbon, nitrogen, and phosphorus elsewhere in the universe. That is being developed through two concepts: the Galactic Habitable Zone, (GHZ),[47] and the Circumstellar Habitable Zone, (CHZ),[48] in which the probability of water-based planetary life is significant (Fig. 14). Basically, GHZ supports the concept that a habitable ring of stars formed about 8 billion years ago at around 25 000 light years from the core of our Galaxy, embracing less than 10% of the stars in our Milky

Table 1 Kinetics of phosphate ester hydrolysis

Ester	Ion	Cleavage	k 25 °C, s^{-1} or M^{-1} s^{-1}	$\Delta G^{\ddagger}$ 25 °C, kcal mol^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$T\Delta S^{\ddagger}$ 25 °C, kcal mol^{-1}
Monoester						
$H_2O + MeOPO_3H^-$	Monoanion[14]	P–O	2.4×10^{-10}	30.6	30.0	0.6
$H_2O + MeOPO_3^=$	Dianion[15]	P–O	2×10^{-20}	44.3	47.0	2.7
$H_2O + PhOPO_3^=$	Dianion[15]	P–O	6×10^{-14}	36.0	38.0	7.0
Diester						
$H_2O + (MeO)_2PO_2H$	Neutral[16]	C–O	6×10^{-10}	30.0	25.0	5.0
$H_2O + (MeO)_2PO_2^-$	Anion[17]	C–O	1.6×10^{-13}	34.9	25.9	9.0
(or $HO^- + (MeO)_2PO_2H$) neutral						
$HO^- + (MeO)_2PO_2^-$	Anion[14]	C–O	3×10^{-11}	31.7	27.6	4.1
$H_2O + (NpO)_2PO_2^-$	Anion[8]	P–O	7×10^{-16}	38.1	29.5	8.6
$HO^- + (NpO)_2PO_2^-$	Anion[8]	P–O	1.4×10^{-15}	37.7	29.5	8.0
Triester						
$H_2O + (MeO)_3PO$	*Neutral*[14]	C–O	2×10^{-8}	28.1	22.6	5.5
$HO^- + (MeO)_3PO$	Neutral[15]	P–O	1.4×10^{-4}	22.7	15.4	7.3
$HO^- + (EtO)_3PO$	Neutral[14]	P–O	9×10^{-6}	24.3	14.1	10.2
Monoesterase						
Yersinia PTPase	Ref. 9	P–O	1000	—	—	—
Diesterase						
Staphylococcus nuclease	Ref. 18	P–O	95	14.7	10.8	3.9

Table 2 Candidate elements, oxyacids, and their esters

	Relative elemental abundance (Number of atoms relative to silicon)					Oxyacid				Oxyacid esters				
Element	Earth crust	Meteorite	Solar system	Universe	Man	Oxyacid	pK_{a1}	pK_{a2}	pK_{a3}	Diester stability/$t_{1/2}$	Diester charge	Bond cleaved	Monoester stability/$t_{1/2}$	Monoester charge	Bond cleaved
Si	1.000	1.000	1.000	1.000	1.000	H_4SiO_4	9.5	> 13	–	< 1 min	0	Si–O	< 1 min	0	—
P	3×10^{-3}	8.6×10^{-3}	8.4×10^{-3}	8×10^{-3}	38	H_3PO_3	2.1	7.2	13.1	10^5 y	–1	P–O	10^{12} y	–2	P–O
V	1×10^{-4}	5.2×10^{-4}	2.9×10^{-4}	3×10^{-4}	7×10^{-5}	$[H_3VO_4]$	3.2	7.8	12.5	< 1 s	–1	V–O	≪ 1 s	–1	V–O
As	2×10^{-6}	1.6×10^{-5}	6.1×10^{-6}	4×10^{-6}	7×10^{-5}	H_3AsO_4	2.2	7.0	11.5	< 2 min	–1	As–O	6 min	–2	As–O
S	8×10^{-4}	0.51	0.445	0.4	6.7	H_2SO_4	< 0	2.0	—	1.7 h	0	C–O	1100 y	–1	C–O

Astrophysics data: K. Lodders. *Astrophys. J.* 2003, **591**, 1220; see also http://www.webelements.com/periodicity/abundance_universe/index.html; HVO_4 and esters: A. S. Tracey and M. J. Gresser, *Can. J. Chem.*, 1988, **66**, 2570.

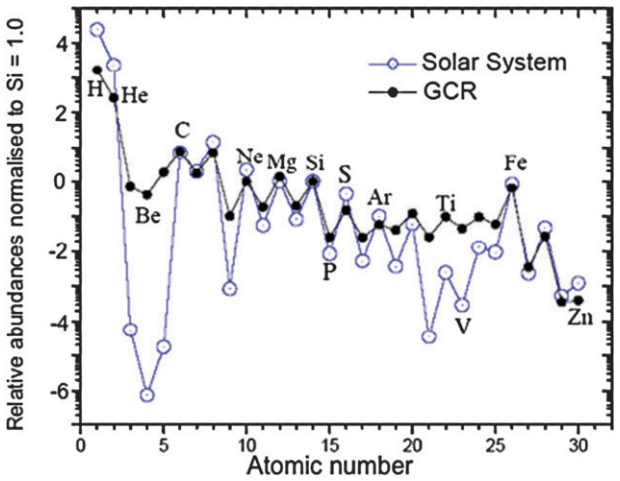


Fig. 13 Relative abundances of the lighter elements in the solar system and in the universe. Reproduced with permission from Professor J. S. George, *Astrophys. J.*, 2009, **698**, 1666.

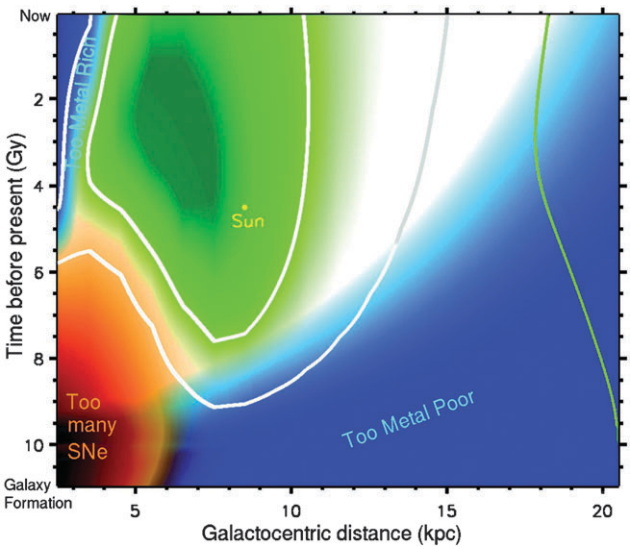


Fig. 14 The Galactic Habitable Zone (GHZ) in the disk of the Milky Way based on the star formation rate, metallicity (blue), sufficient time for evolution (gray), and freedom supernova explosions that would extinguish life (red). The white contours encompass 68% (inner) and 95% (outer) of the origins of stars with the highest potential to harbour complex life today. Reproduced with permission from Professor Charles Lineweaver, ANU.

Way. Planets within this zone occupy a notional shell of space surrounding such a star where the surface temperature can maintain liquid water. This is sometimes called the "comfort zone". Many of these planets will be much older than Earth, maybe by 1 billion years! One of the major results from such work is the identification of Gliese 581d as the outermost of four planets of Gliese 581, a star that is "only" some 20 light years away from our sun in the constellation of Libra. Because of its mass, nearly 8 times that of Earth, this planet is classified as a super-Earth. In late April 2009, new observations by the original discovery team concluded that the planet is within the habitable zone where liquid water and, therefore, life could exist.

Will man in time find phosphate-based life there?

Conclusion

Phosphoric acid has a unique ability among the elemental oxyacids to form condensed polymers, both chains and rings, that are proven phosphorylating agents under mild conditions.[40] They can lead directly to phosphate mono- and diesters that recent research has shown to combine extreme stability to spontaneous hydrolysis with susceptibility to rapid manipulation by phosphoryl transfer enzymes. While the evolutionary catalytic role of the 2′-hydroxyl group in the emergence of ribozyme catalysis in an RNA world has been thoroughly developed,[49] the covalent catalytic role of the carboxyl group in primordial phosphoryl and carbonyl transfer has been largely neglected and merits further study, especially as it makes a key contribution to catalysis of phosphoryl transfer in several enzymes. Such catalytic processes are yielding their secrets by studies on transition state complexes using a combination of X-ray and NMR structural analysis. Many of them originated at the dawn of life to juxtapose the stability of phosphate esters for genomic purposes with their ready manipulation to develop regulatory and control processes. Since there appears to be no oxyacid from an alternative element in the Periodic Table capable of bridging these two extremes, there is a strong case for the contention that phosphate will be found to command comparable roles in life wherever it exists in the universe.

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References

1 J. Baddiley, A. M. Michelson and A. R. Todd, *Nature*, 1948, **161**, 761.
2 A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 1955, 2632.
3 H. G. Khorana and A. R. Todd, *J. Chem. Soc.*, 1953, 2257.
4 A. R. Todd, *Proc. Natl. Acad. Sci. U. S. A.*, 1959, **45**, 1389–97.
5 A. R. Todd, in *"Where there's life there's phosphorus"*, ed. M. Kageyama, K. Nakamura and T. Oshima, Japan Sci. Soc. Press, Tokyo, Japan, 1981, p. 275.
6 J. M. Sturtevan, J. A. Gerlt and F. H. Westheimer, *J. Am. Chem. Soc.*, 1973, **95**, 8168.
7 B. Blagoev, S. E. Ong, I. Kratchmarova and M. Mann, *Nat. Biotechnol.*, 2004, **22**, 1139.
8 G. K. Schroeder, C. Lad, P. Wyman, N. H. Williams and R. Wolfenden, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 4052.
9 Z.-Y. Zhang, Y. Wang and J. E. Dixon, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 1624.
10 J. Kumamoto, J. R. Cox and F. H. Westheimer, *J. Am. Chem. Soc.*, 1956, **78**, 4858.
11 E. A. Dennis and F. H. Westheimer, *J. Am. Chem. Soc.*, 1966, **88**, 3432.
12 F. H. Westheimer, *Chem. Rev.*, 1981, **81**, 313.
13 F. H. Westheimer, *Science*, 1987, **235**, 1173.
14 C. A. Bunton, D. R. Llewellyn, K. G. Oldham and C. A. Vernon, *J. Chem. Soc.*, 1958, 3574.
15 C. Lad, N. H. Williams and R. Wolfenden, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 5607.
16 C. A. Bunton, M. M. Mhala, K. G. Oldham and C. A. Vernon, *J. Chem. Soc.*, 1960, 3293.
17 R. Wolfenden, C. Ridgway and G. Young, *J. Am. Chem. Soc.*, 1998, **120**, 833.
18 E. Serpersu, D. Shortle and A. S. Mildvan, *Biochemistry*, 1987, **26**, 1289.
19 The software package MOE (Molecular Operating Environment, available from the Chemical Computing Group - http://www.chemcomp.com/) was used for calculation of the electrostatic surface and creation of the graphics.
20 S. Rahlfs, S. Koncarevic, I. Rimma, M. M. Boniface, S. Savvides, R. H. Schirmer and K. Becker, *Mol. Biochem. Parasitol.*, 2009, **163**, 77.
21 A. C. Hengge, *Adv. Phys. Org. Chem.*, 2005, **40**, 49; W. W. Cleland and A. C. Hengge, *Chem. Rev.*, 2006, **106**, 3252; R. H. Hoff and A. C. Hengge, *J. Labelled Compd. Radiopharm.*, 2007, **50**, 1026.
22 I. Schlichting and J. Reinstein, *Biochemistry*, 1997, **36**, 9290.
23 N. J. Baxter, G. M. Blackburn, J. P. Marston, A. M. Hounslow, M. J. Cliff, W. Bermel, N. H. Williams, F. Hollfelder, D. E. Wemmer and J. P. Waltho, *J. Am. Chem. Soc.*, 2008, **130**, 3952.
24 C. T. Driscoll and W. D. Schechter, in *Aluminium and Health: a critical review*, ed. H. J. Gitelman, Marcel Dekker, NY, 1989, pp. 30–35; R. B. Martin, *Coord. Chem. Rev.*, 1996, **149**, 23; H. Sigel, C. P. DaCosta and R. B. Martin, *Coord. Chem. Rev.*, 2001, **219–221**, 435.
25 But only when interpreted as an MgF_3^- complex.
26 M. Bowler, submitted.
27 J. M. Denu, J. A. Stuckey, M. A. Saper and J. E. Dixon, *Cell*, 1996, **87**, 361.
28 M. Zhang, M. Zhou, R. L. Van Etten and C. V. Stauffacher, *Biochemistry*, 1997, **36**, 15.
29 G. M. Blackburn and M. J. Brown, *J. Am. Chem. Soc.*, 1969, **91**, 525.
30 S. A. Khan, A. J. Kirby, M. Wakselman, D. P. Horning and J. M. Lawlor, *J. Chem. Soc. B*, 1970, 1182.
31 There is a strong case for re-examination of all of the 34(?) protein structures in the PDB that have been hitherto assigned as AlF_3 simply on interpretation of electron densities. At structural resolutions of ≥ 1.0 Å it is not possible to differentiate AlF_3^0 from MgF_3^- by X-ray diffraction. Either PIXE or ^{19}F NMR chemical shifts must be employed.
32 N. J. Baxter, M. W. Bowler, T. Alizadeh, M. J. Cliff, A. M. Hounslow, B. Wu, D. B. Berkowitz, N. H. Williams, G. M. Blackburn and J. P. Waltho, *Proc. Natl. Acad. Sci. USA*, 2010, in press.
33 N. J. Baxter, A. M. Hounslow, M. W. Bowler, N. H. Williams, G. M. Blackburn and J. P. Waltho, *J. Am. Chem. Soc.*, 2009, **131**, 16334.
34 A. P. Madhusudan, N. H. Xuong and S. S. Taylor, *Nat. Struct. Biol.*, 2002, **9**, 273.
35 K. M. Holtz, B. Stec and E. R. Kantrowitz, *J. Biol. Chem.*, 1999, **274**, 8351.
36 C. A. Smith and I. Rayment, *Biochemistry*, 1996, **35**, 5404.
37 Y. Wang, L. Liu, Z. Wei, Z. Cheng, Y. Lin and W. Gong, *J. Biol. Chem.*, 2006, **281**, 39642.
38 M. T. Murphy, V. V. Flambaum, S. Muller and C. Henkel, *Science*, 2008, **320**, 1611.
39 *cf.* J. J. Cowan, C. Sneden, S. Burles, I. I. Ivans, T. C. Beers, J. W. Truran, J. E. Lawler, F. Primas, G. M. Fuller, B. Pfeiffer and K.-L. Kratz1, *Astrophys. J.*, 2002, **572**, 861.
40 R. Lohrmann and L. E. Orgel, *Science*, 1971, **171**, 490; R. Österberg, L. E. Orgel and R. Lohrmann, *J. Mol. Evol.*, 1973, **2**, 231.
41 W. B. Huttner, *Ann. Rev. Physiol.*, 1988, **50**, 363; Y.-B. Ouyang, W. S. Lane and K. L. Moore, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 2896.
42 I. N. Smirnova, A. S. Shestakov, E. B. Dubnova and A. A. Baykov, *Eur. J. Biochem.*, 1989, **182**, 451.
43 D. A. Bies, *J. Chem. Phys.*, 1955, **23**, 428.
44 S. E. Woosley, A. Heger and T. A. Weaver, *Rev. Mod. Phys.*, 2002, **74**, 1015.
45 D. Arnett, *Supernovae and Nucleosynthesis*, Princeton University Press, Princeton, New Jersey, 1996; J. Oró, T. Mills and A. Lazcano, *Adv. Space Res.*, 1995, **15**, 81.
46 ACE News, 2004, October 6th, Advanced Composition Explorer, NASA, USA.
47 C. H. Lineweaver, Y. Fenner and B. K. Gibson, *Science*, 2004, **303**, 59.
48 W. von Bloh, C. Bounama, M. Cuntz and S. Franck, *Proc. Internat. Astronom. Union*, 2007, **30**, 503.
49 G. F. Joyce, *Nature*, 2002, **418**, 214, and refs therein. L. E. Orgel, *Crit. Rev. Biochem. Mol. Biol.*, 2004, **39**, 99.

