Review

Metallic Fluoride Complexes as Phosphate Analogues for Structural and Mechanistic Studies of Phosphoryl Group Transfer Enzymes

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Received: 30-09-2009

Abstract

There have been intensive efforts to try to understand the details of phosphoryl transfer reactions extending from non-enzymatic (or enzyme model) systems to the mechanisms of the enzyme catalysed reactions. As phosphate analogues, few metallic fluorides AlF_x, BeF_x and MgF_x affect the activity of a variety of phosphoryl transfer enzymes, and it is accepted that these small inorganic complexes are useful chemical probes for structural and mechanistic studies in enzymology because they are able to mimic phosphoryl group in ground state (BeF_x) as well as in transition state (AlF_x,MgF_x). Al^{3+} and Be^{2+} tend to form stable complexes with different fluoride anions (x = 1 to 4) spontaneously in aqueous solution but Mg^{2+} does not. BeF_x geometry is strictly tetrahedral resembling the phosphate ground state when bound to an acyl group of protein active site (phosphorylated acyl groups are unstable otherwise), or the Michaelis complex when BeF_x concominantly with nucleoside diphosphate replaces γ-phosphate group in nucleoside triphosphate sites. AlF_x and MgF_x are identified as enzymatic analogues of phosphoryl transition state where both are able to form different coordination geometries within the enzyme active sites: trigonal bipyramidal (AlF_3 and MgF_3^-) or octahedral (AlF_4^- or MgF_4^{2-}). The geometry and charge of MgF_3^- are the best suited to mimicking the trigonal planar PO_3^- moiety of phosphoryl transfer transition state but MgF_3^- does not, unlike aluminum and beryllium fluoride complexes, exists in solution and can be assembled and stabilized in suitable active site only. Therefore it is particularly interesting to characterize as a potentially highly accurate transition state analogue and may be the best reagent of choice for studying phosphoryl transfer reactions in future.

Keywords: phosphoryl transfer, metallic fluoride, transition state analogue

1. Introduction

Nucleophilic substitutions at phosphate monoesters group (*phosphoryl group*, where for simplicity we classify displacement at the γ-phosphate group of a nucleotide triphosphate with the reactions of phosphate monoesters because an unsubstituted phosphoryl group is transferred in all these cases) comprise one of the most important classes of reactions in biology because phosphoryl group reactions play a central role in metabolism, regulation, energy housekeeping and cell signaling of any live organism.[1–5] As phosphate esters are extremely stable in neutral aqueous solutions at ambient temperature ($t_{1/2} \sim 1.1 \times 10^{12}$ years for alkyl phosphate dianion hydrolysis, $t_{1/2} \sim 31$ million years for dialkyl phosphate anion hydrolysis),[6] efficient catalysis is crucial for the control of all these cellular processes. The enzymes are unique catalysts of enormous catalytic power that can accelerate phosphoryl transfer reaction rates by factors up to 10^{21},[6,7] and are consequently able to reduce these reactions half-lives to a viable time scale. Phosphoryl transfer reactions are catalysed by regulatory enzymes, ATPases, protein, and small molecule phosphokinases, protein, and small molecule phosphatases or phosphomutases.[2,5] All these enzymes are among the most proficient catalysts known today[7–9] but our understanding of the origins of their prowess as catalysts is far from complete[10,11] and the mechanisms of enzyme action are particularly challenging to rationalize because even when the preferred mechanistic routes of these enzymes are clear,[2,5] we are still faced with ascertaining the reasons for their catalytic rate enhancement.

To obtain a comprehensive and quantitative understanding of enzyme catalysis, the interactions of an enzyme active site with its substrate as it passes through its transition state (TS) need to be defined[12]. A snapshot of an enzyme in a high-energy state would be immensely useful, as it would allow the very interactions that bring about the catalysis to be observed. However, is this realistic given how elusive high-energy intermediates (INTs) and TSs inevitably are? No physical or spectroscopic method is available to observe the structure of TSs of enzymatic reactions directly. Thus long-lived transition state analogues (TSAs) that bind tightly in an enzyme active site and act usually as efficient slow tight-binding inhibitors have been of paramount importance in defining the structural and energetic framework for enzymatic catalysis.[13–15] Structures of TSA complexes of phosphoryl transfer enzymes[16–18] can be used to deduce whether the mechanism of the phosphoryl transfer reaction occuring in the enzyme active site is associative or dissociative. Since the two mechanisms differ in charge distribution and bond order of the TS, the location of positively charged amino acids and the distance of the phosphate analogue to the leaving groups are crucial. However, to provide a reliable basis for extrapolation from TSA structures to TS structures, the TSA must accurately reflect the geometry and charge distribution of the true TS but the inhibition constant of a TSA is not a sufficient good measure to evaluate this fit, which should be based on correlation between the inhibition constant and catalytic parameters.[19]

Several related metallic fluoride complexes have been used as models for scissile phosphoryl group and currently provide the best available approximations for the TSs of phosphoryl substitution reactions or unstable phosphorylated protein intermediates.[17,18] The use of the small metallic fluoride molecules has led to a quantum jump in our understanding of the biophysical mechanisms of enzyme catalysis underlying such diverse biological processes as metabolism, signal transduction and cellular regulation, the common denominator of which is the catalysis of phosphoryl transfer reactions. This review discusses the chemical mechanisms of phosphate transfer reactions, the biochemistry of enzymes that catalyse these reactions and basic chemistry of metallic fluoride complexes in relation to phosphate analogy, their structural and chemical properties that are fundamental for their prowess to be efficient models of phosphoryl transfer TSs in many enzyme active sites.

2. Mechanistic Considerations on Phosphate Substitution Reactions

Phosphoric acid may be esterified in one, two, or three positions, forming monoester, diester, or triester (triesters do not occur naturally), respectively. Phosphate transfer reactions are substitution reactions and they have been described in terms of a continuum of mechanisms with two limiting cases where two different high-energy INTs exist on the reaction pathway.[1,20,21] A fully *dissociative* mechanism (see Fig. 1A), which is a realistic possibility only for phosphate monoesters, proceeds via a highly reactive trigonal metaphosphate INT which is captured by an acceptor group. The metaphosphate PO_3^- anion has never been directly observed in solution,[22] but it exists as a stable entity in gas phase, where it is surprisingly unreactive.[23] Other oppositional extreme, a fully *associative* (two-step addition-elimination) mechanism (see Fig. 1B) involves a transitory trigonal bipyramidal INT – phosphorane. Between these extremes are *concerted* (two-step addition-elimination) mechanisms (see Fig. 1C) with trigonal bipyramidal TS but no INT. The addition-elimination mechanisms occur in some reactions of triesters and diesters[1,20,21] and it has been speculated to occur in some enzymatic reactions of phosphate monoesters, too.[24]

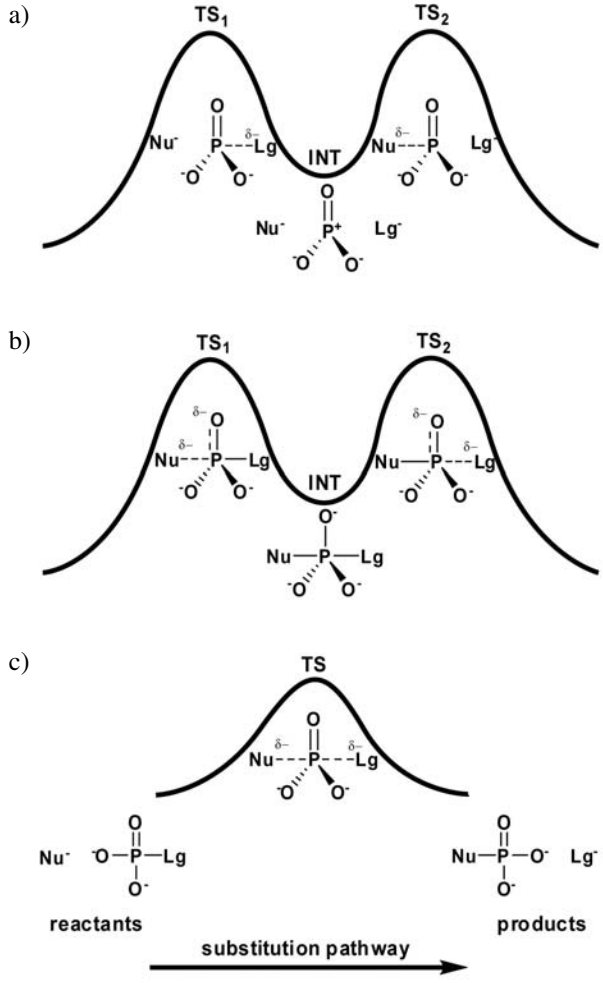


Figure 1. Three possible chemical mechanisms (reaction paths) for phosphoryl transfer. Hypothetical free enthalpy diagrams for each path highlight the fact that an intermediate is formed in the case of (A) dissociative (metaphosphate) and (B) associative (phosphorane) mechanisms, whereas there is (C) no intermediate in the concerted reaction.

The continuum of mechanisms is best appreciated by considering the axial distances from the entering and leaving oxygens to the reaction center phosphorus in the trigonal bipyramidal INT or TS (Fig. 2), which measure the extent of bonding of the entering and leaving atoms on phosphorus[25]. In a fully dissociative mechanism (classical S_N1 mechanism), the axial P–O distances would both be ≥ 3.3 Å, the van der Waals sum of phosphorus (1.9 Å) and oxygen (1.4 Å), indicating only contact but no bonding. The relationship between bond distance D(n) and fractional bond number (n) has been given by Pauling[26] as

$$D(n) = D(1) - 0.60 \log_{10}(n) \quad (1)$$

in which D(1) is the single bond distance, 1.73 Å for the P–O covalent bond.[26] A classical S_N2 mechanism would have 0.5 bonds concerted to both the entering and leaving groups in the trigonal bipyramidal TS and could be therefore described as 50% associative. From eq. 1, a P–O bond that is half formed would have an axial bond distance of 1.91 Å.

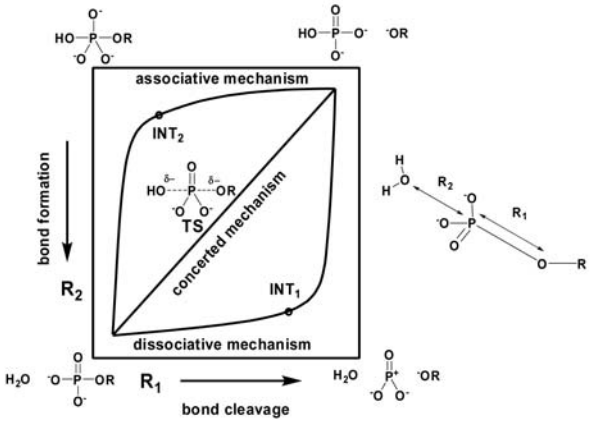


Figure 2. A schematic reaction coordinate diagram for the hydrolysis of phosphomonoesters. The reactants are shown at bottom-left but products at upper-right. The figure provides a clear definition of different paths, reaction intermediates and also defines the reaction coordinates R_1 (the distance between the reacting phosphate and leaving group) and R_2 (the distance between the reacting phosphate and attacking nucleophile).

The very short lifetime of the TSs or high energy metastable INTs on the reaction pathway is currently well below the time scale of crystallographic analysis, so the structure of these complexes is not amenable to direct study by this method. The direct observation of TSs for simple organic reactions has required ultrafast lasers with femtosecond resolution[27] but other physico-chemical techniques, e.g. ^{18}O kinetic isotope effect (KIE),[21,28,29] linear free-energy relationships (LFER),[22,30–32] stereochemistry by using ^{16}O, ^{17}O, ^{18}O labelled chiral phosphate group,[2,33,34] vibrational spectroscopy[35] or computational approaches[36,37] are usually available to observe and study the reaction mechanisms of phosphate transfer in solution or enzyme active sites. Solution phosphate transfer reactions can proceed by all three, mechanistically distinct, nucleophilic-substitution pathways: dissociative, concerted and associative.[1,20,21] These differ in the timing of the formation of phosphorus nucleophile bond and cleavage of the phosphorus leaving group bond. The prevailing chemical pathway is determined by the nature of nucleophile, characters of electrophile (phosphate mono-, di- or triester) and leaving group,[1,2,20,21] and also by the solvent and its pH.[38]

It has long been known that the dianions of phosphate monoesters hydrolyze by dissociative mechanism[1,20,21] where the bond to the leaving group is largely broken before the incoming nucleophile and phosphorus atom bond formation results. Thus, the rate constant varies tremendously with the pK of the leaving group but the role of the nucleophile is diminished as the sensitivity of reaction rate to the pK of the entering nucleophile is low. The activation entropies are close to zero for this type of reactions, and there is a substantial ^{18}O KIE for the P–O bond being cleaved.[2,20,21] These evidences have established that the formation of a highly reactive metaphosphate-like species is central in this dissociative reaction mechanism[5] whereas there is no evidence for true metaphosphate anion PO_3^- INT in phosphate monoester solvolysis reaction in aqueous solution at least.[22,23] Phosphoanhydrides are analogous to phosphate monoesters in that they posses a single phosphoryl substituent, and it has been shown that phosphoanhydrides like ATP also reacts via dissociative mechanism.[39,40] The contrast is an associative mechanism, which occurs in phosphate triester substitution reactions, in which a pentavalent-like species (phosphorane) is generated.[20,21,41] The pentacoordinate phosphorane need not to be a TS, however, but rather may be an INT (especially for cyclic esters in which steric crowding is reduced) with a sufficient lifetime to allow pseudorotation.[2,42] In associative TSs, there is a significant degree of bond formation between the incoming nucleophile and the attached phosphorus before leaving group departure. Therefore for triesters, the reaction rates are equally sensitive to the pK of the nucleophile and the leaving group. As the degree of protonation of the phosphate monoester is increased at lower pH, one observes an increase in the sensitivity of reaction rate to the pK of the nucleophile, and under such conditions the TSs of phosphate monoesters evidently begin to assume some of the characteristics of associative process, too.[21]

Phosphate diesters are more stable than either mono- or tristers, so that the phosphodiester linkages that join the nucleotides of DNA are highly resistant to spontaneous hydrolysis.[6] If the leaving group has a pK around neutrality, however, hydrolysis is facile and more easily followed. Cyclic phosphodiesters with five-membered rings are also much more reactive, as are diesters containing a

hydroxyl in position to attack and form a cyclic phosphodiester. Thus RNA, in contrast to DNA, is readily cleaved in either acid or base as a result of attack of the 2'-hydroxyl of the ribose to give a cyclic 2',3'-phosphodiester[43]. For reactions of diester anions, LFER[41,44] and ^{18}O KIE[29] values range between those for reactions of monoester dianions and those of triesters. These data are consistent with the idea that all of the phosphodiester reactions exhibit TSs that are intermediates between dissociative metaphosphate like moiety and associative phosphorane like species. Phosphodiester substitution reactions seem to be concerted and do not involve phosphorane INTs except for those phosphodiester reactions, which appear to involve acid catalysis[2,20,21]. In summary, the high-energy states for uncatalysed phosphate transfer reactions across the three classes of phosphate esters generally follow a trend from metaphosphate-like INTs for monoesters and anhydrides, to a more synchronous concerted reaction TS for diesters, to phosphorane-like INTs for triesters.

Although a full and convincing explanation at the quantum mechanical level has not yet been made as to why for example dissociative TS should be preferred for nonenzymatic phosphate monoester substitution reactions[36], the simplest explanation in that case is based on the concept that the negative charges on two of the phosphate oxygens repel incoming nucleophiles and stabilize metaphosphate-like species. More difficult to address is how enzymes catalyse phosphoryl transfer reactions with such extreme efficiency, how relevant are mechanisms of phospho-transfer reactions in solution to reaction processes catalysed by enzymes and what then controls the chemical pathway within a protein catalytic scaffold? The rules governing selection of a pathway for a given enzyme-reactant pair are unfortunately still not known and their discovery cannot be predictable yet[36]. Therefore looking for probes of the structural source of enzyme catalysis is a great challenge in enzymology[13]. The greater the rate enhancement that an enzyme produces (enzyme proficiency), the greater is its affinity for the altered substrate in the TS compared to with its relatively modest affinity for the substrate in the ground state[8,9]. That principle has furnished a basis for the design of TSAs, stable molecules exploiting that special affinity[13,14].

3. Classes of Enzymes Involving Reactions at Phosphoryl Group

The enzymes that handle phosphate monoesters and displacement at the γ-phosphoryl group of nucleotide triphosphates fall into three main categories[2]: the *phosphatases*, where water is acceptor of the phosphoryl group (these include enzymes such as alkaline phosphatase that are merely hydrolytic, and enzymes such as the ATPases where the free energy available is coupled to some other metabolic process); the *phosphokinases*, where a nucleotide triphosphate is the phosphoryl donor and some molecule other than water is the acceptor; and the *phosphomutases*, for which the acceptor is another functional group on the donor molecule.

3. 1. Phosphatases

Phosphatases catalyse the hydrolysis of protein and small molecule phosphate monoesters producing inorganic phosphate as one of the final products. Phospho-protein phosphatases are generally grouped into two main subclasses based on substrate specificity and genetic homology[45,46]: the protein phosphatases (PPs) specifically hydrolyze serine/threonine phosphoesters, while protein tyrosine phosphatases (PTPs) hydrolyze phosphotyrosine. Less specific small molecule phosphatases are alkaline phosphatases (APs), acid phosphatases and purple acid phosphatases (PAPs)[5].

From reaction mechanism point of view there are two groups of phosphatases that act by different kinetic mechanisms. One group of phosphatases, typified by bacterial APs, acid phosphatases and PTPs, follows a two-step overall kinetic mechanism and uses an active site nucleophile (*Ser* in APs, *His* in acid phosphatases and *Cys* in PTPs) to displace the alcohol leaving group from the substrate to form a phosphoenzyme intermediate, which is subsequently hydrolyzed by nucleophilic addition of water. There are numerous evidences for the phospho-enzyme intermediate like overall retention of configuration on chiral phosphate esters, burst transient kinetics, site specific mutagenesis as well as structural data obtained by x-ray crystallography and ^{31}P NMR[2,5,47,48]. However, the chemistry of catalysis is not the same for all these phosphatases with the common denominator in phosphorylated enzymes during the reaction pathway as APs use binuclear metal catalysis (Fig. 3A) but acid phosphatases and PTPs act in the absence of metal cofactors (Fig. 3B)[5]. In any case the rate enhancement by enzymes that act through a double displacement mechanism is not simple to analyze in terms of TS affinity or TS stabilization.

A second group of phosphatases catalyses direct attack by water on phosphorus to displace the alcohol leaving group[49] and therefore lends itself directly to the estimation of TS affinities. Enzymes that bring about direct water attack include PAPs and PPs[5], in which a similar binuclear metal center coordinated with seven invariant amino acids (Fig. 3C) activates a two metal ions bridging (coordinated) hydroxide for taking an active part in the substitution reaction as either as the nucleophile or as a general base for nucleophilic attack of water molecule[24,50,51]. The reaction occurs with inversion of configuration at phosphorus, which support direct phosphoryl transfer to water[52]. It could be assumed that phosphoryl transfer reactions catalysed by these enzymes follow the similar single step mechanism as uncatalysed solution reactions and therefore the catalysis should be energeti-

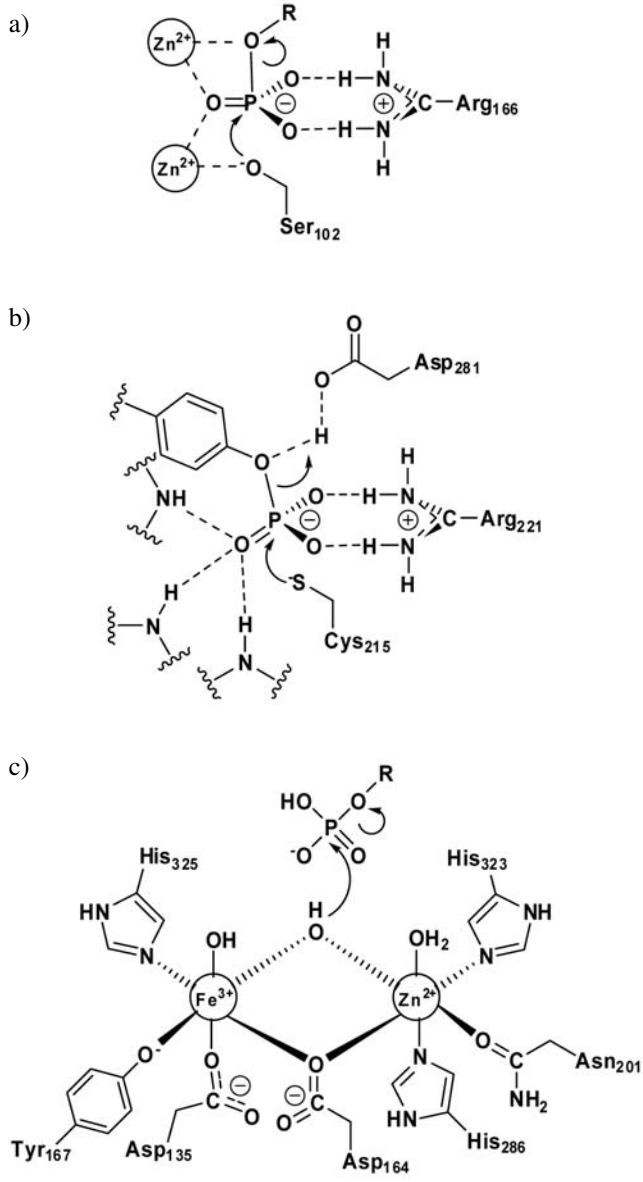


Figure 3. Model of the interactions in the reaction catalysed by: A) alkaline phosphatase, B) protein tyrosine phosphatase and C) purple acid phosphatase.

cally most efficient because the dissociative mechanistic pathway in solution is stabilized only. However, in many cases, it is often suggested (and sometimes stated explicitly) that phosphoryl transfer enzymes stabilise an associative type TS[53–55]. Some model complexes (as dinuclear cobalt (III) complex shown in Fig. 4 which bears an obvious resemlance to the active site of protein phosphatase 1) were used in analysing the characteristics of alternative associative mechanisms that have been proposed for the metalophosphatase catalysed reactions[56] but the detailed understanding of how these reactions proceed, and the corresponding enzyme-catalysed processes, is still the subject of debate.

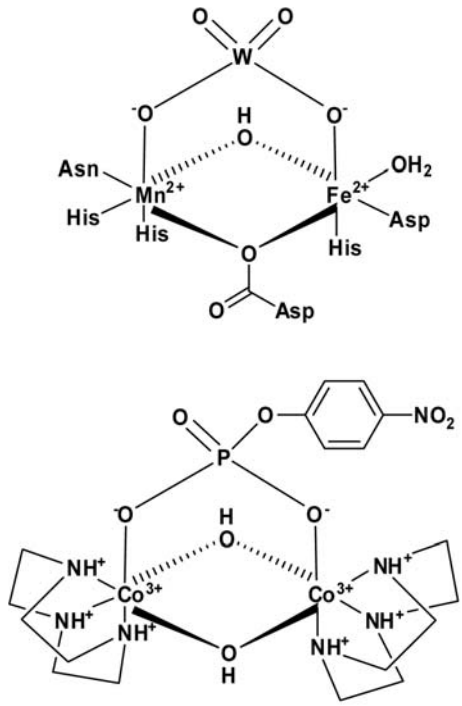


Figure 4. Schematic representation of the active site of protein phosphatase 1 with bound tungstate compared to the model dinuclear Co(III) complex.

3. 2. Phosphokinases

Phosphokinases belong to an ubiquitous group of enzymes that participate in a variety of cellular pathways. By definition, the more common name kinases is applied to enzymes that catalyse the transfer of the terminal γ-phosphate unit from a nucleotide triphosphate to an acceptor other than water[2,5], which can be a small carbohydrate, lipid, or protein substrate. Although all kinases catalyse essentially the same phosphoryl transfer reaction, they display remarkable diversity in their substrate specificity, structure, and the pathways in which they participate. There are more than 17,000 available kinase sequences that are classified into 30 distinct families based on sequence similarity, and fall into seven general structural folds for which three-dimensional structures are known[57]. It is very interesting that such a diversity in sequence and difference in structural folds accomplish almost the same catalytic mechanism of phosphate group transfer from a nucleotide triphosphate to a substrate[25]. However, there are many kinases which differ in chemistry of the phosphoryl acceptor group[5]. The reactions with alcohols have favorable equilibrium constants and thus permit phosphorylation of sugars and other small molecules so that they can enter into the further metabolic pathways. The phosphorylation of serine, threonine, and tyrosine hydroxyls on proteins which plays important regulation role is catalysed by protein kinases[25,58] and is energetically favo-

rable reaction, too. In contrast to the phosphorylation of hydroxyls groups, the reactions of ATP with an amino or guanidinium nitrogen or even with carboxyl acid are unfavorable, and the corresponding kinases catalyse these reactions in the reverse direction (e.g. creatine kinase or pyruvate kinase).

Most kinases catalyse direct transfer of the PO_3^- unit to the acceptor (Fig. 5) and their kinetics follows sequential mechanism where phosphoryl transfer reaction contains the following three principal components[59]: binding and orienting the phosphate donor (ATP); binding and orienting the phosphate acceptor (substrate); and catalysis of the chemical reaction. The transfer of the γ-PO_3^- unit of ATP to a substrate with inversion of stereochemical configuration is a convincing evidence that the acceptor group of the substrate directly displaces the phosphate in a single step[5]. Of course, more complex mechanism involving multiple covalent intermediates are possible theoretically for interpreting the stereochemical inversion, but the active sites of kinases do not appear to present the appropriate nucleophiles for such mechanisms. However, there are rare kinases like nucleoside diphosphate kinase that utilize ping-pong kinetics mechanism in which a nucleoside triphosphate donor phosphorylates a histidine, and the phospho-histidine form of the enzyme then phosphorylates a nucleoside diphosphate acceptor[5,57].

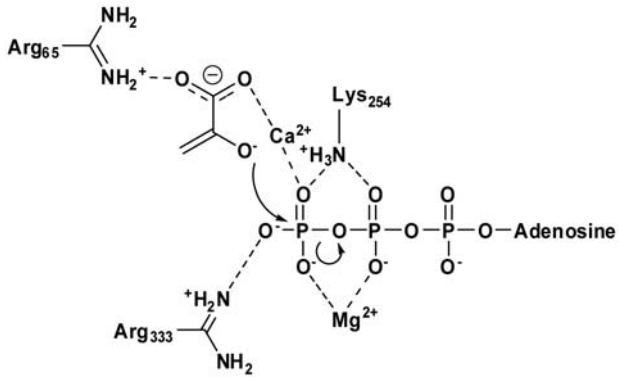


Figure 5. Model of the interactions in the reaction catalysed by a kinase.

Almost all kinases require a divalent metal cation in order to function[2,5,59]. Mg^{2+} usually activates ATP for catalysis by weakening the bond between the β and γ-phosphate group for phosphoryl transfer (see Fig. 5). Metal ions are usually cooordinated by a conserved glutamate, aspartate, or other hydroxyl-containing residues in the active site[25,59]. In some cases, however, the magnesium cations are positioned by coordinated water molecules and have no direct liganding to the enzyme. Several kinases utilize additional metal cofactors such as a secondary magnesium cation, a manganese cation, calcium ions (like shown in Fig. 5), or a potassium cation.

There has been considerable debate within literature as to whether kinase-catalysed phosphoryl transfer reactions operate primarily with an associative (S_N2-like) or dissociative (S_N1-like) TS. Various biochemical approaches have been used to tackle this question with enzymes in solution, yielding different results depending on the particular protein. Positional isotope exchange experiments on pyruvate kinase indicate that an associative TS is operational[60], while kinetic studies on nucleoside diphosphate kinase[61] and secondary ^{18}O isotope experiments with hexokinase[62] in both cases suggest a dissociative TS. Therefore the question about the catalysis evolved and used by kinases active sites remains still unsolved.

3. 3. Phosphomutases

In the reactions catalysed by the phosphomutases, the phosphoryl group is transferred to a different site in the same substrate, although the reaction is usually not truly intramolecular[2,5]. There are two mechanistic classes of phosphomutases[2]. The first class, exemplified by phosphoglucomutases (PGMs), shows a requirement for the bisphospho-substrate as a cofactor, and the enzymes catalyse the intermolecular transfer of phosphoryl groups amongst the two monophosphorylated substrates and bisphosphorylated cofactor (Fig. 6). The cofactor is evidently required to maintain the enzyme in its active phosphorylated form, this phosphoryl group being transferred to either of the two monophosphorylated substrates or, much slowly, to water. The α- and β-PGMs catalyse the interconversion of α/β-D-glucose-1-phosphate to D-glucose-6-phosphate where α-PGM acts on α-C(1) anomer, and β-PGM on the β-C(1) anomer. The extensive studies on rabbit muscle α-PGM have been done by Ray and co-workers[63–67] but β-PGM from *Lactococcus lactis (L. lactis)* has excited a great curiosity recently[68–75]. These enzymes are members of different protein families but both employ Mg^{2+} and α- or β-D-glucose-1,6-bisphosphate as cofactors and form a phosphoenzyme intermediate, by the reaction of an active-site nucleophile, which is *Ser* in α-PGM and *Asp* in β-PGM. They differ significantly in the stability of phosphoenzyme intermediate ($t_{1/2}$ ~ 240 days for rabbit muscle α-PGM[76] and $t_{1/2}$ ~ 30 s for β-PGM[70,73]) and conformational cycling and binding of their substrates during the catalysis. As α-D-glucose-1,6-bisphosphate molecule can reorient itself within the active site of α-PGM[77], the dissociation and rebinding of β-glucose bisphosphate analog is absolutely required for β-PGM[70,73,74]. In contrast to the phosphoglucomutases, the phosphoglycerate mutase from wheat germ catalyses a cofactor-independent intramolecular phosphoryl group transfer. No phosphoenzyme has been detected directly but it has been presumed from sterochemical studies that a phosphoenzyme must be an intermediate as overall retention at phosphorus has been observed[2]. However, it seems that phosphorylated enzyme is of relatively high energy

and that glycerate binds extremely tightly to it. In summary, for the cofactor-dependent enzymes like PGMs the phosphorylated substrate (glucose-1,6-bisphosphate) is occasionally lost from the active site of dephosphorylated enzyme, whereas for cofactor-independent enzyme, the corresponding intermediate (e.g. glycerate) is never released from the active site of phosphorylated enzyme.

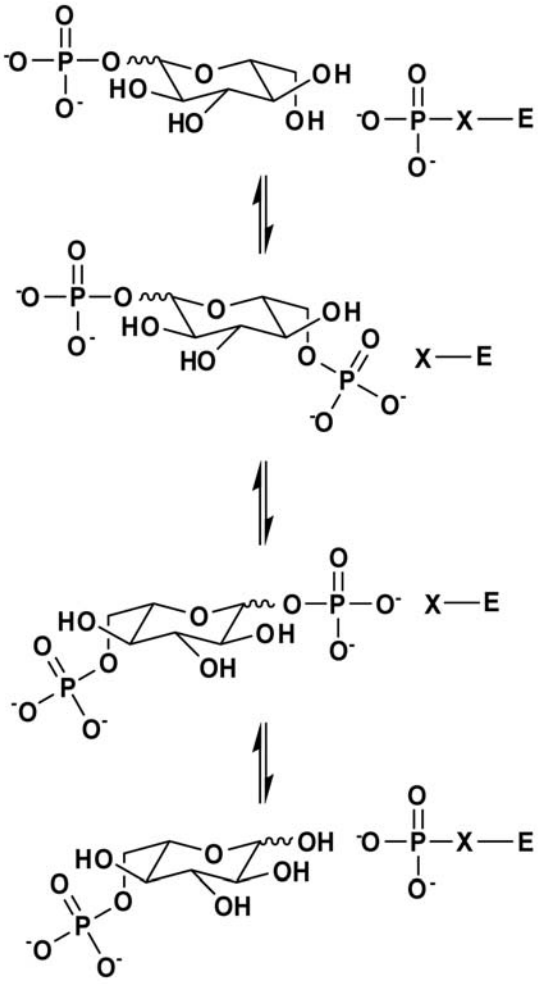


Figure 6. The reaction scheme of phosphoglucomutase that catalyse the intermolecular transfer of phosphoryl groups amongst the two monophosphorylated substrates and biphosphorylated cofactor.

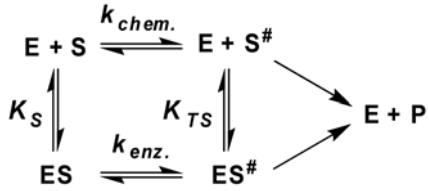
Phosphoglucomutases have become a model enzymes for studying phosphoryl transfer reactions catalysed in the defined matrix of their active sites, recently. β-PGM from *L. lactis* was identified as the first enzyme with determined crystal structure of the active phosphorylated form of any phosphoglucomutase in the presence of its natural substrates trapped in a high energy state mimicking TS[72] and therefore it could illuminate the enzyme-catalysed transfer of phosphoryl group. The claim of Lahiri *et al.*[72] to have identified the pentacovalent phosphorus INT of a phosphoryl transfer reaction addresses an important, long disputed problem: whether the enzyme-catalysed transfer of phosphate is dissociative or associative. The apparent observation of pentaoxy-phosphorane has suggested that the transfer occurs through the associative, addition-elimination process and contrasts with the solution mechanism. However, another interpretation of the high-resolution crystallographic data of Lahiri *et al.* has been suggested[68,78,79] which assumes that this β-PGM structure is likely to contain a TSA, rather to represent the high energy phosphorane INT. This change in interpretation is crucial, because the structural analog can be interpreted with equal validity as a mimic of the TS for a concerted reaction, as a phosphorane INT, or as a metaphosphate species in a dissociative process. The difference between these systems is simply a few tenths of an angstrom in the apical bond distances[56] and cannot be resolved solely on the basis of a model structure. Therefore, skeptisism exists among the scientific community which believe that the catalytic mechanisms of phosphoglucomutases as well as other phosphoryl transfer enzymes remain unidentified.

4. Enzymatic Transition States and the Transition State Analogues

Catalysis is defined as preferential stabilization of a reaction's TS, relative to its ground state[80,81]. Thus, detailed knowledge of the TS character for both non-enzymatic and enzymatic reactions is essential to decipher enzymatic catalysis. For reactions of small molecules in solution, not much can be learned about the nature of reaction INTs by looking at ensembles of reactants battered by random solvent collisions and in a multitude of energy states and extents of progress to product. Enzyme-catalysed reactions, in contrast, occur in the defined and precise environment. The ensemble of electrostatic, hydrogen bonding and steric interactions that occur between enzyme and substrate along a reaction pathway increase the probability that TS will be formed[82]. These interactions are defined by the structure of the reactant and by the catalytic scaffold of the enzyme where some catalytic residues are conserved in all members of the enzyme family because they are used for catalysis of the core chemistry. The unique ability of enzymes to synchronize transiently multiple interactions at specific site with reactants, intermediates, and products is the source of the enormous rate acceleration accomplished by enzymes (up to 10^{21}-fold)[8]. However, the life-time of TSs within the time span of a bond vibration (10^{-13} s) contrasts sharply with typical enzyme catalytic rates of 1 to 10^3 s^{-1}. This contradiction comes from the limits of diffusional encounter between enzyme and substrate in solution and the relatively long times needed to rearrange protein structure and position the substrate for productive chemistry[82].

An enzyme proficiency as a catalysts can be appreciated by dividing the second-order rate constant for its action

on the substrate ($k_{enz.}/K_S$) by the rate constant of the same reaction, measured in neutral aqueous solution in the absence of enzyme ($k_{chem.}$)[9] (Scheme 1). Catalytic proficiency, defined in this way, measures an enzyme's ability to lower the activation barrier for the reaction of a substrate in solution. Catalytic proficiency, expressed in units of concentration, represents the lower limit of the enzyme's affinity for the altered substrate in the TS (K_{TS}, see Scheme 1) but the fleeting lifetime of an enzymatic TS prevents a true thermodynamic equilibrium from forming a tightly bound TS complex. For phosphatases, the most proficient enzymes identified thus far, the maximal formal dissociation constants of the altered substrate in the TS are approximately 10^{-26} M[8], and these and other phospho-transfer enzymes that are extremely proficient by the above criterion are expected to be unusually sensitive to a special class of mechanism based reversible inhibitors often called TSAs, designed to resemble the altered substrate in the TS. The transition metal oxoanion vanadate (VO_4^{3-}) is a close structural and chemical mimic of phosphoryl group as oxygen atoms tend to adopt a pentacoordinate, trigonal bipyramidal geometry around the central vanadium (V) atom[83]. This mimicry has been observed many times in x-ray crystal structures of enzymes complexed with vanadate[16]. As would be expected for such a TS mimic, vanadate is a potent inhibitor of many phosphoryl transfer enzymes[84]. However, the lack of a correlation between inhibition constants and catalytic parameters for wild type and mutated enzymes suggests that vanadate is not a true TSA[85–87].



Scheme 1

None of the TSAs, however, approach the expected binding of the TS (K_{TS}) based on Scheme 1. Dissociation constants in the picomolar – 10^{-12} M – range are not uncommon[88,89] and analogues in the femtomolar – 10^{-15} M – range have been described[90] (some inhibitors, made by 'click chemistry', have also reached the femtomolar range but are not necessarily related to the TSs of the enzymes[91] an have consequently little relevance to catalysis). That this is the case is not surprising. It is impossible to get a perfect analogue, since bonds are being made and broken in the true TS and TSA capture the simultaneous enzyme molecular excursions that generate the dynamic TS. In the presence of a TSA, the protein structure collapses around the analogue into a static, thermodynamically equilibrated potential energy well and we are faced with the problem of establishing how well the complex of enzyme and TSA resembles the geometry of the non-equilibrium TS that characterize the activation energy saddle point.

It is often postulated that the enzymes do not energetically stabilize TSs undergone in solution only but in contrast can change the nature of the TSs e.g. a phosphate monoester in enzyme active site would be rendered diester-like by metal-coordination and shows an associative instead of dissociative mechanism[53–55,60,72]. If one assumes that compared to the ground state, the peripheral oxygen atoms bear more charge in an associative TS and less charge in a dissociative TS, it is really expected that coordination of an ubiquitous divalent metal cation to oxygens of the transferred phospho-group would inhibit dissociative pathway but accelerate an associative process. However, the experimental data obtained by different phosphate transfer enzymes puzzle with ambiguous interpretations[5] and one cannot generally answer the question: do the reaction mechanism remains the same within the enzyme walls as in solution or a different low-energy path has been found by several enzyme evolutionary driving forces? The studies that endeavour to solve this elusive puzzle continue to be the focal point of bioorganic chemistry but an actual image of each chemical species formed during the reaction pathway in the enzyme active site should be ultimate goal which would be able to unambiguously prove the correct mechanism of phosphate group substitution catalysed by an enzyme. X-ray crystallography is the conduit to such images and it has been reported in many papers recently that x-ray study may have trapped high energy INTs (phosphorane or metaphosphate) of phosphoryl transfer in an enzyme active site[72,92–95]. However, it is striking that the structural differences between the phosphorane INT and a slightly dissociative TS are only about 0.5 Å between the entering and leaving groups within a similar trigonal bipyramidal geometry. Thus, these images of enzyme-high energy INT complexes are unlikely to be able to provide the resolution necessary to make mechanistic distinction of this subtlety. There are also doubts that a trapped INT[72] is truly the objects itself at all. In other words, a metallic fluoride TSA can be assembled in the enzyme active site (by chemicals usually needed in crystallographic procedures) where it can delude as an illusive trapped INT[68,70,78,79]. Anyhow, it has been known for many years that several metallic (Al, Be, Mg) fluoride complexes have been able to mimic a phosphate in enzymology, indeed[17,18], and the concept of a metallic fluoride complex as phosphoryl transfer TSA (aluminum or magnesium fluoride) or phosphate ground state analogue (beryllofluoride) has become widely accepted[68–70,96–106].

5. Basic Chemistry of Al, Be, Mg and their Fluoride Complexes

Fluorine (F) is a group 7B halogen next to oxygen in the periodic table (Fig. 7). Both atoms have the same si-

ze and the same valence orbitals. Fluorine exists as a diatomic molecule with remarkably low dissociation energy. As a result it is the most chemically reactive non-metal and also the most electro-negative element. Consequently it has strong affinity to combine with other elements to produce chemical compounds known as fluoride. Although there are several sources of fluoride in nature the main one for life organisms is surface water. The latter is contaminated with fluoride because the original source of fluoride is fluoride rich rocks containing fluorite (CaF_2), cryolite (Na_3AlF_6) or fluorapatite ($Ca_5(PO_4)_3F$) and when water percolates through these rocks it leaches out the fluoride ions (F^-).

electrons are in the same shell. Aluminum is the most abundant metal on earth but somewhat 'hidden' element. It is concealed in minerals such as bauxite ($Al_2O_3 \times 2H_2O$) and cryolite (Na_3AlF_6) but with the advent of acid rain, Al^{3+} escapes from minerals, dissolve in fresh water, and thus become available to living organisms.

Al^{3+} is the most accessible oxidation state for aluminum, and in aqueous solution, Al^{3+} forms different species with water components at different pH[113–115]. The Al^{3+} level is usually very low in natural water, due to the fact that free Al^{3+} instantly precipitates as insoluble $Al(OH)_3$ hydroxide. In strong acid (pH < 3) Al^{3+} exists mainly as the octahedral $[Al(H_2O)_6]^{3+}$ ion, whereas in basic conditions (pH >

IIA	IIIB				VIIB
4 +2	5	6	7	**8 –2**	**9 –1**
Be	B	C	N	**O**	**F**
$1s^2 2s^2$				$1s^2 2s^2 2p^4$	$1s^2 2s^2 2p^5$
12 +2	**13 +3**	14	**15 +3,+5,-3**	16	17
Mg	**Al**	Si	**P**	S	Cl
$1s^2 2s^2 2p^6 3s^2$	$1s^2 2s^2 2p^6 3s^2 3p^1$		$1s^2 2s^2 2p^6 3s^2 3p^3$		

Figure 7. A section of the Periodic Table. For the elements related to phosphate or its analogues their symbols and corresponding data are highlighted. Up-left of each symbol are atomic numbers, up-right oxidation states and electron configurations are given at the bottom of each symbol.

Fluoride has been found to inhibit the activity of many important enzymes but in contrast some enzymes, such as adenylyl cyclase, lactate dehydrogenase, glycogen phosphorylase, and many others regulatory proteins such as G-proteins and response rugulators, are stimulated by fluoride in millimolar concentrations[107]. The main reason of fluoride biological effects may be explained empirically by the chemical characteristics of fluorine which has an even greater capacity than oxygen for forming hydrogen bonds and can bind to a protein molecule through hydrogen bonds between F atoms and nearby hydrogen donor groups on amino acid side-chain. Consequently, the native regular architecture of hydrogen bonds in the protein active sites may be destroyed by fluoride itself but it should be noted that the ability of fluoride to participate in hydrogen bonds is an extensively studied complex issue[108,109]. However, another concern on fluoride biochemistry is that fluoride shows synergistic effects with some metallic cations such as Al^{3+}, Be^{2+} and Mg^{2+}[110–112]. Each of these ions form small inorganic complexes with fluoride showing their own structure and chemistry but with common denominator that these complexes have been recognized to act as phosphate analogues and may be useful 'chemical trick' probes in phospho-transfer enzymes investigations[17,18,96–106].

Aluminum (Al) is a group 3B metal and is close to phosphorus in the periodic table (Fig. 7), their valence

7), a tetrahedral $[Al(OH)_4]^-$ structure prevails[115]. In the biochemically critical pH range of 4.3 to 7.0, the ion structures are less clear but recently a kinetic evidence supports a five-coordinate $[Al(H_2O)_4OH]^{2+}$ ion as a predominant form of aluminum hydroxide under ambient conditions[113,114]. This results make Al^{3+} an extra ion in contrasts with other trivalent metal aqua ions, for which there is no evidence for stable pentacoordinate hydrolysis products.

As a hard metal ion Al^{3+} interacts most strongly with hard donors, and therefore its complexes with fluoride ligands are very stable. The fluoride complexes of Al^{3+} have been intensively studied[115–118]. An Al–F bond is the same length as a P–O bond in phosphate (1.5–1.6 Å). Like phosphorus, aluminum has a possible coordination numbers of 1–6, due to the possible hybridization of its outer shell 3p electrons with the 3d orbitals[118]. Despite the existence of higher order fluoroaluminate species in the solid state, there is little evidence for the existence of Al–F complexes with fluoride coordination number equal or higher to 5 in aqueous solution[117,118]. However, the most interesting Al–F compounds in enzymology are AlF_3 and/or AlF_4^- and it has been shown that at ambient temperature and physiological pH aluminum ion is always octahedrally coordinated to *i* fluoride ligands and *6-i* water molecules but tetrahedral AlF_4^- ion can be solubilized in organic solvents and crystallized as organocation salts or isolated as anhydrous crystals only[115,119]. The latter is very interesting because a variety of measurements indicate

that $[Al(OH)_4]^-$ in aqueous solution is tetrahedral[120]. Given that OH^- and F^- are isoelectronic and isochoric and show many parallelisms in their chemistry, in both the solution and solid states, it is somewhat surprising that their interactions with Al^{3+} should be so dissimilar in geometry of complex formations as well as in kinetics of ligand exchange. Anyhow, it is believed that aluminofluorides can adopt several different geometries in a protein active site: tetrahedral, trigonal bipyramidal and octahedral[102,104].

Beryllium (Be) is a group 2A lightweight metal (Fig. 7) and is a relatively rare element. Because of its small size, the beryllium atom has a high ionization energy and thus does not tend to form the Be^{2+} ions. Therefore it forms essentially partial covalent bonds with other elements. Beryllium is mined from naturally occuring poorly soluble silicates including beryl ($Be_3Al_2(SiO_3)_6$) and bertrandite ($Be_4(OH)_2Si_2O_7$). Coal combustion is the chief reason for its presence in broader environment but fresh water contains less than 0.001ppm Be because at neutral pH condensation reactions ensue and highly insoluble precipitates of beryllium hydroxide $Be(OH)_2$ appear. The chemistry of beryllium differs considerably from that of the other members of its group in periodic table such as magnesium, but although beryllium is much lower mass than aluminum its chemistry resembles that of aluminum due to similar electro-positivity. The other striking characteristic of beryllium is its tendency to form compounds in which it has an incomplete octet of valence electrons, a tendency shared only by aluminum. Because the octet rule can be violated, a number of different compounds of beryllium with oxygen (or fluorine) ligands, like H_2O for example, can be made. Beryllium is also the most toxic non-radioactive metal because it can displace H^+ in many strong hydrogen bonds in which beryllium as a 'tetrahedral proton' is thermodynamically preferred[121].

The chemistry of beryllium in aqueous medium is very interesting. It is well known that the beryllium cation in water solution exists as tetraaqua $[Be(OH_2)_4]^{2+}$ cation under strong acidic conditions only. When the pH in solution increases, the hydrolysis reaction takes place and various beryllium positively charged hydroxocomplexes are formed ($[BeOH]^+$, $[Be_2OH]^{3+}$, $[Be_3(OH)_3]^{3+}$, $[Be_5(OH)_6]^{4+}$, $[Be_6(OH)_8]^{4+}$)[122,123]. With increasing pH condensation reactions ensue and finally precipitates of beryllium polymeric hydroxide appear (pH 6–9), which redissolve as the pH is increased further. In alkaline hydroxyberyllate solutions the identification of negatively charged $[Be_4(OH)_{10}]^{2-}$ has been reported[124].

The close relation between hydroxide and fluoride anions suggested complementary NMR studies of aqueous beryllium fluoride solutions[125]. It has been identified that water molecules compete successfully with fluoride anions for a place in the coordination sphere of the Be^{2+} cation. Be–F bonds (1.55Å) have the same lenghts as Al–F and P–O bonds. In contrast to that of phosphorus or aluminum, however, beryllium bonding is strictly tetrahedral[126,127]. The external 2s electron orbitals hybridize with the 2p orbitals to give tetrahedral sp^3 bonding, like that of carbon and, as for carbon, the 3d orbitals are much too distant to hybridize significantly and allow no other bonding geometry. Beryllofluorides are tetrahedrally coordinated with *i* fluoride anions and *4-i* water molecules but biologically most relevant species is $[BeF_3(H_2O)]^-$ which is able to mimic phosphate in ground state[17]. There has been no evidence of the existence of condensed di- or polynuclear fluoroberyllates in any of the experiments. It should be pointed out that the well resolved ^9Be–^{19}F NMR spectra clearly demonstrate that fluoride exchange between different beryllium centers is very slow on the NMR time scale in comparison with aluminum (in the range of seconds)[125].

Magnesium (Mg) is a group 2B metal next to aluminum in the periodic table (Fig. 7). The most important minerals of magnesium are dolomite ($CaMg(CO_3)_2$) and magnesite ($MgCO_3$) that are relatively widespread in nature. Magnesium generally binds oxygen atoms in ligands rather than nitrogen or sulfur but may form alkyl- or arylmagnesium-halides (Grignard reagents), too. The latter are very important organometallic chemical tools that act as which attack carbon atoms that are present within polar bonds (e.g., a group) to yield a carbon-carbon bond.

Because of magnesium electron configuration similarity with aluminum it also has a strong tendency to bind six ligands in a regular octahedral arrangement[122]. Among possible ions it readily binds water; the hexa-aquated magnesium ion, $[Mg(H_2O)_6]^{2+}$ is common in crystal structures, even when there is a strong anion available as a counterion which might have been thought to be more suitable as ligand (F^- for example). The affinity of Mg^{2+} for water may be partly connected with the size of the cation in that the six oxygen atoms in the inner coordination sphere are in contact with each other, as well as, with the magnesium ion. Some other metal ions of similar size do not show this affinity for water. Thus, the normal liganded state of magnesium is rigidly octahedral with six oxygen atoms (often from water molecules) sorrounding the cation. Therefore it is not surprising that MgF^+ is the only one fluoride complex found in aqueous solution[68] but several magnesium fluoride complexes with different geometries can be found in active sites of proteins: trigonal bipyramidaly (MgF_3^-)[68] or octahedraly (MgF_4^{2-}) coordinated[106].

6. AlF_x, BeF_x and MgF_x Complexes as Phosphate Analogues in Enzymology

6. 1. Metallic Fluoride Complexes (MF_x) Analogy with Phosphate

Fluoride anions have long been known to stimulate the activity of adenylyl cyclase[128] and GTP binding pro-

teins (G-proteins).[129] It was later demonstrated that the effects primarily attributed to fluoride are caused by synergistic action with alluminium.[130] This fact had at first been ignored because aluminum is a normal (but also 'hidden') component of glass from which it is etched in traces by a solution with fluoride. The requirement for aluminum is highly specific; of 28 other metal tested, only beryllium promoted activation of the guanine nucleotide binding regulatory component of adenylate cyclase by fluoride.

Aluminofluorides and beryllofluorides – usually written AlF_x and BeF_x to indicate that a mixture of species can be formed and the exact one responsible for any given effect may not be known – as small inorganic molecules mimic the chemical structure of a phosphate[17] but an important functional difference exists between phosphate group and analogous fluoro-metallic complexes. While in phosphate the oxygens are covalently bound to the phosphorus and do not exchange with oxygen from solvent hydroxyl, in the fluoro-metallic complexes ionic bonds are formed between electropositive metal and the highly electronegative fluorine. In aqueous solution the fluoride anions bound to the metal cation are exchangeable with free fluoride, hydroxyl ions or water molecules.[117,118,122] The complexes are not permanent and equilibria exist between the various possible AlF_x and/or BeF_x complexes.[112]

As unique chemical tools in biological studies, AlF_x and BeF_x affect the activity of phosphoryl transfer enzymes and have been succesfully used in the study of their structures and catalytic mechanisms.[96–106] A variety of such enzymes and their complexes with AlF_x and BeF_x have been crystallized.[131] The ions AlF_4^-, BeF_3^- and AlF_3 molecule have a propensity to occupy phosphate-binding sites because they approximate the shape and charge of phosphoryl group in flight (AlF_x) or in ground state (BeF_x), too. However, aluminum and beryllium fluoride complexes are neither isosteric with trigonal planar PO_3^- moiety of a phosphoryl transfer TS: AlF_4^- is a square planar ion but BeF_3^- is generally non-planar, nor isoelectronic with negatively charged PO_3^- species: trigonal planar AlF_3 is indeed chargeless. It has been suggested that the pH may influence whether an AlF_4^- or AlF_3 species forms in the enzyme active site[102], although the reliability of the assignment of the AlF_3 species concerned has been questioned and some reports strongly suggest that structures assigned as AlF_3 are likely to be MgF_3^-,[78,101,132] as magnesium is often present under experimental conditions[18,68–70,72,78] and anionic charge is prioritized over geometry in both AlF_x and MgF_x TSAs of phosphoryl transfer enzymes.[69] MgF_3^- presents both the correct geometry and the charge to mimic PO_3^- group in flight but it does not, unlike aluminum and beryllium fluoride complexes, exists in solution because of its low formation constant[133] and can be assembled and stabilized in suitable active site only.[18,68,69] Therefore it is particularly interesting to characterize as a potentially highly accurate TSA.

6. 2. BeF_x Complexes as Phosphate Analogues

The pentacoordinated bipyramidal geometry is excluded for BeF_x and consequently beryllofluoride complexes can mimic phosphate in ground state only. Therefore, beryllofluoride complexes, of which physiologically most active is $[BeF_3(H_2O)]^-$ (BeF_3^- shortly), are used in mechanistic studies of many purine nucleotide binding proteins, such as G-proteins, F_1-ATPase and myosin,[96,105,134,135] or in investigations of phosphorylation of aspartate residues in two large protein families: the haloacid dehalogenase superfamily of hydrolases and response regulators.[103,136,137] In case of nucleotide binding proteins BeF_3^- acts as γ-phosphate analogue (Fig. 8A), i.e., high affinity binding of the beryllofluoride analogue requires the presence of a nucleoside diphosphate in the catalytic nucleotide site of the enzyme but it has been shown that an analogue can be formed in solution, too.[138] The lability of the acyl phosphate linkages in aspartate-phosphorylated proteins (half lives in seconds to hours)[68,70] has hindered structural studies of their phosphorylated (active) form but BeF_x complexes are persistent phosphate analogues that can be used to facilitate determination of the structure of these protein active forms (Fig. 8B). It is expected that BeF_3^- binding induces a conformational change very similar to that caused by phosphorylation.[103,136,137] However, it is always dangerous to try to deduce energetics of catalysis from mimicking structure, so direct measurements of changes in affinity need always to be done to ve-

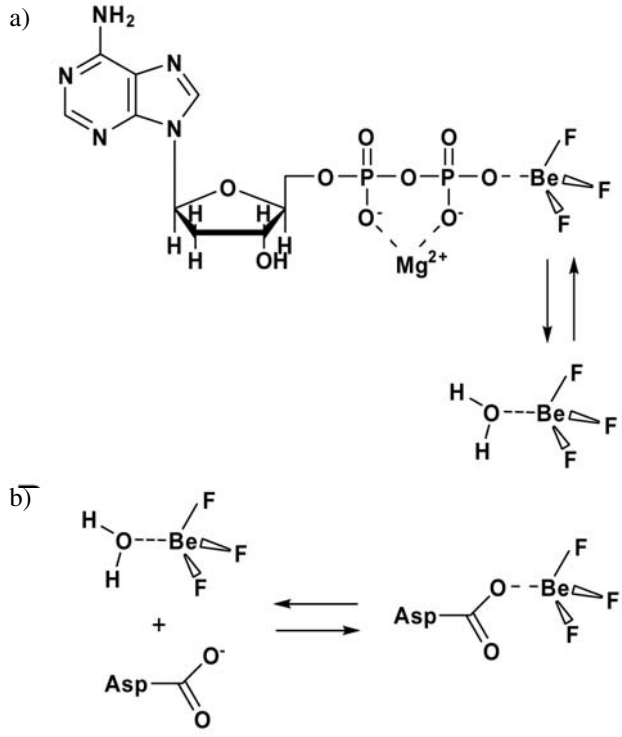


Figure 8. Reaction of BeF_3^- with a) β-phosphate group of a nucleotide diphosphate and b) an acyl group site in protein active site.

rify that the changes seen with BeF_3^- do indeed mimic those of phosphorylation, as expected.

6. 3. AlF_x Complexes as Phosphate Analogues

A breakthrough on the mechanism of AlF_x on G-proteins was achieved by Bigay.[96] Their work on transducin focused on the point that, to allow activation by AlF_x, a GDP is required to bind to the nucleotide site of a G-protein. Analogues of GDP were accessible only if the terminal oxygen on the β-phosphate remains unsubstituted. This findings led them to conclude that AlF_x interacts directly with the oxygen on the β-phosphate of GDP. This is the position where a γ-phosphate would bind if it were a GTP. Isolated Gα-protein normally keeps a GDP molecule permanently bound when AlF_x interacts with Gα-protein through binding to the β-phosphate of GDP. The bound AlF_x (or BeF_x, too) simulates the presence of the bound γ-phosphate of GTP and therefore confers on the protein the structure of the active Gα · GTP state. The most revealing structure concerning GTPase catalysis was provided by the crystal structure of Giα GDP AlF_4^-[97]. This structure (Fig. 9A) shows that AlF_4^- is located at the γ-phosphate binding site and linked to the β-phosphate. However, the central Al atom exhibits an octahedral, hexacoordinated geometry, rather than expected tetrahedral orientation. Four fluorine atoms bind around aluminum in the equatorial plane (90° between each other) but GDP β-phosphate oxygen and a hydrolytic H_2O molecule occupy the axial positions (perpendicular to the fluorine plane). The close resemblance of this structure and charge with the described bipyramidal TS of phosphate indicates that AlF_4^- can be a TSA for phosphoryl transfer reaction catalysed by trimeric G-proteins or several other proteins[102].

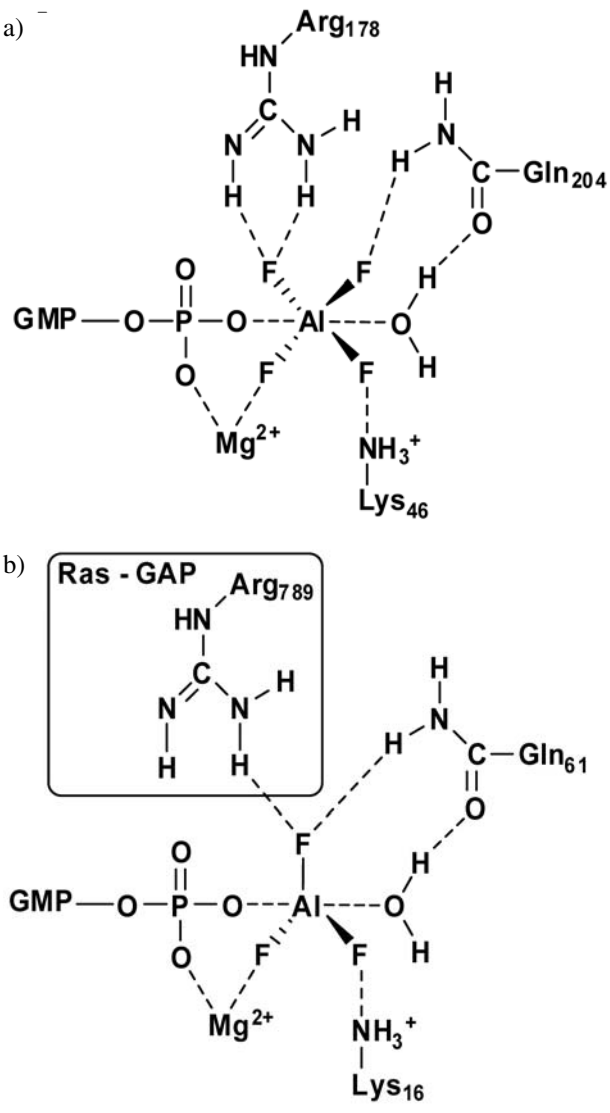


Figure 9. Model of the interactions of AlF_x TSA in active site based on the crystal structure of A) Giα · GDP · AlF_4^- (octahedral coordination), and b) Ras · GDP · AlF_3 · GAP (trigonal bipyramidal coordination).

Small GTP-binding proteins are a superfamily of monomeric G-proteins that are Ras-like. These proteins have low intrinsic GTPase activity. Thus, Ras proteins require their GTPase activating proteins (GAP) to accelerate the GTP hydrolysis process. Aluminofluorides affect the activity of the latter Ras GAP complex but rather than attaining an octahedral geometry in the case of trimeric G-proteins, aluminum fluoride (AlF_3) assumes a trigonal bipyramidal structure in Ras GAP (Fig. 9B).[139] This conformation found in other phosphoryl transfer enzymes[101,140] more closely resemles the true TS of γ-phosphate. The reason for two different conformations (octahedral AlF_4^-, trigonal bipyramidal AlF_3) was explained to be caused by experimental conditions. By changing the pH value in UMP/CMP-kinase crystallization buffer, Schlichting and Reinstein discovered that the bonding configuration of AlF_x can switch from AlF_4^- in acid pH to AlF_3 in alkaline pH.[102] Factors other than pH, such as F concentration, may also have effects on AlF_x configuration.

6. 4. MgF_x Complexes as Phosphate Analogues

Heterotrimeric G-proteins can also be activated by fluoride in the absence of aluminum or beryllium. Higashijima et al.[141] showed that high concentrations of magnesium could replace the requirement for aluminum. This observation was extended by Antonny et al.[142,143] who kinetically concluded that this was due to MgF_3^- bound to Gα GDP but MgF_x complexes as phosphate analogues were somehow lost in obscurity and unnoticed for many years. The main reason could be the fact that magnesium (in contrast to aluminum or beryllium) does not form fluoride complexes with two or more F^- anions in aqueous solutions[68]. It was unbelievable that a MgF_x complex unformed in solution could be assembled and found in enzyme

active sites only. The formation of MgF_3^- inside the protein active site seems entropically very unfavourable process and trigonal planar coordination of MgF_3^- is very unusual, too. However, several kinetic studies insinuated that aluminum independent fluoride binding was attributed to the formation of a complex containing magnesium fluoride.[142–145] The first structural evidence that MgF_3^- existed as TSA was determined in 2002[18] and it was confirmed that investigated crystals of small G-proteins truely contained MgF_3^- and not AlF_3 by proton-induced X-ray emission (PIXE) spectroscopy. The same magnesium fluoride TSA was determined by NMR (Fig. 10)[68,69] and kinetic studies of β-phosphoglucomutase, too.[70] Furthermore, it is possible that some x-ray structures previously interpreted as containing AlF_3 might in fact contain MgF_3^-[102] because high millimolar concentrations of magnesium is usually present in structural study cases. It is known that MgF_4^{2-} complex can be formed in protein active sites[106,146] but of all metallic fluorides MgF_3^- complex would be expected to mimic the TS of phosphoryl (PO_3^-) group most closely in charge and in geometry. Therefore MgF_3^- may be the best reagent of the choice for studying phosphoryl transfer reactions because it might be formed at places of the highest TS affinity of the most proficient enzymes only.

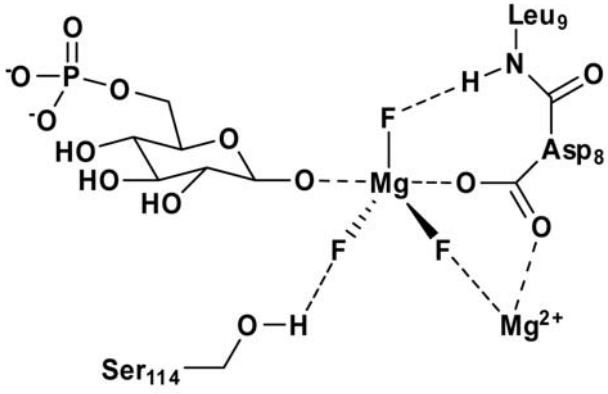


Figure 10. Model of the interactions of MgF_3^- TSA in active site based on the structure β-phosphoglucomutase.

7. Conclusion

Metallic fluorides are noteworthy and widely accepted as phosphate analogues among the structural biologists. They enable the insight into many feasible mechanistic steps of phosphoryl transfer enzymes or possible conformational structures of otherwise unstable phosphorylated regulatory proteins. Although much is now known about different enzyme structures containing metallic fluorides mimicking phosphate ground state or phosphoryl transition state, more needs to be learned about their reliability to reflect real image of enzyme catalysis. Therefore, enzyme kineticists might be more deeply involved into the future studies of metallic fluorides as structural/mechanistic phosphate analogues. The main goal of enzyme kinetics should be to examine the correlation between the metallic fluorides inhibition constants and enzymes catalytic parameters. However, it is still believed that the crystal structure of an enzyme cannot reveal the whole of its catalytic mechanism or with other words ''Making a model of a horse from photographs does not necessarily tell us how fast it can run''[147].

8. Acknowledgement

This work was supported by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia (Grant P1-170).

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Povzetek

Encime, ki katalizirajo prenos fosforilne skupine, lahko zagotovo uvrstimo med najbolj učinkovite znane katalizatorje. Zaradi tega potekajo izredno intenzivne raziskave teh encimov na ravni preučevanja njihove strukture in reakcijskih mehanizmov, ki se odvijajo v njihovih aktivnih mestih. Znano je, da fluoridi aluminija (AlF_x), berilija (BeF_x) in magnezija (MgF_x) vplivajo na aktivnost mnogih encimov, ki katalizirajo prenos fosforilne skupine, saj se lahko na različne načine vežejo v njihova aktivna mesta in tam nadomestijo (posnemajo) fosfatno skupino. V encimatiki jih poznamo kot fosfatne analoge. Že majhne količine ionov Al^{3+} in Be^{2+} tvorijo v vodnih raztopinah stabilne anorganske komplekse z različnim številom fluoridnih ionov (x = 1 do 4), a nasprotno ioni Mg^{2+} nimajo te lastnosti. Geometrijska razporeditev ligandov je pri spojinah BeF_x vedno tetraedrična. Takšna je tudi pri fosfatni skupini v osnovnem energijskem stanju in zaradi tega so kompleksi BeF_x uporabne kemijske sonde za študij fosforilacije karboksilnih skupin v proteinih ali Michaelisovega kompleksa v encimih, ko BeF_x nadomesti γ-fosfatno skupino koencima nukleozid trifosfata. Kompleksi AlF_x in MgF_x so poznani kot analogi prehodnega stanja fosforilne skupine. Geometrijska razporeditev ligandov v aktivnem mestu encimov je pri obeh spojinah lahko trikotno-bipiramidalna (AlF_3, MgF_3^-) ali oktaedrična (AlF_4^-, MgF_4^{2-}). Pri tem MgF_3^- najbolje posnema tako geometrijo kot tudi naboj fosforilne PO_3^- skupine v prehodnem stanju. Vendar pa kompleks MgF_3^- sploh ni obstojen v vodni raztopini in se lahko tvori in stabilizira le v ustreznem proteinskem miljeju. Prav zaradi tega izgleda, da bi lahko bil kompleks MgF_3^- najbolj primeren analog prehodnega stanja fosforilne skupine in v prihodnje najboljša izbira pri raziskavah encimov, ki katalizirajo prenos te skupine.