Stereochemical, Mechanistic, and Structural Features of Enzyme-catalysed Phosphate Monoester Hydrolyses

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1 Introduction

The transfer of phosphoryl groups from one entity to another is of central importance to the cellular control of hundreds of metabolic pathways. These include the oxidation of glucose to provide energy for the cell, the activation of nucleosides to provide the building blocks for DNA and RNA, the biosynthesis of amino acids and aminoacyl t-RNA molecules to provide the building blocks for proteins, and the switching-on and turning-off of many enzymes involved in signal transduction and in controlling cellular metabolism. Several of these processes are still poorly understood at a molecular level and the next ten years promise to be particularly exciting as the intricacies of neurotransmission, intracellular signal transduction, and receptor site mediated response are delineated.

Kinase enzymes are responsible for transferring phosphoryl groups to phosphoryl group acceptor moieties within molecules. These sites are usually, but not always, O-atoms. The accepting O-atom can be part of a wide range of different functional groups including alcohols, enols, phenols, carboxylates, and phosphates, The structures and sizes of the acceptor molecules are diverse. Kinase enzymes exist to phosphorylate small molecules such as acetate, pyruvate, D-glucose, and adenosine as well as large molecules, for example, the serine, threonine, and tyrosine residues of a vast array of proteins. Usually adenosine 5′-triphosphate (ATP) serves as the phosphorylating agent in these reactions and the O-atom of the acceptor molecule (X = OH) directly displaces adenosine 5′-diphosphate (ADP), an excellent leaving group, from the γ-phosphorus atom of ATP (Scheme 1).

Phosphatase or phosphohydrolase enzymes are responsible for the removal (hydrolytic cleavage) of phosphoryl groups from an equally diverse range of molecules (Scheme 2). This short article reviews and compares what we know about the mechanism and structure of several different phosphatases and highlights and discusses how nature has arrived at different solutions to what is essentially the same chemical problem in the hydrolysis of different phosphate substrates. We shall concern ourselves only with monophosphate ester hydrolyses where the leaving group is an alcohol.

2 Phosphate Monoester Hydrolysis

2.1 Non-Enzymic Reactions

In essence, as for the carboxylic ester, there are two fundamental mechanisms by which a phosphate monoester can be hydrolysed. In the first, the C-1 carbon atom of the alcohol can serve as the electrophile so that the entire phosphate group is replaced by the O-atom of a water molecule or hydroxide. In the second, water or hydroxide attacks the phosphorus atom and displaces the alcohol or alkoxide with an intact C–O bond *via* phosphorus–oxygen bond fission. In principle these two mechanisms can be easily distinguished by performing hydrolyses in ^{18}O-water, since they give different isotopically labelled products; in practice, acid-catalysed water–phosphate O-atom scrambling can complicate analyses (Scheme 3).

The alkyl–O fission mechanism is only important at very low pH where the leaving group is neutral phosphoric acid. For alkyl esters possessing alkyl groups that form stable carbocations, for example benzyl and t-butyl phosphate and α,D-glucose 1-phosphate, the reactions procede *via* an S_N1 ionization process. Conversely at very low pH, methyl phosphate and primary and secondary alkyl phosphate esters are hydrolysed *via* two competing processes, S_N2 alkyl–oxygen bond cleavage and phosphorus–oxygen bond cleavage.[1] Above pH 1.5, where the leaving phosphate group possesses at least one negative charge, hydrolyses proceed *via* phosphorus–oxygen bond cleavage mechanisms. This mode of cleavage is followed by all enzyme catalysed phosphate monoester hydrolyses reported to date, and thus the phosphorus atom serves as the electrophile.

2.2 Enzyme Models and the Role of Metal ions

Many phosphatase enzymes employ metal ions as cofactors to lower the activation energy for P–O bond fission. The exact mechanism by which this is achieved depends on the specific enzyme. However, it is known from model studies that metal ions can contribute in three distinct ways. First, the association of a metal ion, for example Mg^{2+}, with water or an alcohol lowers its pK_a. This is achieved because the conjugate base,

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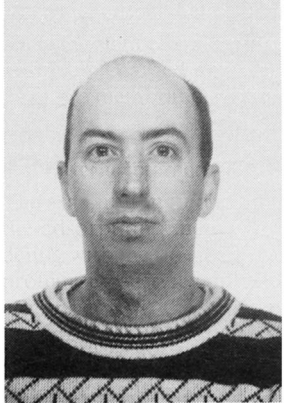
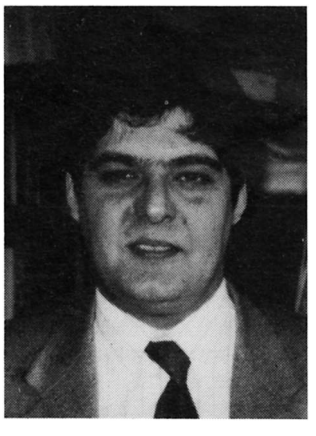
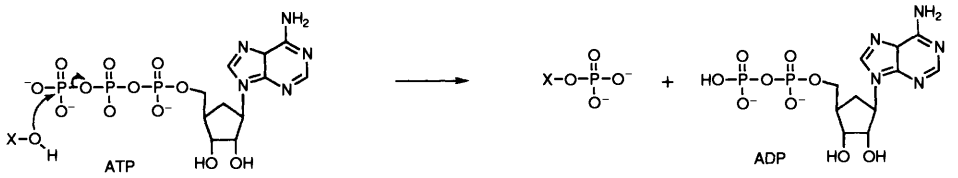
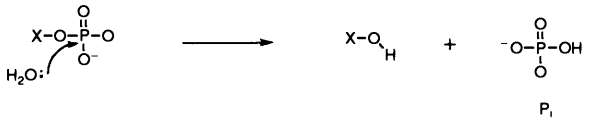


After working at Wellcome in Beckenham, David Gani obtained B.Sc. and D.Phil. degrees at the University of Sussex. In 1983 he moved to Southampton as a holder of a Royal Society University Fellowship and in 1990 took the Chair of Organic Chemistry at St. Andrews. He is currently pursuing several programmes at the interface of biology and chemistry. These include studies of the mechanism of the enzyme inositol monophosphatase, the design and synthesis of inhibitors for viral proteinases, the synthesis of cyclic peptide inhibitors of the protein phosphatases, and the mechanisms of methylaspartase, glutamate mutase, and threonine synthase.

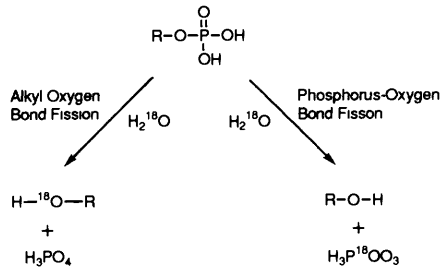




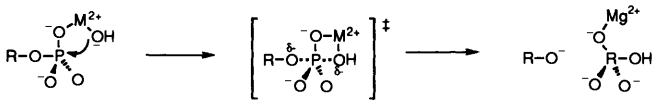
Scheme 1



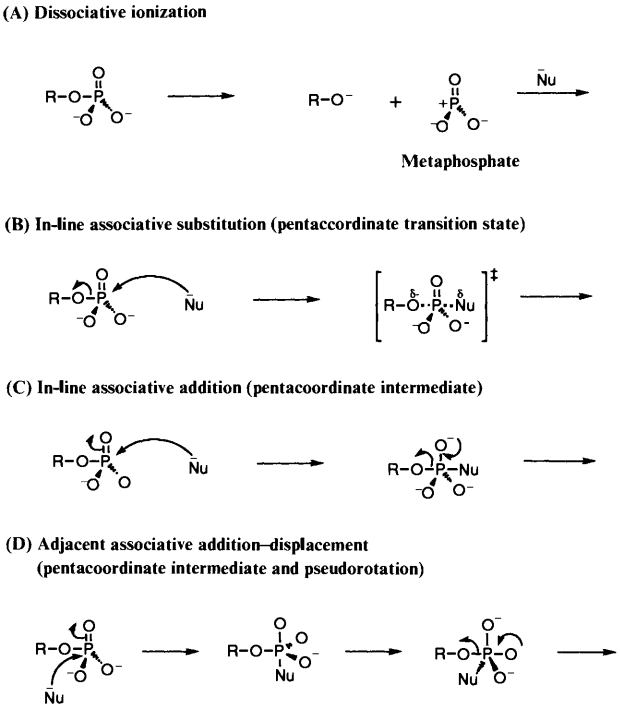
Scheme 2



Scheme 3



Scheme 4



Scheme 5

hydroxide or alkoxide, can dissipate its charge through a binding interaction with the metal ion. Since these conjugate bases are better nucleophiles than the parent acids, attack on the elecrophilic P-atom is enhanced relative to the uncatalysed reaction. Metal ions can also promote reaction by positioning the nucleophile correctly for attack on the P-atom. This can be achieved by forming four-membered cyclic transition states (metallophosphodioxetanes) as shown in Scheme 4. This arrangement provides a further advantage in enhancing the electrophilicity of the P-atom through Lewis acid coordination of the metal ion to one of the O-atoms that becomes equatorial in the trigonal bipyramidal transition state. Yet further advantage can be obtained by allowing another metal ion to bind to the O-atom of the leaving group. Jencks has examined many aspects of metal ion catalysis relevant to phosphatase action in model reactions and reference to the original work is recommended.[2] In essence, enzymes appear to utilize fully the catalytical potential of metal ions but, as is highlighted below, there are many subtle differences in the exact way that this is achieved.

2.3 Stereochemistry and Mechanism of Phosphate Ester Hydrolysis

Before progressing to consider the details of individual phosphatase enzymes, it is worth examining which stereochemical options are available for the transfer of a phosphoryl group from an alcohol to an incoming nucleophile For phosphatases, following the stereochemical course is far from trivial.[3,4] The inorganic phosphate product molecule possesses four equivalent O-atoms and there are only three stable isotopes of oxygen available which could be used to label the species Resorting to the use of sulfur as a surrogate for one of the oxygen ligands in phosphorothioate substrates, so that the product is a chiral [^{16}O,^{17}O,^{18}O]thiophosphate, superficially resolves the problem but, almost certainly, changes the kinetic and possibly, the chemical mechanism of the reaction (see below) Fortunately, the use of phosphorothioate substrates probably does not alter the stereochemical outcome of enzyme-catalysed processes.[5]

Of the four possible mechanisms available for phosphoryl transfer in free solution (Scheme 5, A—D) the first (A) should lead to racemization and two (B and C) should lead to inversion of configuration Mechanism A is a dissociative ionization, analogous to an S_N1 process in carbon chemistry and the nascent trigonal metaphosphate species could, if free, react with a nucleophile on either face to give a racemic product However, within the environment of an active site, the out-going charged nucleofuge might shield one face of the metaphosphate species and suppress front face attack such that inversion would be observed.

Mechanism B, an in-line associative process, is entirely analogous to an S_N2 displacement in carbon chemistry and would give inversion of configuration. The transition state would be pentacoordinate and the nucleofuge and nucleophile, which occupy the apical positions, would be only partially bonded to the central P-atom

Mechanism C is also an in-line associative process but gives rise to a pentacoordinate stable intermediate with fully formed bonds The displacement occurs with inversion of configuration, as for mechanism B

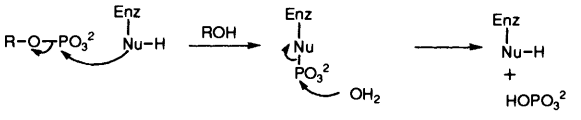
Finally, mechanism D gives rise to retention of configuration *via* an adjacent associative mechanism Here the pentacoordinate stable intermediate must pseudorotate (swap ligands about the P-atom between apical and equatorial positions) so that the nucleofuge leaves from an apical position [6] At the present time

there is evidence to support the operation of each of the four mechanisms and no compelling reasons to discount any Clearly differentiating between mechanisms A,B, and C in Scheme 5 within the confines of the active-site of an enzyme is not possible on stereochemical information alone

2 3 1 Alkaline Phosphatase

Alkaline phosphatase is a non-specific phosphomonoesterase which shows maximum activity at pH 9—10, hence its name The enzyme occurs in both procaryotes and eucaryotes and is, without question, the most well studied of the phosphatases The enzyme from *E coli* is a homodimer (M_r = 94000 Daltons) of 449 amino acid residue subunits and contains two active-sites Each active-site binds two Zn^{2+} ions and one Mg^{2+} ion [7] The amino acid sequence of the *E coli* enzyme shows significant homology (*ca* 25—30%) to mammalian alkaline phosphatases and the primary structure of the mammalian enzymes have been fitted to the *X*-ray crystal structure of the bacterial enzyme All of the key metal ion binding interactions in the *E coli* enzyme are preserved through identical or analogous interactions in the mammalian enzymes and it is, therefore, reasonable to believe that the enzymes operate by analogous mechanisms

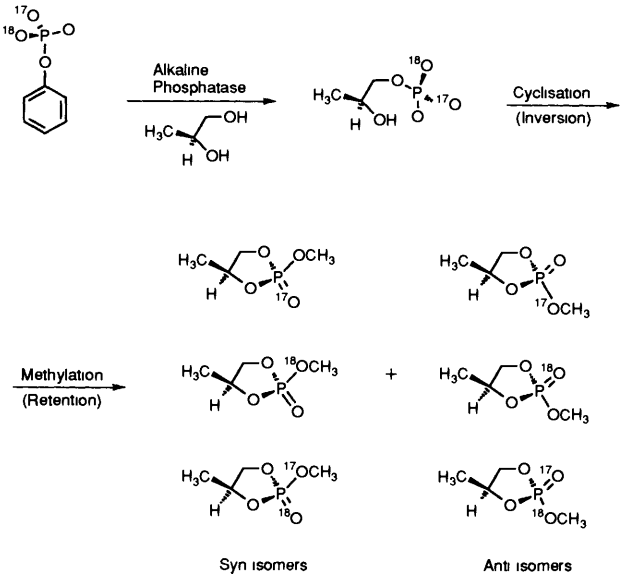
Early studies suggested and then showed that the enzyme catalyses a two-stage ping-pong (on–off) phosphate ester hydrolysis reaction In such mechanisms the phosphoryl group is transferred to a group on the enzyme – which is then subsequently hydrolysed – rather than directly to water (Scheme 6) Evidence for the proposal stemmed from several observations (i) The enzyme gave similar values of k_{cat} for a range of structurally diverse substrates [Note these k_{cat} values are suppressed by common late rate-limiting steps, dephosphorylation (at low pH) and P_i dissociation (at high pH)] (ii) The enzyme was able to catalyse the transfer of a phosphate group from one alcohol to another (transphosphorylation) (iii) The enzyme was able to catalyse ^{18}O-label exchange from ^{18}O-water into the product, inorganic phosphate (P_i), in the absence of the other product (alcohol) (iv) The enzyme displayed burst-phase kinetics for the release of the product alcohol (faster than its steady-state rate of formation) at low temperature (v) A phosphoryl enzyme was shown to be formed from free enzyme and P_i at low pH and subsequent studies identified a serine residue in the protein as the phosphorylated moiety



Scheme 6

Thus, the enzyme displaces the alcohol from the P-atom using an active-site serine residue and, at some subsequent time, a water molecule displaces the serine side-chain from the phosphorylated enzyme (E–P) to give an E P_i complex and, then, ultimately the free enzyme and P_i(Scheme 7, path A) Alternatively, in the presence of an alternative alcohol, the alcohol can displace the serine side-chain of E–P to regenerate a new enzyme–substrate complex which can, of course, react to give the alcohol and E–P or dissociate to give the free enzyme and a new substrate (Scheme 7, path B)

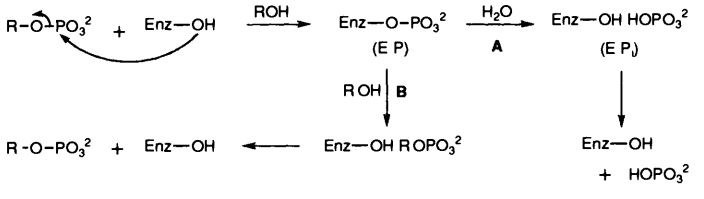
A consequence of the highly symmetric mechanism utilized by alkaline phosphatase is that the absolute stereochemical configuration at phosphorus is retained upon transphosphorylation, as was demonstrated by Knowles [8] In the original work phenyl phosphate, chiral at phosphorus, underwent transphosphorylation to give (2*S*)-1-phosphopropan-1,2-diol which was converted into the cyclic diester form prior to configurational analysis by mass spectrometry (Scheme 8) [Note that since these original studies ^{31}P-NMR spectroscopy techniques have superseded mass spectrometric analysis [4]] More recent work has shown that a mutant enzyme in which the active-site serine residue (Ser102) is replaced by cysteine also gives retentive transphosphorylation Careful consideration of the transphosphorylation' process (Scheme 8) reveals that either two retentions or two inversions would give overall retention Thus unfortunately, one can say nothing about the stereochemical course of the individual phosphoryl transfer steps Nevertheless, some insight has come from crystallographic studies



Scheme 8

In 1991 Kim and Wyckoff reported on an *X*-ray crystal structure of *E coli* alkaline phosphatase complexed to P_i at 2 0 Å resolution [9] This structure was a considerable refinement on an earlier structure at 2 8 Å resolution and allowed a rather thorough examination of the interactions of the protein and the inorganic phosphate molecule with the zinc and magnesium metal ions (Figure 1)

Zinc ion number one ($Zn^{2+}1$) is penta-coordinated by the imidazole N-atoms of His331 and His412, both carboxylate O-atoms of Asp327, and one of the phosphate O-atoms $Zn^{2+}2$ is tetrahedrally coordinated by imidazole N-atom of His370, one of the carboxylate O-atoms of Asp51 and Asp369, and a second of the phosphate O-atoms Thus, both Zn^{2+} ions interact with P_i The Mg^{2+} ion is octahedrally coordinated with the remaining carboxylate O-atom of Asp51, one of the carboxylate O-atoms of Glu322, the hydroxy group of Thr155, and three water molecules Thus, the Mg^{2+} ion does not interact with P_i The



Scheme 7

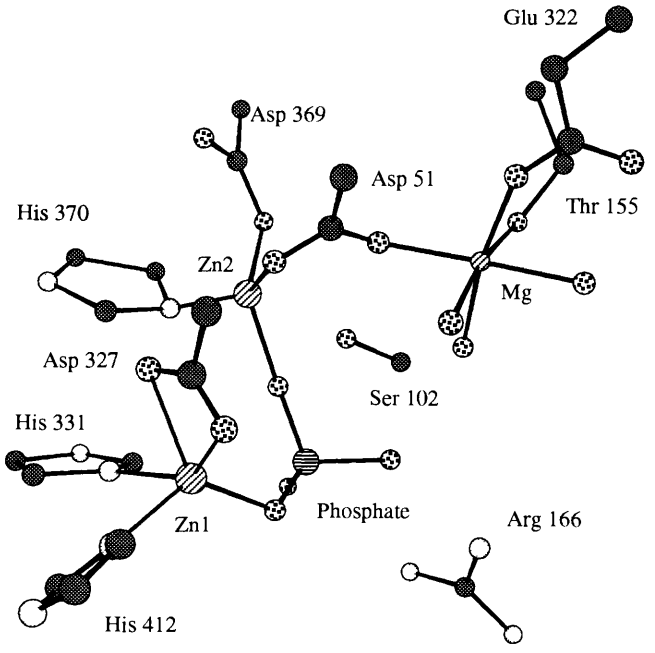


Figure 1 Structure of alkaline phosphatase.

remaining two O-atoms of the P_i molecule are tightly held by the two guanidium amino groups of Arg166. Each P_i–O-atom also forms two additional H-bonding interactions, on one hand, with two water molecules (one of which is coordinated to Mg^{2+}), and on the other hand with the amide group of Ser102 and with a water molecule.

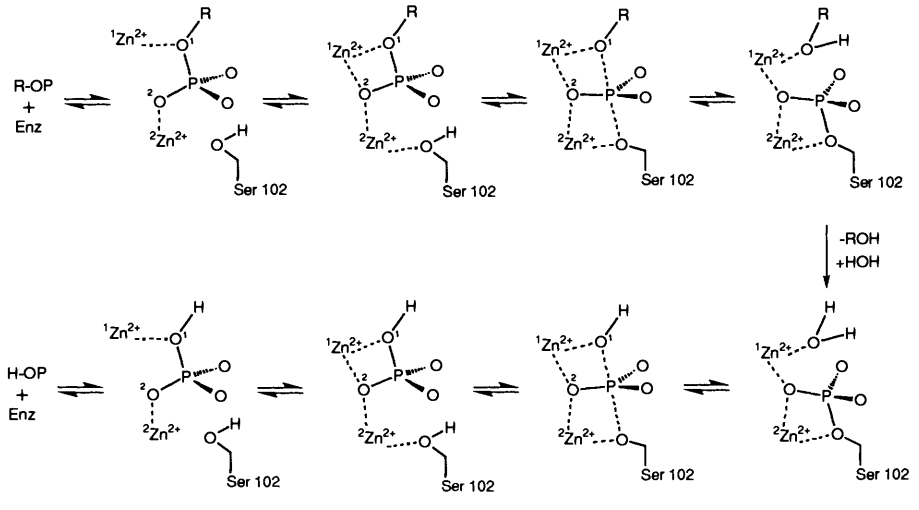
Kim and Wyckoff were also to obtain a structure for a Cd-substituted enzyme at 2.5 Å resolution which contained a phosphorylated active-site serine residue.[9] Together the two crystal structures were used to construct a detailed description of the catalytic mechanism (Scheme 9). According to this, the substrate, ROP, first binds to the enzyme such that the phosphate O-atom bearing the alkyl group (O1) coordinates to $Zn^{2+}1$. One of the other phosphate O-atoms (O2) coordinates to $Zn^{2+}2$ such that the hydroxymethyl O-atom of Ser102 is placed diametrically opposite to the alcohol O-atom of the leaving group. The phosphoryl transfer then begins to proceed by the hydroxymethyl O-atom coordinating to $Zn^{2+}2$. This action causes the phosphate O2-atom to bridge the two Zn^{2+} ions and allows the hydroxy group of Ser102 to deprotonate and attack the P-atom. The result is the formation of a pentacoordinated phosphorus intermediate or transition state which then collapses to an inverted tetrahedron through the departure of the $Zn^{2+}1$ chelated alkoxide O-atom (O1) (Scheme 9). Two important mechanistic features of the phosphoryl transfer reaction are defined by the sequence of events so far. First, the transfer to Ser102 occurs with inversion of configuration and secondly, either of the two in-line associative mechanisms discussed above (Scheme 5, B or C) could operate.

Following the cleavage of the O1–P bond, the alkoxide becomes protonated, leaves the coordination sphere of $Zn^{2+}1$, and then dissociates from the active site. A solvent-derived water molecule then enters the active site, chelates to $Zn^{2+}1$, deprotonates, and then attacks the phosphoryl serine moiety through an in-line displacement. Ultimately through the reverse sequence of steps to those described above, the non-covalent enzyme.P_i complex is formed and P_i dissociates from the active site to complete the catalytic cycle (Scheme 9).

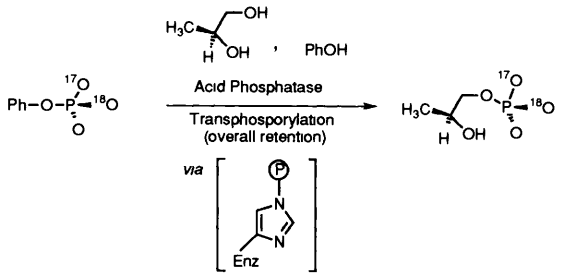
2.3.2 Acid Phosphatase

Very much less is known about acid phosphatases, a family of non-specific enzymes of molecular weight 40 000–60 000 Da. The enzymes have been isolated from a variety of sources including yeast, human lysosomes, and human prostate as well as plants and bacteria. These enzymes show pH optima at pH 2.5 and do not require metal ion cofactors for catalysis. The enzymes from different sources show limited amino acid sequence homology although certain regions of the proteins are highly conserved, in particular, a motif near the N-terminal which includes Arg-His-Gly-Xaa-Arg-Yaa-Pro (positions 16–22 in the *E. coli* enzyme).[10] The enzymes display many characteristics indicative of a ping-pong phosphorylated enzyme intermediate mechanism. These include: (i) the formation of a phosphoprotein containing a covalently modified histidine residue;[11] (ii) burst-phase kinetics for the release of the product alcohol; (iii) transphosphorylation activity; (iv) an ability to catalyse ^{18}O-label exchange from the solvent into P_i. The stereochemical course of the transphosphorylation reaction catalysed by the enzyme was determined in an analogous manner to that for alkaline phosphatase and overall retention of configuration for the two transfer steps was observed.[12] Again it must be stressed that the result provides no information on the stereochemical course of the individual steps.

Recent studies on the *E. coli* enzyme have tested the role of the conserved residues between different species. The alterations of Arg16 to alanine or His17 to asparagine gave inactive proteins. By analogy to the role played by the equivalent residues in phosphoglycerate mutase it is believed that Arg16 interacts with both His17 and His303 at the active-site and that these histidine



Scheme 9



Scheme 10

residues serve as the phosphoryl acceptor moiety (Scheme 10) and as a proton donor, respectively [13] The alteration of His303 to alanine residues gave an enzyme with very low activity, in accord with its proposed role, as did the alteration of the putative substrate binding residue, Arg92 (to alanine) The alteration of Asp304 to Ala changed the rate limiting step from the hydrolysis of the phosphoenzyme intermediate in the wild-type and His303Ala mutant to formation of the phosphoenzyme intermediate Thus, Asp304 rather than His303 may be involved in protonating the departing O-atom of the alcohol These latter results indicate how difficult it is to rationalize the effects of specific mutations in the absence of good *X*-ray crystal data Hopefully such data will become available shortly to facilitate a detailed comparison with alkaline phosphatase

2 3 3 Purple Acid Phosphatase

Purple acid phosphatases occur in bacteria, plants, and animals and hydrolyse aryl phosphate monoesters, phosphoric anhydrides, and the phosphoserine residues of phosphoproteins The most extensively studied examples are those isolated from porcine uterus (uteroferrin) and from bovine spleen Both enzymes are monomeric glycoproteins of about 35000 Daltons which show ~90% amino acid sequence homology Purple acid phosphatases contain two Fe ions at the active site The oxidized form contains two antiferromagnetically coupled Fe^{3+} ions and is purple in colour and inactive Reduction gives the active $Fe^{3+}Fe^{2+}$ form which is pink [14] Interestingly, purple acid phosphatase from kidney bean contains Fe^{3+} and Zn^{2+} at the active site

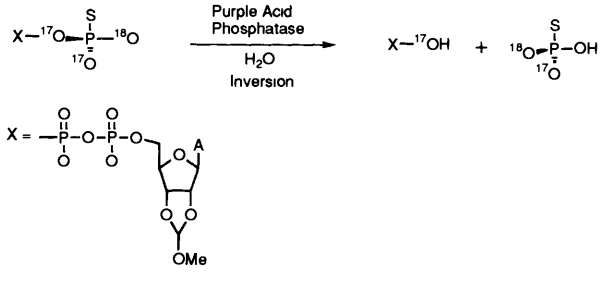
Although little active-site structural information is available, until very recently it was believed that the enzymes operated *via* a phosphorylated enzyme intermediate [15] Evidence to support such a mechanism stemmed from the observation of (i) burst-phase kinetics, (ii) transphosphorylation activity, and (iii) the retention of ^{32}P-label by the enzyme after exposure to the substrate [γ-^{32}P]-ATP However, it was subsequently shown that the enzyme catalyses the transfer of the γ-phosphorothio group of γ-chirally labelled 2′,3′-methoxylidine-γ-phosphorothio-ATP to water with inversion of configuration at phosphorus (Scheme 11) [16] The most likely implication of this result is that the hydrolytic mechanism involves the direct displacement of the leaving group from the terminal P-atom by water in a single step A mechanism consistent with the kinetically determined roles of the metal ions in binding to and activating water and the substrate is shown in (Scheme 12)

2 3 4 Inositol Monophosphatase

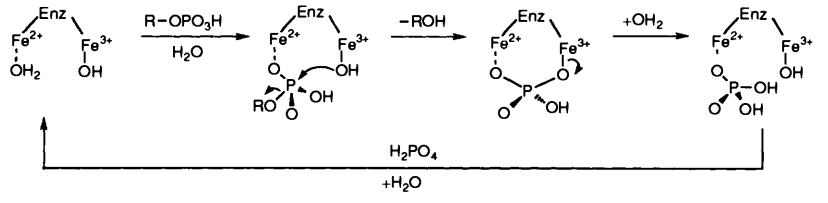
Inositol monophosphatase catalyses the hydrolysis of both enantiomers of *myo*-inositol 1- and 4-phosphate and a range of nucleoside 2′-phosphates The enzyme has attracted considerable interest in recent years because it is believed to be an important target for lithium (cation) therapy in the treatment of manic depression [17] Indeed, the enzyme is inhibited by Li^+ in the millimolar ranges used in therapy and is even more sensitive to Li^+ at the physiological concentrations of the product (P_i) in the brain (2—5 mM)

The enzyme has been purified to homogeneity from a number of mammalian sources including rat, bovine, and human brain The proteins are very similar in size – homodimers of about 28000 Dalton subunits – and the bovine and human brain enzymes show only minor differences in amino acid sequence These enzymes have been the most intensively studied

Inositol monophosphatase shows an absolute requirement for a divalent metal ion and activity is supported by Mg^{2+}, Mn^{2+}, and Zn^{2+} ions Ca^{2+}, Gd^{3+} and a number of other divalent and trivalent metal ions are competitive inhibitors for Mg^{2+} Li^+ on the other hand inhibits uncompetitively with respect to substrate at low concentration (double reciprocal plots of initial rate, v, *versus* substrate concentration, [*S*], give a parallel line pattern for different concentrations of Li^+) and non-competitively with respect to Mg^{2+} (double reciprocal plots of v, versus $[Mg^{2+}]$ converge on the abscissa {the negative portion of the x-axis}) These early results indicated that Li^+ did not simply compete for the Mg^{2+} binding site but prevented the release of P_i from the enzyme or retarded the breakdown of a possible E–P intermediate [18] Further work demonstrated that there was a burst-phase release of inositol and defined the order of product dissociation from the enzyme as inositol first and



Scheme 11



Scheme 12

phosphate last. It was also demonstrated that Mg^{2+} binds to the enzyme after the substrate and dissociates from the enzyme before P_i is released.[19] However, all attempts to identify a phosphorylated enzyme species failed. Good evidence for the operation of a ternary complex mechanism involving the direct displacement of the alcohol by a nucleophilic water molecule was obtained when it was shown that the enzyme-catalysed exchange of ^{18}O-label into P_i depended absolutely on the presence of the inositol.[20] Further evidence against the operation of a phosphorylated enzyme mechanism was obtained when it was shown that inositol phosphorothioates were processed by the enzyme at only slightly lower rates than the natural substrates. Phosphatases that operate *via* E–P intermediates, including alkaline phosphatase, process phosphorothioate substrates extremely slowly. Thus, it was emerging that inositol monophosphatase operated by a very different mechanism to those of the well studied phosphatases described above.

Elegant hydroxy group deletion studies defined many of the important binding interactions of the substrate with the enzyme and demonstrated that the 3-OH and 5-OH groups of the inositol ring were not important for either binding or catalysis. Significantly, it was established that the 2-OH and the 4-OH groups and 1-O-atom of the inositol skeleton were important for binding to the enzyme while the 6-OH group was in some way involved in catalysis (Figure 2).[21] The publication of an *X*-ray crystal structure for a Gd^{3+} sulfate form of the protein allowed many of the findings from the substrate hydroxy group deletion studies to be rationalized by modelling.[22] The structure indicated clearly that the sulfate O-atoms could chelate to the metal ion which was quite deeply buried in the enzyme. In these studies it was reasonably expected that the Gd^{3+} ion would bind in place of the Mg^{2+} ion and that the sulfate anion would serve as a surrogate for enzyme-bound P_i. However, many kinetic properties of the enzyme remained unaccounted for, including the different Mg^{2+} binding orders (timings) predicted by the crystal structure (Mg^{2+} should bind before the substrate) and observed from the kinetic studies (Mg^{2+} should bind after the substrate). Since many laboratories had recognized that the activity of the enzyme did not show simple saturation kinetics with increasing Mg^{2+} concentration, it appeared that there might be two Mg^{2+} binding sites.

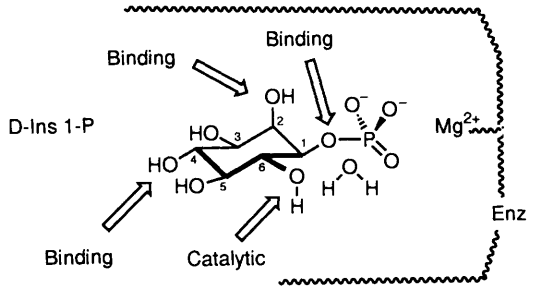


Figure 2 Schematic representation of enzyme–substrate interactions for inositol monophosphatase.

Attempts to identify the position of the additional Mg^{2+} binding site were pursued in two laboratories using either chemical and kinetic approaches or *X*-ray crystallographic methods. In the chemical approach the enzyme's ability to process nucleoside 2′-phosphates and 2′-thiophosphates was probed using the Mg^{2+} and the Mn^{2+} forms of the enzyme.[23] The results of these studies and the finding that adenosine was unable to mediate the exchange of ^{18}O-label from water into P_i (unlike inositol, see above) was used to determine the active conformation for adenosine 2′-phosphate at the active-site of the enzyme. In this structure the 4′-hydroxymethyl group and the adenine moiety occupied unfavourable axial positions in the ribofuranosyl ring and it was reasoned that only chelation of the ether O-atom and the (leaving) 2′-O-atom of the ribofurunosyl moiety by a metal ion to form a five-membered metallocycle would sufficiently stabilize the conformation. Thus, a role for the second Mg^{2+} became evident (Figure 3a). Comparison with the analogous conformations for inositol phosphates indicated that the 1-O atom (the nucleofuge) and the catalytically essential 6-OH group of D-*myo*-inositol 1-phosphate should chelate to the second Mg^{2+} ion (Figure 3b).

The finding that two Mg^{2+} ions were required and their identified actual positions in the active complex explained several unanswered questions, including why a catalytically important 6-OH site existed in the inositol skeleton, why there were discrepencies in the perceived binding orders for Mg^{2+}, and why Li^+ inhibition changed from simple uncompetitive at low [Li^+] to mixed at high [Li^+]. Subsequent studies using substrates and inhibitor analogues provided further evidence for the operation of a two-metal-site mechanism (see Figure 3).

Independently, *X*-ray crystallographic studies of protein–substrate complexes using inhibitory metals to prevent reaction provided an almost identical picture of the active-site interactions (Figure 4).[24] Indeed, only one difference in the proposed

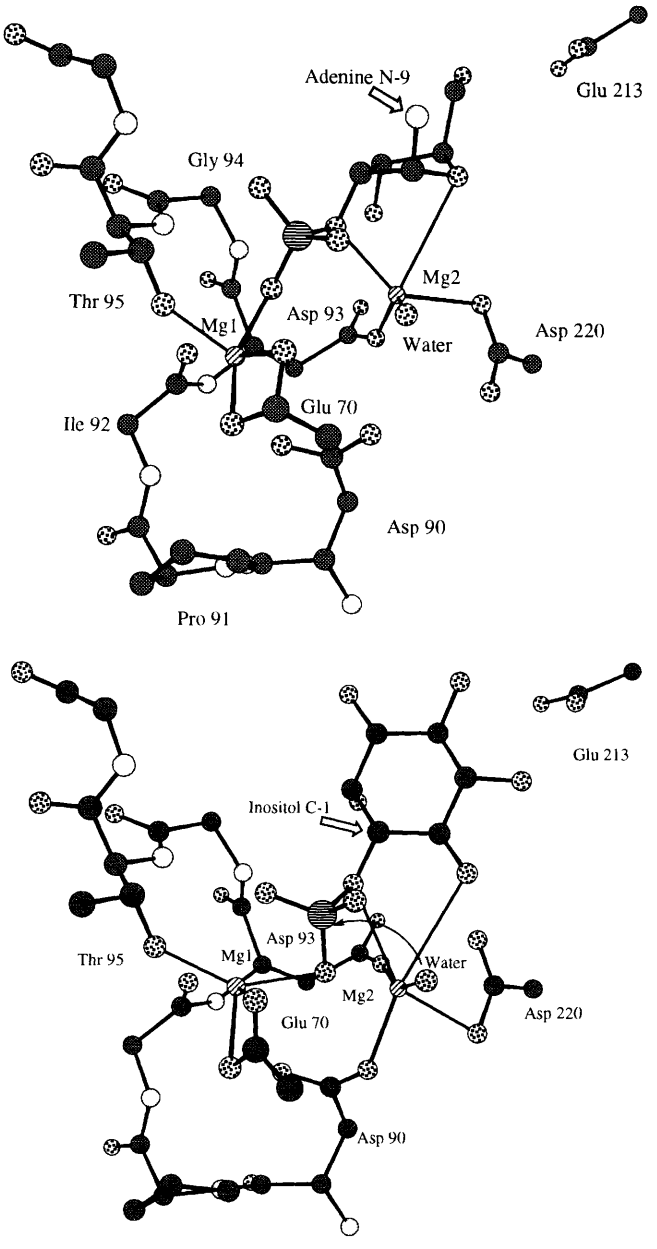


Figure 3 (Upper) Structure of the complex of inositol monophosphatase and 2′-AMP. (Lower) Structure of the complex of inositol monophosphatase and D-*myo*-inositol phosphate with the attacking nucleophile bound to $Mg^{2+}2$.

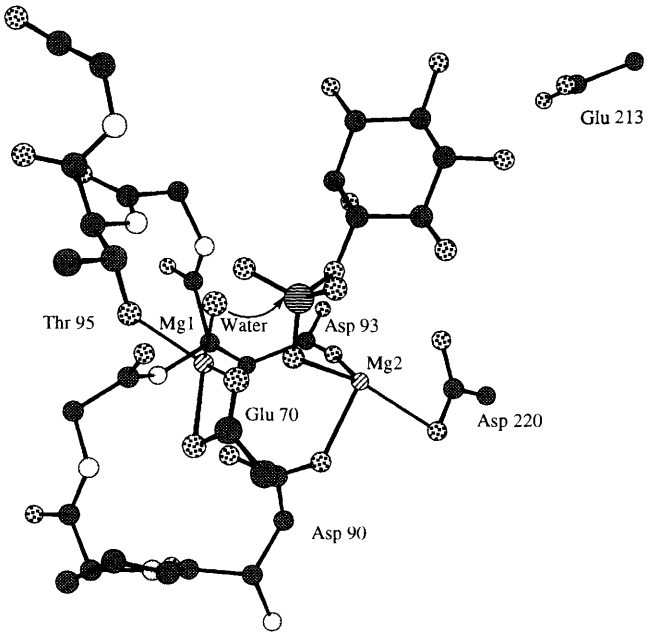


Figure 4 Structure of the complex of inositol monophosphatase and D-*myo*-inositol phosphate with the attacking nucleophile bound to $Mg^{2+}1$.

mechanisms existed. This concerned which of the two-metal-ion sites provided activation, through chelation, for the nucleophilic water molecule. On the basis of the rapid rates of ^{18}O-exchange from water into P_i in the presence of inositol and the established orders for metal ions binding to the enzyme, Cole and Gani argued that the second Mg^{2+} ion should activate the water molecule.[23] This was because both $Mg^{2+}2$ and its associated water molecules could readily dissociate from the enzyme and exchange with the solvent and rebind without the substrate debinding. The *X*-ray study placed the nucleophilic water molecule on the first Mg^{2+}. The two different sites for the nucleophile would give different stereochemical courses for phosphoryl transfer. A location on $Mg^{2+}1$ would give inversion of configuration through an in-line diplacement whereas attack by a water molecule chelated to $Mg^{2+}2$ would give retention *via* an adjacent association and pseudorotation.

2.3.5 D-*Fructose 1,6-Bisphosphate 1-Phosphatase*

Fructose 1,6-bisphosphatase hydrolyses the 1-phosphate ester group of D-fructose 1,6-bisphosphate to give fructose 6-phosphate and P_i. The enzyme is a homotetramer of 35000 Dalton subunits. Kinetic studies show that only the α-anomer is hydrolysed, but a slow, non-enzymatic interconversion of the α- and β-anomers ensures that the complete hydrolysis of a mixture of the anomers occurs over time. The enzyme is inhibited allosterically by AMP and activated by divalent metal cations such as Mg^{2+}, Mn^{2+}, and Zn^{2+}. As is the case with inositol monophosphatase, these ions become inhibitory at high concentration. Kinetic studies of metal binding have shown that between one and three Zn^{2+} ions per monomer are able to bind. For Mn^{2+}, in the absence of substrate, only a single ion is able to bind, but a second ion can bind in the presence of the substrate or a substrate analogue. There is still some uncertainty regarding the binding of Mg^{2+} where there appears to be two binding sites at pH 7.2 and 9.1 in the absence of EDTA, but only a single site in the presence of EDTA. Current opinion holds that two metal ions are required for catalysis[25] and that the $E.Mg^{2+}.S$ complex possesses a very low affinity for a second Mg^{2+} ion. This property might explain why crystal structures have been determined showing two Zn^{2+} ions or two Mn^{2+} ions at the active site, but only single occupancy for Mg^{2+}.

As was the case for inositol monophosphatase, no phospho-enzyme intermediate has been detected. Fructose bisphosphatase is able to catalyse ^{18}O exchange from ^{18}O-labelled P_i into the solvent in the absence of the product, fructose 6-phosphate, although the reaction is extremely slow – 160-times slower than in the presence of fructose 6-phosphate ($t_{1/2}$ = 14 min).[26] The ^{18}O-exchange reaction requires the presence of a divalent cation, shows the same broad specificity for Mg^{2+} as the hydrolysis reaction, and a maximum rate when the enzyme is saturated with both P_i and fructose 6-phosphate. These properties together are indicative of a direct displacement ternary complex mechanism. Benkovic and co-workers have shown that the hydrolysis reaction proceeds with inversion of configuration at phosphorus[27] which further refines the geometry of displacement by water or hydroxide as of the in-line type (mechanisms B or C of Scheme 5).

Several crystal structures of fructose bisphosphatase have been determined with a variety of metal cations and substrate analogues bound at the active site. The absence of any likely protein-based nucleophile in the vicinity of the 1-phosphate (Figure 5) supports the view that this enzyme, like inositol monophosphatase, conducts hydrolysis by means of a direct displacement.

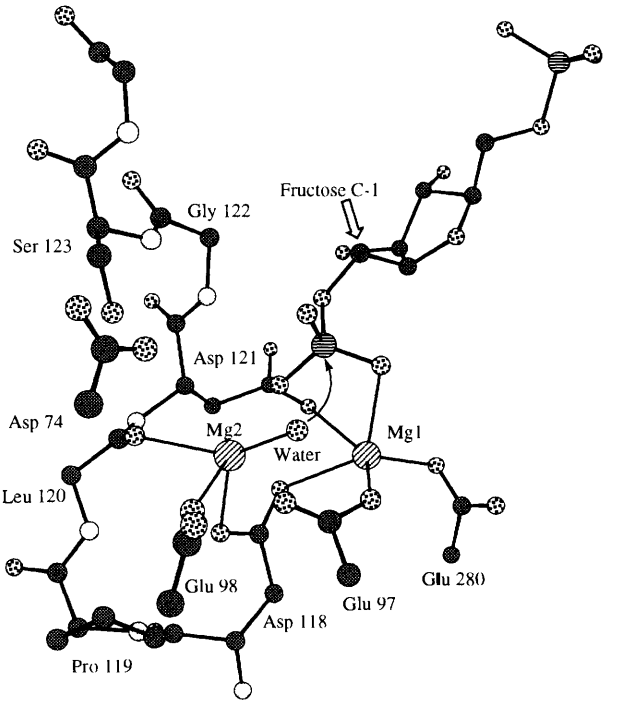


Figure 5 Structure of the complex of fructose bisphosphatase and D-fructose 1,6-bisphosphate.

Despite the clear similarities in mechanism between fructose bisphosphastase and inositol monophosphates described above, the two enzymes show very little sequence and structural homology. Their different substrate specificities account for much of this, but a comparison of the metal-binding sequences is of special interest. The crystal structure of inositol monophosphatase, shows the sequence 90—96 (Asp-Pro-Ile-Asp-Gly-Thr-Thr) forming a kinked structure, binding the metal (Gd^{3+}) with the side chain of Asp90, the backbone carbonyl group of Ile92 and the side chain of Thr95. The corresponding sequence in fructose bisphosphatase, 118—124 (Asp-Pro-Leu-Asp-Gly-Ser-Ser) takes up an identical conformation (Figure 5) and contributes to the binding at both metal sites. The side chains of Asp118 and Asp121 coordinate to the $M^{2+}1$ site (the only site shown to bind Mg^{2+}), while the side chain of Asp118 also coordinates to the $M^{2+}2$ site, along with backbone carbonyl group of Leu120 and the side chain of Ser123 (Figure 3a). Side chains of acid residues from elsewhere in the chain complete the

coordination shells at the two sites, but do not show any sequence homology with equivalent residues from inositol monophosphatase

While the $M^{2+}2$ site of fructose bisphosphatase corresponds to the known metal ion binding site of inositol monophosphatase, the $M^{2+}1$ site closely correlates to the second Mg^{2+} binding site of inositol monophosphatase, suggesting that these two enzymes are mechanistically very similar However, in contrast to the situation for inositol monophosphatase, the leaving O-atom of the substrate phosphomonoester does not appear to be chelated by either of the metal ions If it is chelated in the active complex it would be through an interaction with the ion in the $M^{2+}1$ site rather than with the second binding ion as in the case for inositol monophosphatase

2 3 6 5'-Nucleotidase

5'-Nucleotidase catalyses the dephosphorylation of a range of nucleoside 5'-phosphates where the preference for given substrates varies with the source of the enzyme 5'-Nucleotidase activity was first discovered in the venom of snakes and has since been isolated from a wide variety of species including, bacteria, plants, and mammals [28] 5'-Nucleotidase activities can be split in two classes based on their substrate binding affinities Low K_m nucleotidases bind IMP, AMP, and GMP at micromolar concentrations, while high K_m nucleotidases bind the same substrates at millimolar concentrations Both classes of 5-nucleotidase are homotetramers The low K_m forms possess subunit masses of ~40000 Da while those of the high K_m forms range from 42—69000 Da depending on source Both types require divalent cations for activity and show a preference for Mg^{2+} However, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, and Ni^{2+} divalent cations support catalysis with reduced activity Both types of 5'-nucleotidase are activated by ATP but the two forms differ in their pH optima The high K_m forms are maximally active at pH 6 5 while the low K_m forms show pH optmium at 7 5—9 0, depending on the structure of the 5'-nucleotide substrate

Though very similar in many respects, the two classes of 5'-nucleotidase are thought to serve different physiological roles By virtue of its high substrate binding affinity, the low K_m enzyme is believed to be largely responsible for the dephosphorylation of AMP The principal substrate target for the high K_m enzymes is believed to be IMP

At the present time little is known about the mechanisms of the hydrolysis reactions catalysed by the enzymes Furthermore, no sequence nor structural information has yet been reported for either class of enzyme Nevertheless, the 5'-nucleotidases show features most closely related to those of fructose 1,6-bisphosphatase and inositol monophosphatase For example, the enzyme from *Crotalus atrox* venom does not catalyse transphosphorylation, nor does it catalyse the exchange of ^{18}O-label from water into P_i Thus, its properties are consistent with those expected for a ternary complex mechanism in which water directly attacks the electrophilic P-atom In keeping with the stereochemical course of all proven single-step enzyme-catalysed phosphoryl group transfers reported to date, the *Crotalus atrox* venom enzyme catalyses hydrolysis with inversion of configuration at phosphorus [29]

2 3 7 Protein Tyrosine and Protein Serine, Threonine Phosphatases

Protein phosphatases are a diverse group of enzymes responsible for the dephosphorylation of a range of phosphoproteins Many are involved in the regulatory control of cellular processes as diverse as cell growth and proliferation, protein, cholesterol and fatty acid biosynthesis and glycolysis/gluconeogenesis The protein phosphatases can be categorized into two large groups, those that dephosphorylate phosphotyrosine residues within proteins and those that dephosphorylate phosphoserine or phosphotheonine residues within proteins

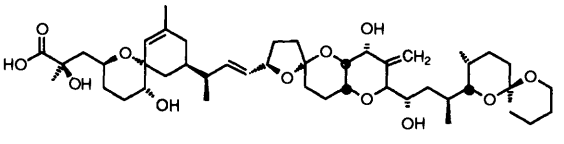
High molecular weight phosphotyrosyl protein phosphatases, whether associated with receptors or not, share a conserved catalytic domain of 240 amino acid residues [30] On the other hand, a group of low molecular weight cytoplasmic phosphotyrosyl protein phosphatases, thought to be important in the intracellular phosphoprotein dephosphorylation, do not show sequence homology to the larger enzymes

The kinetics and mechanism of the low molecular weight forms have been studied extensively Early work indicated that the enzyme catalyses transphosphorylation and a stereochemical study showed that the reaction occurred with overall retention of configuration Further work with the bovine heart enzyme demonstrated the existence of a phosphorylated enzyme intermediate and recently it was shown that the thiomethyl group of a cysteine residue (Cys12) served as the active-site nucleophile [31]

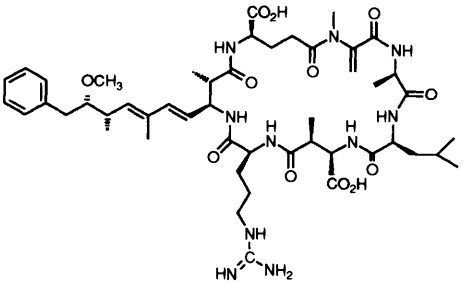
The high molecular weight phosphotyrosyl protein phosphatases also operate *via* the intermediacy of a phosphorylated enzyme but, in contrast to the low molecular weight form, the active site nucleophile is the imidazole side-chain of a histidine residue [10] Very recently the results of structural studies on the high and low molecular weight forms have been published [32]

The phosphoserine–phosphothreonine protein phosphatases are a ubiquitous group of enzymes which constitute the catalytic domains of several multiprotein complexes The enzymes are classified by their sensitivities to various protein inhibitors (inhibitors 1 and 2) and by their cofactor dependencies [33] Phosphoprotein phosphatase 1 (PP1) contains a catalytic domain of ~35000 Da and is inhibited by inhibitor-1 and inhibitor-2 The type-2 enzymes are not significantly affected by inhibitors 1 and 2, but the catalytic domains of PP2A (~35000 Da) and PP2B, calcineurin, (60000 Da) show significant sequence homology to PP1 PP2C (46000 Da) appears not to be related PP1 and PP2A do not appear to require divalent metal ions for activity, whereas PP2B requires Ca^{2+} and binds calmodulin, and PP2C requires Mg^{2+} for activity Catalysis by PP1, PP2A, and to a lesser extent PP2B is inhibited by the tumour promoter okadaic acid (1) but PP2C is insensitive PP1 and PP2A are also inhibited by microcystin (2) and nodularin (3)

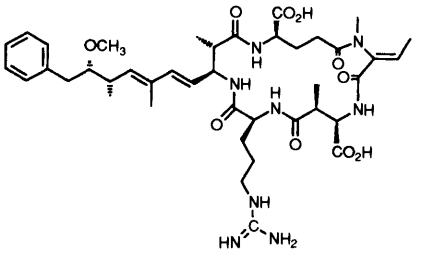
While almost nothing is known about the hydrolytic mechanism for any of the enzymes, a recent sequence comparison of purple acid phosphatase with PP1 and PP2A has indicated that certain regions of these enzymes are similar Based on this comparison and the fact that purple acid phosphatases are able to dephosphorylate proteins possessing phosphoserine and phosphothreonine residues, it has been proposed that PP1 and PP2A are iron–zinc metalloenzymes [34] These ideas have yet to be substantiated



Okadaic Acid
(1)



Microcystin-LR
(2)



Nodularin
(3)

2 3 8 Overview

It is evident from this cursory examination of the mode of action of the monophosphate ester hydrolase enzymes that nature has evolved many different mechanisms to cleave P—O bonds Interestingly, the less specific enzymes, including alkaline and acid phosphatase, operate *via* phosphorylated enzyme intermediates At a chemical level, this may reflect the fact that there is very little on which to bind in catalysing a non-specific hydrolysis – just the phosphate group Therefore, in order to standardize the catalytic problem, the enzyme might be expected first to transphosphorylate internally, to release the alcohol product, and then to tackle the hydrolytic step on a species which is identical regardless of the starting substrate Specific enzymes can, of course, bind as much of the substrate as is required for stabilizing the transition state for the direct hydrolysis by water (or hydroxide) in a single step

While the type and geometrical arrangement of the metal ions used in phosphohydrolase catalysis varies widely, in each case their roles are very similar to position and activate the nucleophile, to position and enhance the electrophilicity of the P-atom, and to provide Lewis acid catalysis for the leaving alkoxide (where necessary) The reported stereochemical courses of all of the systems examined above show that single phosphoryl transfer steps occur with inversion and, where retention occurs, there are an even number of transfer steps Whether inositol monophosphatase, which acts *via* a direct displacement, will follow this trend remains to be seen

3 References

1 T C Bruice and S J Benkovic, in 'Bioorganic Mechanisms', Vol 2, W A Benjamin, New York, 1966, Ch 5, pp 1 109
2 D Herschlag and W P Jencks, *Biochemistry*, 1990, **29**, 5172
3 J R Knowles, *Annu Rev Biochem*, 1980, **49**, 877
4 H G Floss, M -D Tsai, and R W Woodard, *Top Stereochem*, 1984, **15**, 253
5 P A Frey, *Adv Enzymol Relat Areas Mol Biol*, 1989, **62**, 119
6 S L Buchwald, D H Pliura, and J R Knowles, *J Am Chem Soc*, 1984, **106**, 4916
7 R A Anderson, W F Bosron, F S Kennedy, and B L Vallee, *Proc Natl Acad Sci USA*, 1975, **72**, 2989
8 S R Jones, L A Kindman, and J R Knowles, *Nature*, 1978, **257**, 564
9 E E Kim and H W Wyckoff, *J Mol Biol*, 191, **218**, 449
10 K Ostanin, E H Harms, P E Stevis, R Kuciel, M -M Zhou, and R L Van Etten, *J Biol Chem*, 1992, **267**, 22830
11 R L Van Etten, *Ann New York Acad Sci*, 1982, **390**, 27
12 S L Buchwald, M S Saini, J R Knowles, and R L Van Etten, *J Biol Chem*, 1984, **259**, 2208
13 L A Fothergill-Gilmore and H C Watson, *Adv Enzymol Relat Areas Mol Biol*, 1989, **62**, 227
14 J W Pyrz, J T Sage, P G Brunner, and L Que, *J Biol Chem*, 1986, **261**, 11015
15 J B Vincent, M W Crowder, and B A Averill, *J Biol Chem*, 1991, **266**, 17737
16 E G Mueller, M W Crowder, B A Averilland, and J R Knowles, *J Am Chem Soc*, 1993, **115**, 2974
17 D Gani, C P Downes, I Batty, and J Bramham, *Biochim Biophys Acta*, 1993, **1177**, 253
18 J K Shute, R Baker, D C Billington, and D Gani, *J Chem Soc Chem Commun*, 1988, 626
19 A P Leech, G R Baker, J K Shute, M A Cohen, and D Gani, *Eur J Biochem*, 1993, **212**, 693
20 G R Baker and D Gani, *Biomed Chem Lett*, 1991, **1**, 193
21 R Baker, C Carrick, P D Leeson, I C Lenon, and N Liverton, *J Chem Soc Chem Commun*, 1991, 298
22 R Bone, J P Springer, and J R Atack, *Proc Natl Acad Sci USA*, 1992, **89**, 10031
23 A G Cole and D Gani, *J Chem Soc Chem Commun*, 1994, 1139
24 S J Pollack, J R Atack, M R Knowles, G McAllister, C I Ragan, R Baker, S R Fletcher, L L Iversen, and H B Broughton, *Proc Natl Acad Sci USA*, 1994, **91**, 5766
25 Y Zhang, J -Y Liang, S Huang, H Ke, and W N Lipscomb, *Biochemistry*, 1993, **32**, 1844
26 T R Sharp and S J Benkovic, *Biochemistry*, 1979, **18**, 2910
27 P L Domanico, J F Rahil, and S J Benkovic, *Biochemistry*, 1985, **24**, 1623
28 R Itoh, *Comp Biochem Physiol*, 1993, **105B**, 13
29 M -D Tsai, *J Am Chem Soc*, 1980, **102**, 5416
30 H Charbonneau and N K Tonks, *Ann Rev Cell Biol*, 1992, **8**, 469
31 P Cirri, P Chiarugi, G Camici, G Manou, G Raugei, G Cappugi, and G Ramponi, *FEBS Lett*, 1993, **214**, 647
32 (*a*) J A Stuckey, H L Schubert, E B Fauman, Z -Y Zhang, J E Dixon, and M A Saper, *Nature*, 1994, **370**, 571 (*b*) X-D Su, N Taddei, M Stefani, G Ramponi, and P Nordland, *Nature*, 1994, **370**, 575 (c) D Barford, A J Flint, and N K Tonks, *Science*, 1994, **263**, 1397
33 P Cohen, *Annu Rev Biochem*, 1989, **58**, 453
34 J B Vincent and B A Averill, *FEBS Lett*, 1990, **263**, 265