Enzymatic Mechanisms of Phosphate and Sulfate Transfer

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1. Introduction

Many biological molecules contain phosphate or sulfate, and the enzymatic reactions that transfer these groups play important roles in metabolism. DNA and RNA are phosphate diesters, while many intermediates in metabolism exist as phosphate monoesters. Phosphorylation of proteins is an important control mechanism. While triesters are not naturally occurring biological molecules, enzymes have evolved to hydrolyze these man-made toxic compounds. ATP and similar molecules contain phosphoanhydrides, which liberate considerable free energy upon their hydrolysis and, thus, provide the energy needed for muscle movement and the biosynthesis of other bonds. Esterification with sulfate serves to solubilize molecules to aid in their excretion, and sulfate monoesters are found among many classes of natural products, possibly aiding in transport. In this review we will briefly present what is known about nonenzymatic phosphate and sulfate transfers, and then we will discuss the kinetic and chemical mechanisms of enzymes that catalyze similar transfers.

1.1. Types of Phosphate Esters

Phosphoric acid may be esterified in one, two, or three positions, forming a monoester, diester, or triester, respectively. Triesters do not occur naturally. However, monoesters and diesters have well-known roles in genetic materials, in coenzymes, and in energy reservoirs, and as intermediates in biochemical transformations, and monoesters formed by protein phosphorylation have key roles in the regulation of a host of processes. Their kinetic stability makes monoesters

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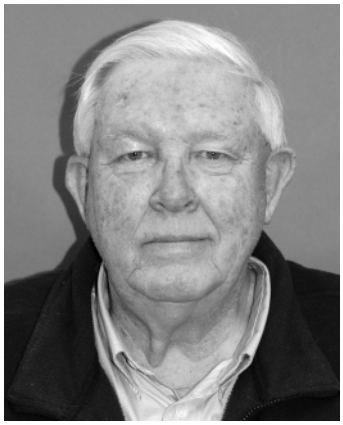

W. Wallace Cleland is a Co-Director of the Institute for Enzyme Research at the University of Wisconsin—Madison and is M. J. Johnson Professor of Biochemistry. He received his B.S. summa cum laude from Oberlin College in 1950 and his M.S. and Ph.D. degrees in 1953 and 1955 from the University of Wisconsin. After two years in the U. S. Army and two more years as a postdoc in Chicago, he returned to Madison and has been a member of the Department of Biochemistry since 1959. He is best known for utilizing kinetic studies to determine enzyme mechanisms, and he also developed the use of dithiothreitol as a reducing agent. His current work involves the use of ^{15}N, ^{18}O, and ^{13}C isotope effects to determine the chemical mechanisms and transition-state structures for selected enzymatic reactions.

Alvan Hengge was born in Cincinnati, OH. After receiving his B.S. degree from the University of Cincinnati (UC) in 1974, he taught high school chemistry and physics from 1975 to 1982 before returning to UC for graduate school and earning a Ph.D. in Organic Chemistry in 1987. This was followed by an NIH postdoctoral fellowship and several subsequent years as an Assistant Scientist in the lab of W. W. Cleland at the Institute for Enzyme Research at the University of Wisconsin. He joined the faculty at Utah State University in 1996, where he is now Professor in the Department of Chemistry and Biochemistry. His major research interests are the investigations of mechanisms of biologically important reactions, particularly phosphate and sulfate ester chemistry.

and diesters particularly suitable for their biological roles. The half-life for the uncatalyzed attack of water on alkyl phosphate dianions is $\sim 1.1 \times 10^{12}$ years ($k = 2 \times 10^{-20}$ s^{-1}) at 25 °C.[1] Protein serine or threonine alkyl phosphates, along with the aryl counterpart tyrosine phosphate, are the substrates for phosphatases involved in signal transduction and regulation. Some of these enzymes produce the largest known enzymatic rate enhancements relative to the uncatalyzed reactions of their substrates ($\sim 10^{21}$-fold). Enzymatic phosphodiester hydrolysis is similarly impressive. The rate for the uncatalyzed hydrolysis of dimethyl phosphate at 25 °C is estimated at 10^{-15} s^{-1}.[2] Staphylococcal nuclease raises the rate of P−O bond fission by 10^{16}-fold, as a lower estimate.[3] These large rate enhancements have led to considerable interest in the details of enzymatic phosphoryl transfer, and to speculations that the enzymatic reaction may differ in crucial aspects from uncatalyzed reactions.

1.1.1. Mechanistic Possibilities

Phosphoryl transfer reactions are substitution reactions at phosphorus, and three limiting mechanisms exist. One is a dissociative, S_N1-type mechanism ($D_N + A_N$ in the IUPAC nomenclature).[4] In such a mechanism, which is a realistic possibility only for monoesters, a stable metaphosphate ion (PO_3^-) forms that is attacked by a nucleophile in a subsequent, rate-determining step. The metaphosphate anion has never been directly observed in solution, but it exists as a stable entity in the gas phase, where it is surprisingly nonreactive.[5] Metaphosphate has been observed in the X-ray structure of fructose-1,6-bisphosphatase grown in an equilibrium mixture of substrate and product,[6] and a moiety that could be described as a stabilized metaphosphate has been observed in the structure of β-phosphoglucomutase (β-PGM) from *Lactococcus lactis* obtained at cryogenic temperature.[7]

Other phosphoryl transfer mechanisms are an associative, two-step addition−elimination mechanism ($A_N + D_N$ in the IUPAC nomenclature[4]) with a phosphorane intermediate, and a concerted mechanism (A_ND_N) with no intermediate. The addition−elimination mechanism occurs in some reactions of triesters and diesters and has been speculated to occur in some enzymatic reactions of monoesters. In the concerted A_ND_N mechanism, bond formation to the nucleophile and bond fission to the leaving group both occur in the transition state.

2. Nonenzymatic Phosphate Transfer

2.1. Uncatalyzed Reactions of Phosphomonoesters

Depending upon pH, a phosphate monoester may be neutral, a monoanion, or a dianion. The neutral form is present only under very acidic conditions, and the reactions of these species have not been subjected to much study. Monoester dianions are less reactive than monoanions, except for esters with a highly activated leaving group such as 2,4-dinitrophenol.[8]

2.1.1. Monoester Dianions

Reactions of monoester dianions exhibit near zero entropies of activation, low Brønsted β_{nuc} but large β_{lg} values,[8,9] and large $^{18}k_{bridge}$ isotope effects.[10,11] These data are indicative of a concerted process with a loose transition state. The collected evidence in favor of this mechanism has been reviewed.[12,13] Despite very loose transition states, there is no evidence for the formation of free metaphosphate. When the phosphoryl group is made chiral using ^{16}O, ^{17}O, and ^{18}O, phosphoryl transfer occurs with inversion of configuration, except in *tert*-butyl alcohol, where racemic *tert*-butyl phosphate forms from chiral *p*-nitrophenyl phosphate.[14,15]

The data and mechanistic conclusions summarized above come from work with aryl phosphomonoesters; as predicted by the large negative β_{lg} value, alkyl ester dianions react at very slow rates, making kinetic investigations difficult. A recent kinetic investigation of methyl phosphate hydrolysis found the rate of the dianion hydrolysis to be below the threshold of detectability, with an estimated rate constant of 2×10^{-20} s^{-1} at 25 °C.[1] This value is close to the rate

predicted from an extrapolation of the Brønsted plot of aryl phosphomonoester dianions, suggesting a similar mechanism for alkyl and aryl esters.

2.1.2. Monoester Monoanions

The hydrolysis of the monoanion of 2,4-dinitrophenyl phosphate is thought to be concerted,[16] but the possibility of a metaphosphate intermediate in monoanion reactions has not been ruled out in general. The hydrolysis of phenyl phosphate monoanion proceeds with inversion of stereochemistry,[14] implying either a concerted mechanism or a discrete metaphosphate intermediate in a preassociative mechanism.

A small β_{lg} (-0.27)[8] indicates little change in effective charge on the leaving group in the transition state, consistent with proton transfer from the phosphoryl group to the leaving group, most likely via one or more intervening water molecules. The kinetic isotope effects (KIEs) in the nonbridging oxygen atoms for the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) and *m*-nitrobenzyl phosphate (*m*NBP) are those expected if the phosphoryl group is deprotonated during reaction.[17] It has been proposed[8] that, for less basic leaving groups, protonation occurs simultaneously with leaving group departure and both processes occur in the rate-limiting step. For more basic leaving groups, a bridge-protonated intermediate forms, followed by rate-limiting P−O bond fission. In either of these scenarios, proton transfer and P−O bond fission could occur in concert with nucleophilic attack or with formation of metaphosphate. Variations in the solvent isotope effects are consistent with the hypothesis that the basicity of the ester group affects the timing of proton transfer.[8,17,18]

The catalytic benefit of leaving group protonation is a major reason the uncatalyzed hydrolysis of phosphomonoesters exhibits a pH optimum at pH ∼4, where the concentration of the monoanion is highest. Protonation of the leaving group is a key feature that is shared in the enzymatic reactions of dianions by many phosphatases. In the latter reactions, the proton comes from an enzymatic general acid.

2.2. Uncatalyzed Reactions of Phosphodiesters

For reactions of diester anions, the Brønsted β_{nuc} and β_{lg} values,[19,20] as well as kinetic isotope effects,[21] are intermediate between those for reactions of monoester dianions and those for triesters. A nucleophile ^{18}O KIE of 1.027 is found for the attack of hydroxide on the diester thymidine-5′-*p*-nitrophenyl phosphate,[22] evidence for direct attack by hydroxide in the rate-limiting step, and together with the presence of normal ^{18}O KIEs in the scissile oxygen atom of such reactions, this points to a concerted mechanism. These data are consistent with the idea that diester reactions exhibit transition states that are intermediate between the loose ones of monoesters and the tight transition states in triester reactions.

P−O bond fission is the usual mode of attack by nucleophiles on phosphodiesters, though there are exceptions. The labile diester methyl-2,4-dinitrophenyl phosphate shows significant amounts of attack at aromatic carbon (nucleophilic aromatic substitution, with loss of methyl phosphate) in competition with attack at phosphorus, most notably with hydroxide and with primary amines.[20] Due to the small size of the methyl group, it is sterically susceptible to nucleophilic attack in phosphate esters; the hydrolysis of the dimethyl phosphate anion occurs almost exclusively by C−O bond fission.[2] But this is an anomaly; an isotope-labeling study of the alkaline hydrolysis of ethyl 4-nitrophenyl phosphate indicated only P−O fission occurs, even at 1 N [$^-$OH].[23]

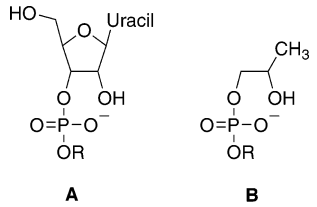

Figure 1. Chemical models for the cyclization of RNA.

The reactions of dinucleotides and related model molecules, such as those in Figure 1, have been studied in detail, because of their biological significance. Phosphoryl cleavage and isomerizations of dinucleotide-3′-phosphate diesters and related compounds have been examined by several groups, most recently by Lönnberg and co-workers.[24,25] Under acidic conditions, isomerization to form a 2′-phosphate diester and cyclization to the 2′,3′ phosphate (Figure 2) proceed at comparable rates via a common intermediate. Near neutral pH, the diester is anionic, and a pH-independent isomerization of the diester is the major reaction. Under alkaline conditions, hydrolysis of the phosphodiester bond is the only reaction observed.[24,25]

A linear free energy analysis was carried out of the cyclization reaction of a series of aryl esters (Figure 1A) under alkaline conditions; these reactions form a 2′, 3′ cyclic phosphate, a model for the first step of the ribonuclease reaction. The data were taken as evidence for a concerted mechanism with a modestly phosphorane-like transition state.

Compared to a β_{lg} of -0.59 for the aryl esters in Figure 1A[26] and to the very similar β_{lg} of -0.56 for those in Figure 1B,[27] the alkaline cyclization of a series of uridine-3′-alkyl phosphates yields a larger β_{lg} value of -1.28.[28] Isomerization to yield uridine-2′-alkyl phosphates is not observed, and the data were interpreted to indicate a concerted mechanism with a later transition state than is the case with the aryl esters. Subsequently, the change in Brønsted slope was reinterpreted[29] to favor a stepwise mechanism, in which the putative dianionic phosphorane intermediate is too short-lived to permit pseudorotation that would allow isomerization.

The ^{18}O kinetic isotope effects have been measured for the isomerization and cyclization reactions of uridine 3′-*m*-nitrobenzyl phosphate.[30] At pH 2.5, an inverse $^{18}k_{nonbridge}$ of 0.9904 on the cyclization is consistent with protonation of the phosphoryl group. The $^{18}k_{nonbridge}$ and $^{18}k_{bridge}$ KIEs of unity for the pH-independent isomerization at neutral pH support a stepwise mechanism with a monoanionic phosphorane intermediate. Isomerization is suppressed in anhydrous *tert*-butyl alcohol, implying that solvent water is required for mediation of the proton-transfer accompanying formation of the phosphorane intermediate. Finally, the $^{18}k_{bridge}$ of 1.0272 for the cyclization at pH 10.5 is consistent with a concerted reaction in which the leaving group departs with a substantial negative charge.[30] This pH-dependent range of mechanisms is consistent with the proposal that a dianionic phosphorane is viable as a transition state but not an intermediate.[31]

2.3. Uncatalyzed Reactions of Phosphotriesters

Triesters are the most reactive of the three classes of phosphate esters. Many of the mechanistic studies on triesters

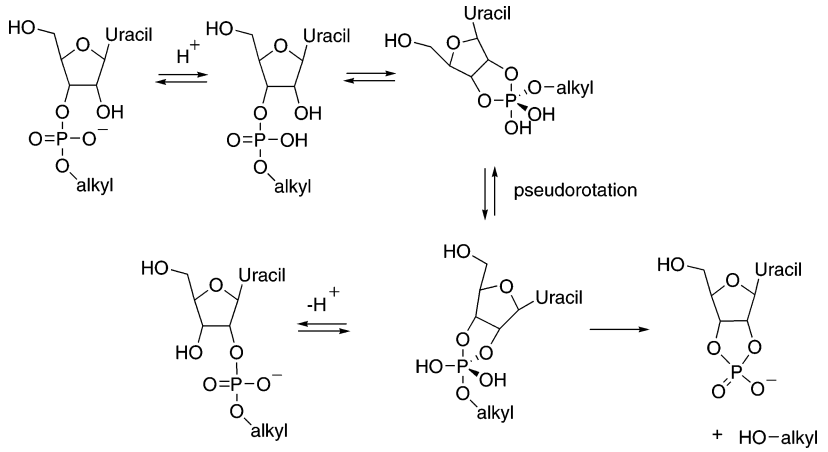

Figure 2. Acid-catalyzed isomerization and cyclization reactions of uridine-3′-alkyl phosphates.

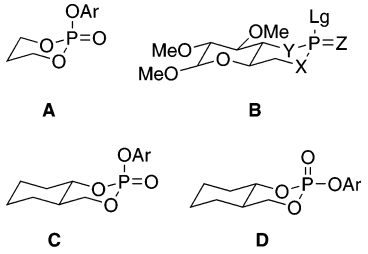

Figure 3. Some of the cyclic phosphotriesters used in mechanistic studies. In part B, the leaving group is denoted by Lg, Z is oxygen or sulfur, and X and Y are oxygen, sulfur, or *N*-methyl.

have been carried out on six-membered ring cyclic triesters (Figure 3).[20] These react at rates that are comparable to those of acyclic triesters, in contrast to five-membered cyclic triesters, which react at much faster rates.

In the reactions of aryl esters in Figure 3A, β_{lg} varies with the basicity of the nucleophile. This and other observations were interpreted to favor, though not require, a two-step mechanism involving a phosphorane intermediate.[20] Stereochemical studies of compounds of the type in Figure 3B, with various nucleophiles and leaving groups, show that retention or inversion of configuration depends not only on the nucleophile and leaving group but also on the solvent, counterions, and other heteroatoms in the six-membered ring.[32] The stereochemical and LFER data to date indicate the existence of a continuum of transition states that bridge concerted and stepwise mechanisms. A study of similar cyclic triesters (Figure 3C and D) that combined LFER studies, stereochemical analysis, and solvent isotope effects[33] also supports the existence of both concerted and stepwise reactions.

For reactions of acyclic triesters with aryl leaving groups, LFER data support concerted mechanisms,[34,35] with transition states that become tighter as leaving group basicity increases. The trend of an increasingly associative transition state as the leaving group basicity increases is also seen in KIEs. ^{18}O isotope effects in the nonbridging oxygen atom and in the leaving group have been measured for the alkaline hydrolysis of a series of acyclic diethyl phosphate triesters. A normal $^{18}k_{nonbridge}$ is observed, reflecting loss of double bond character to the P−O bond. The magnitude of $^{18}k_{nonbridge}$ increases from 1.0063 when the leaving group is *p*-nitrophenol ($pK_a = 7.41$), to 1.025 for *p*-carbamoylphenol ($pK_a = 8.6$), to 1.041 for choline iodide ($pK_a = 13.9$).[36] The calculated isotope effect for full loss of the π bond is 1.04.[36]

2.4. General Trends

The transition states for uncatalyzed phosphoryl transfer reactions across the three classes of phosphate esters generally follow a trend from loose transition states for monoesters, to a more synchronous reaction for diesters, to tight transition states for triesters. It has been noted that the phosphoryl group tends to bear a charge of about −1 across this continuum of transition states.[37] Thus, in monoester dianion reactions, the phosphoryl group sheds nearly a full negative charge to the leaving group in a loose transition state. Diesters have roughly synchronous transition states in which they maintain a uninegative charge, and neutral triesters react by tighter transition states, in which the phosphoryl group accepts negative charge.

2.5. Implications for Enzymatic Catalysis

Comparisons of the structures of enzymes that catalyze phosphoryl transfer show that the only similarity at the active site is the presence of positive charge, in the form either of a binuclear metal center and/or positively charged amino acid side chains. For phosphatases, which catalyze the hydrolysis of phosphomonoesters, it has been suggested that metal ions or cationic side chain sites might change the normally loose transition state into a more associative process by promoting electron withdrawal from the phosphorus atom, thus promoting nucleophilic attack. This would be analogous to the change that results from alkyl substitution; that is, transition states become more associative in the continuum from monoesters to triesters.

While there are many reports of the accelerating effect of metal ions on phosphoester hydrolysis,[38−41] few such reactions have been studied mechanistically to ascertain how the transition state for phosphoryl transfer is affected. In systems that have been investigated, the effect of metal ions has varied.

Magnesium and calcium ions do not alter the loose transition state for the hydrolysis of aryl phosphate monoesters[42] or of ATP.[43] The kinetics of reactions of *p*NPP coordinated monodentately to mononuclear Co(III) complexes are consistent with either a concerted process or an $A_N + D_N$ mechanism.[44,45] KIE experiments with these complexes[46] are more consistent with a concerted mechanism, with a transition state not significantly different from that for uncatalyzed hydrolysis. In these complexes, phosphoryl transfer is to an adjacent, coordinated nucleophile.

KIE data for *p*-nitrophenyl phosphate coordinated to a dinuclear Co(III) complex suggest that the reaction, while

still concerted, has a transition state with significantly more nucleophilic participation than the hydrolysis of uncomplexed *p*NPP.[47] Labeling studies using ^{18}O show that the complex uses a bridging oxide as a nucleophile.[48] For diesters coordinated to the same complex, LFER and KIE data are most consistent with a change to a two-step mechanism in which nucleophilic attack to form a coordinated phosphorane intermediate is followed by rate-limiting breakdown involving bond fission to the leaving group, in contrast to the concerted mechanism followed by the uncomplexed diester.[49−51]

Metal ions may also facilitate catalysis by coordination to a nucleophilic hydroxyl group, facilitating its deprotonation. For example, the aqueous hydrolysis of phosphorylated pyridines is catalyzed by $Mg(OH)^+$ in which[52] a rate enhancement of 10^4 was attributed to the greater nucleophilicity of the metal-bound hydroxide relative to that of water. A classic enzymatic example of this is alkaline phosphatase, in which the serine nucleophile is coordinated to a zinc ion.

A metal-coordinated hydroxide ion can also serve as a base. A dinuclear Zn complex has been shown to catalyze the cleavage of the diester 2-hydroxypropyl-4-nitrophenyl phosphate[53,54] and of RNA[55] by such a mechanism. The absence of a normal solvent deuterium kinetic isotope effect implicated a specific base mechanism in which a zinc-coordinated hydroxide deprotonates the nucleophilic hydroxyl group before the rate-determining step.

The idea that enzymatic phosphoryl transfer of a monoester should differ from uncatalyzed reactions arises, in part, because it is not obvious how an enzymatic active site can stabilize a loose, metaphosphate-like transition state. In such a transition state, the phosphoryl group loses electron density. To preferentially stabilize such a transition state over a ground state, the enzyme would have to destabilize negative charge on the nonbridge oxygen atoms. Yet, the phosphoryl group interacts with cationic groups in all known phosphatases. By contrast, in a transition state for an associative process, electron density on the phosphoryl group increases, giving an obvious means for preferential stabilization by positively charged amino acids or metal ions. However, the phosphoryl group inverts during phosphoryl transfer, and this geometric change has been shown in computational studies of protein tyrosine phosphatases to result, by means of stronger hydrogen-bonding interactions, in preferential enzymatic stabilization of a loose, metaphosphate-like transition state.[56,57]

3. Enzymatic Phosphate Transfer

3.1. Phosphatases

Phosphatases catalyze the hydrolysis of phosphate monoesters, the net transfer of the phosphoryl group to water, producing inorganic phosphate. Phosphatases that transfer the phosphoryl group directly to water possess a binuclear metal center, and the nucleophile is a metal-coordinated hydroxide. Other phosphatases follow a two-step overall kinetic mechanism, forming a phosphoenzyme intermediate that is subsequently hydrolyzed by nucleophilic addition of water. Some, but not all, of the latter enzymes use binuclear metal catalysis as well. Whether or not a phosphoenzyme intermediate lies on the reaction pathway is often tested using a chiral phosphate ester and examining the stereochemical outcome of the overall reaction. Inversion results from direct phosphoryl transfer to water. A ping-pong mechanism with a phosphoenzyme intermediate will yield a final product with retention of configuration.

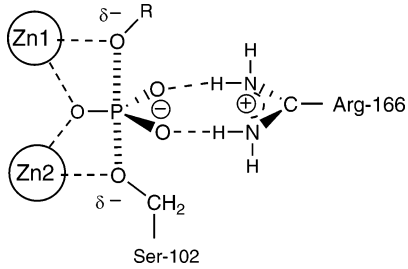

Figure 4. Model of the transition-state interactions in the reaction catalyzed by alkaline phosphatase, based on X-ray structures.[60,62,68]

The stereochemical outcome of phosphoryl transfer has been determined for a number of enzymes using chiral phosphomonoester substrates. In these experiments the phosphoryl group is made chiral either by using ^{16}O, ^{17}O, and ^{18}O or by using a phosphorothioate substrate with two isotopes of oxygen in the nonbridging positions. Summaries of stereochemical results are available in other reviews.[13,58,59]

3.1.1. Alkaline Phosphatase (AP)

Alkaline phosphatases catalyze the nonspecific hydrolysis of the dianion form of phosphomonoesters. These dimeric metallophosphatases are present in most, if not all, organisms. The *E. coli* AP has been extensively studied and may be the most studied two-metal ion catalyst.[60−64] The AP-catalyzed reaction proceeds via a phosphoserine intermediate (Ser-102 in *E. Coli* AP). The hydrolysis of the intermediate to produce inorganic phosphate competes with phosphoryl transfer to other acceptors, such as alcohols or nucleophilic buffers, when present. At pH values > 7, the rate-limiting step in the overall mechanism is release of inorganic phosphate; hydrolysis of the phosphoserine intermediate is rate-limiting at pH < 7.[65,66]

The active site of the AP from *E. coli* contains two Zn^{2+} ions and one Mg^{2+} ion.[60,62] The zinc ions play the most catalytically direct roles; the Mg^{2+} has been suggested to function as the provider of the general base that deprotonates the Ser nucleophile, in the form of a Mg-coordinated hydroxide.[67] All known alkaline phosphatases have this conserved three-metal ion center, as well as an arginine residue (Arg-166 in *E. coli* AP) that plays a role in binding and probably in transition state stabilization (Figure 4).

The substrate coordinates to both zinc ions and also hydrogen bonds to Arg-166. The leaving group oxygen atom is coordinated to Zn1 and is opposite from Ser-102, in position for an in-line attack. Coordination of the Ser-102 oxygen atom to Zn2 facilitates its deprotonation to form the more nucleophilic serine alkoxide; the pH dependence of k_{cat}/K_m with a series of alkyl phosphate substrates indicates that this hydroxyl group has a $pK_a \leq 5.5$ in the free enzyme.[64] Zn1 facilitates P−O bond fission by stabilization of negative charge on the leaving group oxygen atom in the transition state. The roles of the two zinc ions reverse in the subsequent hydrolysis of the intermediate. After ester group departure, water can coordinate to Zn1. The Zn1-hydroxide attacks the phosphoserine intermediate; its departure is stabilized by coordination with Zn2.

For aryl phosphate ester substrates, a nonchemical step such as binding or an associated conformational change is rate-limiting for k_{cat}/K_m. Evidence for this comes from the viscosity dependence of k_{cat}/K_m [69] and from the absence of

isotope effects on k_{cat}/K_m for the AP-catalyzed hydrolysis of *p*-nitrophenyl phosphate.[10] In later work, a β_{lg} value of -0.6 was measured for a small group of alkyl monoester substrates with leaving group pK_a values from 14.1 to 16.2, where phosphoserine formation is rate-limiting.[70]

Unlike the case with aryl phosphate ester substrates, the chemical step of phosphoryl transfer from substrate to enzyme is rate-limiting for k_{cat}/K_m with phosphorothioate substrates. Kinetic results using these substrates have been used to support an assertion that an associative triester-like mechanism, or at least a tight transition state, is operative for AP.[70] The basis for this argument is the so-called thio effect. In uncatalyzed hydrolysis reactions, which have loose transition states, phosphorothioate monoesters react faster than phosphates. In contrast, for triesters, the thio analogues react more slowly. Thus, the fact that AP hydrolyzes phosphorothioates more slowly than it does phosphates was taken to indicate a triester-like transition state.[70,71] Subsequently, it was shown that while wild-type *E. coli* AP catalyzes the hydrolysis of *p*-nitrophenyl phosphate ~70 times faster than *p*-nitrophenyl phosphorothioate, with the R166A mutant the difference drops to about 3. The magnitudes of β_{lg} for phosphorothioate substrates for the native AP (-0.77 ± 0.09) and the R166A mutant (-0.78 ± 0.06) are the same, indicating similar transition states despite the significantly different thio effects, suggesting that the thio effect is not a reliable reporter for the transition state.

AP is subject to product inhibition by inorganic phosphate, with a K_i of ~1 μM. Reactions run under traditional spectrophotometric assay conditions quickly generate concentrations of inorganic phosphate in excess of this. Recently, a sensitive ^{32}P-based assay that avoids this drawback was used for a series of alkyl phosphomonoester substrates.[64] The results confirmed that the chemical step of phosphoryl transfer is rate-limiting for k_{cat}/K_m for these substrates. The value of -0.85 ± 0.1[64] for β_{lg} is somewhat more negative than the earlier value of -0.6.[70] The large leaving group dependence indicates considerable P−O bond fission in the transition state.

The guanidinium group of Arg-166 plays a role in binding and in transition state stabilization but is not essential for catalysis. Replacement of this residue results in an increased K_m and reduced k_{cat}, but enzyme activity remains; R166A is reported to show 2.4% of native activity.[72,73] The k_{cat}/K_m for R166S is decreased ~10^4-fold relative to that for native AP.[74] The β_{lg} value of -0.66 ± 0.1 obtained with the R166S mutant is slightly less negative than the -0.85 ± 0.1 for native AP. If transition-state interactions with the arginine result in a tighter transition state, then native AP should exhibit a less negative β_{lg} than the mutant, the opposite of what is observed.[74] This and other LFER data indicate the AP-catalyzed reaction is characterized by extensive bond fission to the leaving group. Data directly assessing the degree of nucleophile bond formation in the transition state have not been obtained. An indirect assessment of nucleophilic participation comes from the small inverse secondary $^{18}k_{nonbridge}$ KIE on the hydrolysis of glucose-6-phosphate by AP, consistent with a loose transition state.[75]

Mammalian APs have low sequence identity with the *E. coli* enzyme (25−30%) but the active site residues are conserved and the catalytic mechanism is the same. Most of the work on mammalian APs has been done on the human placental (PLAP) and the tissue nonspecific (TNAP) enzymes.[76−78] Structural data reveal the presence of a variable surface region that is absent in nonmammalian APs. Structural studies of PLAP have shown the presence of a peripheral binding pocket 28 Å from the catalytic site. This pocket binds the uncompetitive inhibitor L-phenylalanine, as well as 5′-AMP and *p*-nitrophenyl-phosphonate.[78] This pocket varies in size and electrostatic nature among the mammalian APs and may serve a regulatory role.[78]

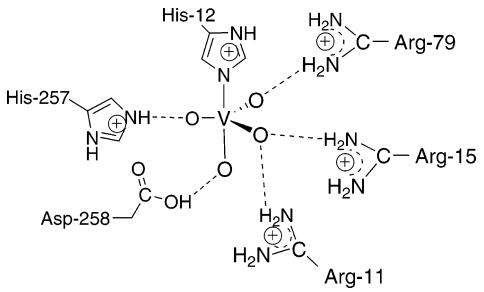

Figure 5. Interactions with active site residues found in the X-ray structure of rat acid phosphatase with bound vanadate.[82]

3.1.2. Acid Phosphatase

As is the case for alkaline phosphatases, the acid phosphatases are nonspecific and catalyze the hydrolysis of phosphomonoesters via a phosphoenzyme intermediate. The acid phosphatases are characterized by a conserved nucleophilic histidine, the absence of metal ion cofactors, and acidic pH optima. The His residue is part of a characteristic amino acid sequence RHGXRXP, and the name histidine phosphatases has been proposed for these enzymes.[79] Though less well studied than the alkaline phosphatase family, mechanistic information has been obtained from X-ray structures,[80−82] mutagenesis studies, and LFER analyses.[83] The overall catalytic reaction proceeds via formation of a phosphohistidine intermediate, giving a net retention of configuration.[84]

An X-ray structure of the rat enzyme with bound vanadate (Figure 5) identifies the likely roles of the conserved His and Asp residues, which are His-257 and Asp-258 in the rat enzyme. In the *E. coli* acid phosphatase, His-303 and Asp-304 correspond to these residues. LFER experiments show that the sensitivity of V_{max} to leaving group pK_a is small for the native enzyme (-0.08), but the D304A mutant gives a β_{lg} of -0.51, consistent with the Asp residue serving as a general acid to protonate the leaving group in the first phosphoryl transfer step, form substrate to enzyme.[83] A logical assumption is that the carboxylate form of the Asp residue then acts as a general base to deprotonate a nucleophilic water molecule in the second step, hydrolysis of the phosphohistidine intermediate.

3.1.3. Purple Acid Phosphatases

The purple acid phosphatases (PAPs) catalyze the hydrolysis of phosphomonoesters via direct attack by a metal-coordinated hydroxide nucleophile. The active sites of PAPs contain seven invariant amino acids coordinating a binuclear metal center. The characteristic purple color of these enzymes arises from a charge-transfer absorption from a tyrosinate ligand to a conserved Fe(III) in the metal center. The second metal ion is always divalent but varies with the source of the enzyme. Mammalian PAPs have Fe(III)−Fe(II) centers, while plant PAPs most typically have Fe(III)−Zn(II) centers. Recently, a PAP was isolated from sweet potato that contains an Fe(III)−Mn(II) center, the first of its kind in any enzyme.[85,86] This novel PAP also differs from others by its

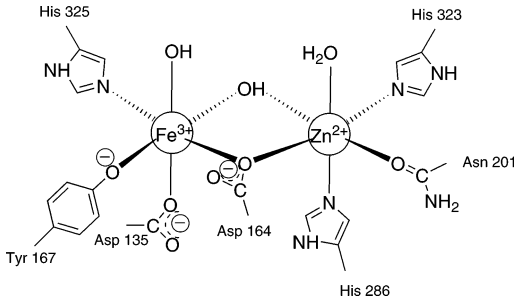

Figure 6. Active site of kidney bean purple acid phosphatase.[89]

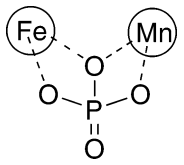

Figure 7. Tripodal mode of coordination of inorganic phosphate to the PAP from sweet potato, unique among the PAP−phosphate structures known to date.

greater catalytic efficiency toward both activated and unactivated substrates,[86] and in its strict requirement for manganese.[85]

The Brønsted β_{lg} values on k_{cat} and k_{cat}/K_m, respectively, have been measured for the PAPs from pig (−0.3 and −0.4), red kidney bean (−0.5 and −0.6), and sweet potato (0.1 and 0.0).[87] For pig PAP, the chemical step was proposed to be rate-limiting on the basis of the large negative β_{lg} [88] The red kidney enzyme is assumed to follow a similar mechanism. The negligible β_{lg} on k_{cat} and k_{cat}/K_m may indicate that the sweet potato PAP utilizes an associative, triester-like transition state or that this PAP has a more effective means for neutralizing the leaving group (for example, using general acid catalysis) as P−O bond fission occurs. The alternative explanation that a nonchemical step has become rate-limiting is unlikely, because of the consistently higher kinetic parameters of the sweet potato PAP relative to the other two forms.[87]

The X-ray structures have been reported for kidney bean PAP,[89] the PAP from rat bone,[90] pig PAP,[91] and the novel sweet potato PAP.[87] Despite little sequence similarity, the enzymes share very similar catalytic sites with seven invariant amino acid ligands to the metal center (Figure 6).

The reaction occurs with inversion of configuration at phosphorus, which supports direct phosphoryl transfer to water.[92] It is generally believed that catalysis begins with the rapid coordination of substrate to the divalent metal ion.[93,94] Subsequent details of the catalytic mechanism, including the identity of the nucleophile, remain to be elucidated. Mechanistic possibilities that have been considered include nucleophilic attack by a bridging hydroxide or for an Fe^{3+}-bound hydroxide to serve as either as the nucleophile or as a general base for nucleophilic attack by water.[38,95,96] Indirect support for the bridging oxygen atom as the nucleophile comes from spectroscopic evidence for the structure of the complex of inorganic phosphate with uteroferrin, a member of the PAP family.[97] Also, the X-ray structure of the sweet potato PAP has phosphate bound in an unusual tripodal fashion (Figure 7), a geometry which suggests the oxygen atom bridging the two metal ions is nucleophile. However, because phosphate is both a product of the reaction and a competitive inhibitor, caution is called for in drawing mechanistic conclusions from such data.

The recombinant human PAP exhibits the bell-shaped pH−rate profile typical of the PAP family. The optimum pH is 5.5, $pK_{a1} = 4.6$, and $pK_{a2} = 6.7$.[98] Spectral changes found at pH < 5.5 indicate that pK_{a1} is due to a metal-bound moiety that is deprotonated in the active enzyme. A plausible candidate for such a group is a metal-bound water, which upon deprotonation could act as the nucleophile in phosphate ester hydrolysis. NMR[99] revealed no differences in the hyperfine-shifted signals between pH 5.5 and pH 7.1, indicating that pK_{a2} does not involve a metal ligand. This ionization may arise from a conserved His residues near the dinuclear site, proposed to serve as a general acid to protonate the leaving group.[100] However, convincing experimental verification of the role of the conserved His residues is lacking.

The identity of the divalent metal ion affects pK_{a1}, consistent with the proposal that the nucleophilic hydroxide bridges the two metals.[101] The EPR spectrum of the Fe^{3+}−Zn^{2+} form of the bovine spleen PAP does not show significant changes upon addition of phosphate at pH 6.5, the optimum pH for k_{cat}, suggesting that phosphate binds only to the spectroscopically silent Zn^{2+}. If phosphate binds in the same manner as a phosphomonoester substrate, this observation suggests that substrate coordinates only to the divalent metal ion in the catalytically active complex.[96]

The role of the trivalent metal ion has been probed by examining the kinetic behavior of the bovine spleen PAP with several trivalent metal ions, including aluminum.[102] The kinetics and pH−rate dependency of the Al^{3+}−Zn^{2+} enzyme were similar to those of the Fe^{3+}−Zn^{2+} and the Ga^{3+}−Zn^{2+} forms. These results are unexpected, in light of the slow ligand exchange properties of trivalent metal ions. This would be disadvantageous in an enzymatic system where rapid product release is desirable. The results suggest that either the enzyme alters the properties of the trivalent metal ion to facilitate ligand exchange or that the catalytic mechanism does not involve phosphate dissociation from the trivalent metal ion. This would favor the mechanistic proposal in which the Fe-bound hydroxide acts as a general base to deprotonate an uncoordinated nucleophilic water molecule. The mode of substrate binding and the identity of the nucleophilic oxygen remain to be conclusively identified in the PAP family.

3.1.4. Phospho-protein Phosphatases

Phospho-protein phosphatases are generally grouped into the protein tyrosine phosphatases (PTPases) and the phosphoserine/phosphothreonine-specific phosphatases based on substrate specificity and genetic homology.[103] As their name implies, the Ser/Thr protein phosphatases dephosphorylate phosphoserine and phosphothreonine residues. These phosphatases have binuclear metal centers similar to those of the purple acid phosphatases, and they also transfer the phosphoryl group directly to water. They are distinguished from the protein tyrosine phosphatases and the dual-specific protein phosphatases, which do not contain metal ions and form a phosphoenzyme intermediate.

Serine/Threonine Protein Phosphatases. Like the PAPs, these enzymes are metallophosphatases that hydrolyze phosphomonoesters by the attack of a metal-coordinated hydroxide. The Ser/Thr phosphatases fall into two structural families, designated PPP and PPM, on the basis of their catalytic domains. The PPP family members share a conserved phosphoesterase motif, $DXH(X)_{25}GDXXDR(X)_{25}GNHE$. This

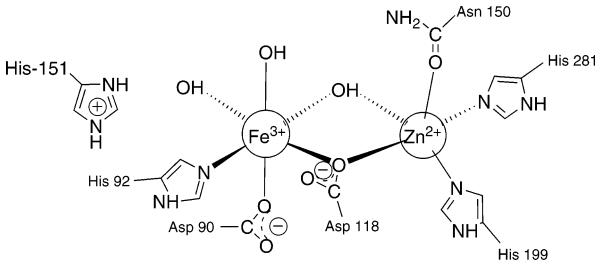

Figure 8. Active site of human calcineurin (PP2B).[106]

family includes the phosphatases known as PP1, PP2A, PP2B (also called calcineurin), and lambda phosphatase (λPP). The identities of the metal ions in PP1 and PP2A *in vivo* are not certain; some evidence indicates that calcineurin has a binuclear $Fe^{3+}-Zn^{2+}$ center, though its best activators *in vitro* are Mn^{2+} and Ni^{2+}.[104] The PPM family is typified by the PP2C subfamily, a group of Mn^{2+}- or Mg^{2+}-dependent Ser/Thr phosphatases in which the metal ligands consist almost entirely of carboxylates,[105] in contrast to the neutral histidines and amide carbonyls that comprise the ligands in the PPP phosphatases. PP2C homologues have been identified in bacteria, plants, yeast, and mammals, and they have a conserved role in negatively regulating stress responses.

X-ray structures have been published of the catalytic subunit of rabbit muscle PP-1 complexed with microcystin[107] and of human PP-1 with tungstate bound.[108] The structures are also available of free human calcineurin (Figure 8) and of human and bovine brain calcineurin complexed with the immunosuppressant complex FKBP12/FK506;[106,109] calcineurin is involved in the regulation of T-cell production and is the target of a number of immunosuppressant drugs.

Kinetic studies with calcineurin yielded a modest solvent isotope effect of 1.35 and also a proton inventory and fractionation factor data that were most consistent with a mechanism involving a single proton transfer from a water molecule coordinated to a metal ion.[110] No transphosphorylation products were found in the presence of alternate nucleophiles, consistent with direct phosphoryl transfer to a metal-coordinated water.[111] No calcineurin-catalyzed oxygen exchange of ^{18}O-labeled water with phosphate could be detected.[112] In a study using *p*NPP as the substrate, product inhibition studies found that both phosphate and *p*-nitrophenol are competitive inhibitors.[110] This pattern is consistent with a random uni−bi (one substrate, two products) mechanism. Taken together, the data indicate direct phosphoryl transfer to a metal-bound water molecule without a phosphoenzyme intermediate.

Because of the similarities of the binuclear metal centers, the same mechanistic possibilities described for purple acid phosphatases exist for the Ser/Thr phosphatases, and as is the case for the PAPs, X-ray structures show a His residue near the active site. A mechanism for PP1 was proposed[108] based on the X-ray structure obtained with bound tungstate, although it must be noted that these crystals were grown at low pH and may not reflect the active structure. The bridging hydroxide was postulated as the most likely nucleophile, and an essentially identical mechanism, with leaving group protonation by His-151, has been suggested for calcineurin.[109]

The catalytic role of the conserved His residue (His-151 in calcineurin, His-76 in λ-protein phosphatase) has been examined using site-directed mutagenesis.[113,114] In both mutants, there was a significant reduction in k_{cat} but only small effects were observed on K_m.[113,114] If the His residue functions as a general acid, its loss should result in a greater dependency of the rate on leaving group pK_a, but this was not observed. The calcineurin mutant showed only a very small change in rate difference between *p*NPP and a phosphoserine substrate despite the large ΔpK_a of the leaving group. With λ-PP, the H76N mutation results in the same 500−600-fold decrease in k_{cat} with both *p*NPP and phenyl phosphate substrates.

Kinetic isotope effects have been measured for both the calcineurin and the λPP-catalyzed reactions.[115−117] For the calcineurin reaction with *p*NPP, phosphoryl transfer is only partially rate-limiting at the pH optimum, becoming more so at pH 8. For λ-PP, phosphoryl transfer is fully rate-limiting, and the measured KIEs are the intrinsic ones. With both enzymes, the KIEs indicate that the dianion is the substrate, which is also the major form present at the pH optima. The $^{18}(V/K)_{nonbridge}$ data for calcineurin and λPP (0.9942 ± 0.0007 and 0.9976 ± 0.0003, respectively) are most consistent with a loose transition state, similar to that seen for the uncatalyzed hydrolysis. The KIEs in the leaving group indicate that partial charge neutralization occurs in the transition state. In the reaction catalyzed by the H76N mutant of λ-PP, the leaving group bears more negative charge, consistent with a role of this residue as a general acid. However, the KIE data indicate only about half a negative charge resides in the leaving group.[21] Thus, the role of the conserved His residue remains to be convincingly demonstrated. It has also been suggested that this residue may serve as a general base to deprotonate the nucleophilic metal-coordinated water.[104]

PP2C is the best characterized member of the PPM family. The X-ray structure of PP2C with bound phosphate[105] shows that the phosphate is not directly coordinated to the metal center; however, it must be noted that this structure comes from crystals grown at pH 5, where the enzyme has very little activity,[118] and thus, it may not represent a catalytically active structure.

The identity of the metal ions of the PPM family *in vivo* is uncertain, but PP2C binds its catalytic metal ions loosely. Typically assayed with Mn^{2+} supplied in the buffer, saturation kinetics show that 10 mM [Mn^{2+}] is needed to achieve full activation.[118] Values of K_{metal} for several divalent ions range from 1.42 mM for Mn^{2+} to 20.6 mM for Mg^{2+}.[118] The highest activity is observed with Fe^{2+}, which results in a 1000-fold higher k_{cat} and V/K than Mg^{2+}. A pH−rate profile of k_{cat}/K_m of the PP2C reaction using Mn^{2+} and Mg^{2+} with *p*NPP as substrate identified two critical ionizations required for activity.[118] A moiety with a p$K_a \sim 7.5$ must be unprotonated, while one with a p$K_a = 9$ must be protonated for catalysis. It was proposed that the moiety with p$K_a =$ 7.5 might be the water molecule that bridges the two metal atoms in the active site. The identity of the other ionizable group is not yet clear.

The catalytic role of His-62 was examined using site-directed mutants.[119] With the substrate *p*NPP, H62Q yielded a 20-fold lower k_{cat} than wild-type PP2C. The pH dependence of k_{cat} was still bell-shaped; however, the k_{cat}/K_m profile showed loss of the basic limb resulting from the group whose pK_a is 9 in the wild-type enzyme. A Brønsted analysis with wild-type PP2C revealed that at pH 7.0 the phosphoryl transfer step is rate-limiting only for substrates with leaving group pK_a values >7.[118] Under such conditions, a β_{lg} of −0.32 was obtained, similar to that exhibited by calcineurin. The H62Q mutant gave a greater dependency ($\beta_{lg} = -0.84$)

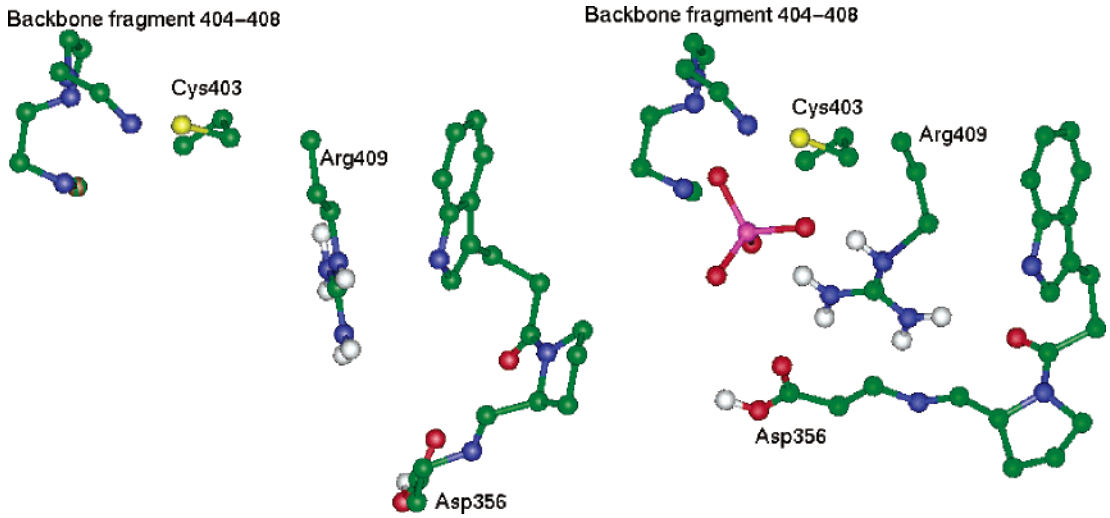

Figure 9. Active site of free *Yersinia* PTPase (left) and when sulfate is bound (right). The Arg side chain rotates to form two hydrogen bonds to the oxyanion, resulting in formation of a new hydrogen bond with the carbonyl oxygen atom of Trp-354. Associated movements of a protein loop bearing Asp-356 bring it into position to function as a general acid during catalysis. The structures shown are from published X-ray structures[127,143] with computer-generated hydrogen atoms added to the Arg and Asp residues. Reproduced with permission from *Biochemistry* **2000**, *39*, 46−54. Copyright 2000 American Chemical Society.

on leaving group pK_a, consistent with a role for His-62 as a general acid.[119]

Protein Tyrosine Phosphatases (PTPases). This group of phosphoprotein phosphatases catalyze the hydrolysis of phosphomonoesters using a conserved cysteine residue to form a phosphoenzyme intermediate,[120−124] and they do not use metal ions. The crucial Cys residue is found in the signature motif (H/V)C(X)$_5$R(S/T). This motif is also found in the low-molecular-weight PTPases and in the dual specific phosphatases, so-called because they dephosphorylate phosphotyrosine as well as phospho Ser/Thr residues.[103,125] These three groups of phosphatases have little sequence similarity other than the signature motif and the placement of the essential cysteine and arginine residues in the active site. The X-ray structures of a number of the enzymes in this group have been reported[126−132] and show that these enzymes share highly similar active sites.

These enzymes have been the focus of intensive mechanistic study that reveals the functions of conserved Cys, Asp, and Arg residues. Mechanistic studies on the *Yersinia* PTP,[133,134] the mammalian PTP1,[135] the low-molecular-weight PTPase Stp1 from yeast,[136] and the human VHR[137,138] have revealed the consistent requirement for the nucleophilic cysteine to be deprotonated for catalysis, and a conserved Asp serves as a general acid to protonate the leaving group in the first step and as a general base to promote attack by water in the subsequent hydrolysis of the phosphocysteine intermediate.[139] The dianion of the phosphomonoester is the substrate.

The active-site cysteine has a very low pK_a: ∼4.7 in the *Yersinia* enzyme[140] and 5.5 in VHR.[137] The low pK_a ensures that it is in the thiolate form under physiological conditions. The interactions responsible for this substantial reduction from the typical thiol pK_a of ∼9 have been most thoroughly examined in the *Yersinia* PTPase. Hydrogen bonding with His-402 has been proposed to decrease the pK_a of Cys-403 by 2.7 pH units,[140] while a hydrogen bond with Thr-410 is responsible for another reduction of about 0.6 pH units.[141] Mutation of the His-402 residue to asparagine or alanine increases the pK_a of Cys-403 to 5.99 or 7.35, respectively.[140] This His residue does not directly hydrogen bond to the Cys residue; thus, the effect is most likely due to the disruption of a hydrogen-bond network within the active site.

The pH−rate profiles for *V*/*K* of the PTPases are invariably bell-shaped, The slope on the acid side of the profile goes to a slope of 2 when conditions allow data to be obtained at sufficiently low pH to permit a full analysis. One of the pK_a values is assigned to pK_{a2} of the substrate and has been shown to vary with the substrate. The enzymatic pK_{a1} is assigned to the conserved Cys nucleophile, and the second pK_a to the conserved Asp. This residue resides on a flexible loop that closes down on the active site cleft upon oxyanion binding. In the *Yersinia* PTP, X-ray structures of the free enzyme compared with those containing bound oxyanions show that the α carbon of the general acid Asp-356 moves by about 6 Å toward the active site upon binding of tungstate or sulfate, into a position ideally suited to protonate the leaving group in the catalytic reaction[127] (Figure 9). Oxyanion binding also causes the guanidinium group of the invariant arginine (Arg-409 in the *Yersinia* enzyme) to shift by about 2 Å to form two hydrogen bonds with the oxygen atoms. The same loop movement is observed in all of the X-ray structures of PTPases with bound oxyanions, as well as in the structure of a catalytically inactive mutant of the yeast low-molecular-weight PTPase complexed with the substrate *p*NPP.[132] Mutagenesis of Arg 409 interferes with the function of general acid catalysis.[142]

Kinetic isotope effects and LFER studies have been used to examine the transition-state structure of the first phosphoryl transfer step, formation of the phosphocysteine intermediate, for *Yersinia* PTP and PTP1,[144] VHR[145] and Stp1,[146] and mutants. For each enzyme, small, inverse $^{18}(V/K)_{nonbridge}$ magnitudes suggest that the transition state is loose, resembling that in uncatalyzed phosphoryl transfer. Near-unity values for $^{15}(V/K)$ observed with all of the PTPases examined, except Stp1, mean that the leaving group is neutral in the transition state, implying that proton transfer to the leaving group is synchronous with P−O bond fission; in the Stp1 case, a partial negative charge remains on the leaving group, suggesting that proton transfer may slightly lag behind P−O bond fission. The magnitudes of the primary $^{18}(V/K)_{bridge}$ KIEs for all of the PTPases are those expected

of a late transition state, in which both P−O bond fission and proton transfer are well advanced.[147]

In all members of this phosphatase family that have been examined, mutation of the general acid Asp to Asn results in the same changes: the magnitudes of both $^{18}(V/K)_{bridge}$ and $^{15}(V/K)$ are significantly higher, indicating extensive bond cleavage to the leaving group, which now bears essentially a full negative charge. Also, $^{18}(V/K)_{nonbridge}$ becomes normal, indicating that the transition state has somewhat more nucleophilic participation, although less than that in reactions of phosphodiesters or triesters.

KIEs and pH−rate dependencies for V/K were measured for the reactions of the dual-specificity phosphatase VHR[148] and the *Yersinia* PTP[149] with the alkyl phosphate *m*-nitrobenzyl phosphate (*m*NBP). The pH−rate profiles are shifted in a manner consistent with the change in pK_{a2} of the substrate, which is 6.2 for *m*NBP versus 5 for *p*NPP, consistent with the dianion form of the substrate as the catalytically active species. KIE data suggested that protonation is more advanced in the reactions with the alkyl phosphomonoester substrate than in those with *p*NPP. This is logical considering the relative basicities of the two substrates; some degree of P−O bond fission must occur to make the proton transfer from Asp thermodynamically favorable. For the more basic *m*-nitrobenzyl alcohol leaving group, this will occur sooner (with a smaller degree of bond fission) than when the leaving group is *p*-nitrophenol. A similar difference in timing of protonation of the leaving group with P−O bond fission was found in the uncatalyzed hydrolysis of the monoanions of *m*NBP and *p*NPP.[17] This leads to the conclusion that the transition states in the PTPase reactions are not significantly different from those in the uncatalyzed hydrolyses of their substrates, with the notable difference that, in the enzymatic reactions, protonation of the leaving group comes from the conserved Asp, while, in the uncatalyzed reaction of the monoanion, the proton comes from the phosphoryl group.

The fact that the phosphocysteine intermediate can phosphorylate alcohols as well as water was used to examine the dephosphorylation of the intermediate in Stp1 using linear free energy relationships.[150] The β_{nuc} value was found to be +0.14, a value indicative of little nucleophilic participation and, thus, a loose transition state.

A number of groups have used computational methods to address the PTPase mechanism. Some of these results led to the proposal of an alternative mechanism, in which the reactive form of the substrate is the monoanion.[151−154] However, computational examinations by others have yielded conclusions in complete agreement with the experimental data.[56,57,155] One such study,[56] using the bovine low-molecular-weight PTPase, found through a structural analysis that the enzyme stabilizes the loose, metaphosphate-like transition state via enhanced hydrogen bonding during the inversion of the phosphoryl group.

3.2. Regulatory Enzymes

3.2.1. His-Asp Phosphorelay Proteins

The reversible phosphorylation of proteins provides the chemical basis for a major means of signal transduction in both prokaryotes and eukaryotes. In eukaryotic cells, proteins are primarily phosphorylated on serine, threonine, or tyrosine residues. In prokaryotes, a so-called two-component mechanism is used, in which a phosphoryl group is transferred from ATP first to histidine and then to an aspartate.[156] It was once thought that the specificities of protein kinases for serine/threonine/tyrosine versus histidine/aspartate were defining characteristics of eukaryotes and prokaryotes, but it is now known that the dichotomy is not absolute.

The two-component signal transduction system is sometimes called the His-Asp phosphorelay. This process occurs in two steps and consists of the overall transfer of a phosphoryl group from a histidine kinase sensor protein to the Asp on a response regulator protein. The sensor histidine kinases contain an ATP binding site, a dimerization domain, and the histidine phosphorylation site; this protein autophosphorylates its histidine residue. The functional forms of sensor histidine kinases are dimers, and mechanistic analyses have shown that each unit in the dimer phosphorylates the histidine residue in the other partner.[157] Recently, the structure of the cytoplasmic portion of the histidine kinase protein from *Thermotoga maritime* in complex with ADBβN has been reported.[158]

Subsequently, the phosphoryl group is transferred from the histidine of the sensor protein to a conserved aspartate of a second protein, the response regulator. The response regulators are sometimes referred to as aspartate kinases but are more properly described as phosphotransferases. Although the simplest of histidine/aspartate phosphorylation systems consist of only the two-protein system described, some systems consist of more than these two proteins.

3.2.2. Ras

The GTP binding proteins, or G proteins, act as molecular switches by catalyzing hydrolysis of GTP to form GDP, and, in so doing, they regulate a number of cellular processes.[159,160] For the Ras family of G proteins, stereochemical analysis has shown that the reaction proceeds with inversion of configuration.[161] The GTPase activity of Ras alone is low, with a half-life of 25 min at 37 °C;[162] this rate is about 100-fold slower than that of typical G proteins. GTPase activating proteins, called GAPs, substantially accelerate the hydrolysis; in the presence of GAP, the GTPase reaction of Ras is 10^5-fold faster. The mechanism of Ras-catalyzed GTP hydrolysis has been the subject of interest, centering on the nature of the transition state and the identity of the catalytic base that activates a water molecule for nucleophilic attack, or whether such a base is even necessary in the transition state.

An X-ray structure of the Ras protein complexed with the GTP analogue GppNp led to the proposal that glutamine-61 acts as a general base to deprotonate the nucleophilic water molecule.[163] It was suggested that Glu-63 assisted in properly orienting Gln-61 and/or increasing its basicity.[164,165]

An amide is an unlikely base, and it was subsequently proposed that the γ-phosphoryl group deprotonates the nucleophilic water molecule. Others have questioned whether a base is needed at all in the transition state. To assess the hypothesis that the γ-phosphoryl group serves as a base, an unusual type of linear free energy relationship was constructed. The γ-phosphoryl group's pK_a in the Michaelis complex was varied by means of the mutation of selected active site residues.[166−168] The pK_a values were measured by titration using ^{31}P NMR or, indirectly, by analysis of the pH−rate profile of the GTP hydrolysis reaction. A correlation between k_{cat} and the γ-phosphoryl group pK_a was found, giving a Brønsted slope of 2.1; in the presence of the activating protein GAP, the slope increased to 4.9.[166,168] These results are consistent, though not exclusively so, with the

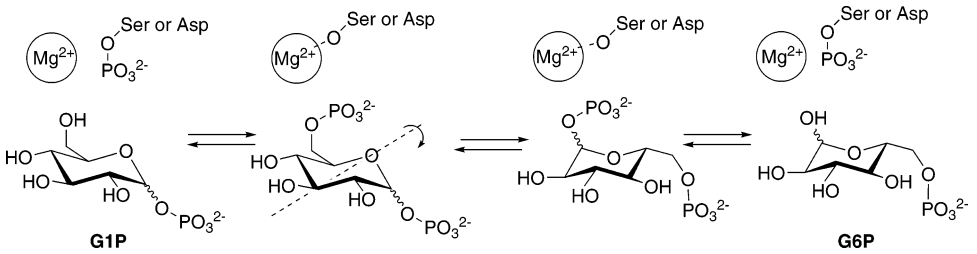

Figure 10. Representations of the four enzyme−substrate complexes on the reaction pathway of phosphoglucomutases (PGMs): G1P = glucose-1-phosphate; G6P = glucose-6-phosphate.

notion that the γ-phosphoryl group serves as a base. It has alternatively been suggested that the correlations result from a strengthening of cooperative hydrogen bonding interactions between cationic residues and the γ-phosphoryl group as its basicity increases, which further stabilize the transition state.[3] The small, inverse solvent isotope effect ($^{D}k = 0.7$)[166] implies that deprotonation occurs before or after the rate-limiting step, since a proton in flight in the transition state would be expected to result in a significant normal solvent isotope effect.

Model studies of the uncatalyzed reactions of GTP and related molecules led to a challenge to the assumption that a base is necessary in the transition state.[169] Transfer of the γ-phosphoryl group from ATP, GTP, and pyrophosphate to a series of alcohols gives a β_{nuc} of 0.07, indicative of minimal nucleophilic participation in the transition state.[43] Phosphoryl transfer to water from a series of phosphoanhydrides give a Brønsted β_{lg} of -1.1, indicating considerable bond fission to the leaving group.[43] Together, these results indicate a loose, metaphosphate-like transition state for the model reactions.

In such a transition state, the greatest change in charge distribution is an increase in negative charge density on the $\beta-\gamma$ bridging oxygen atom. In the structure of Ras with bound GppNp, the $\beta-\gamma$ bridge oxygen is exposed at the bottom of a crevice, an observation that led to the prediction[169] that the GAP proteins might stimulate GTPase activity by donation of a hydrogen bond from an Arg residue to the $\beta-\gamma$ bridge oxygen. This was confirmed by the X-ray structure of Ras-GDP-AlF_3 in a complex with GAP-334.[170] Using a fluorescent GTP substrate analogue, it was shown that mutation of this so-called arginine finger residue to lysine reduces k_{cat} by 3 orders of magnitude.[162]

In a time-resolved FTIR study,[171] the Ras reaction was initiated by the photolysis of a bound caged form of GTP. In the FTIR of unbound GTP, the coupling between the vibrations of the three phosphoryl groups is no longer present in bound GTP; instead the individual vibrations of the α-, β-, and γ-phosphate groups are observed, suggesting that binding to Ras forces GTP into a restrained conformation. The binding also causes shifts in the frequencies of the vibrations of all three phosphoryl groups that indicated a shift of negative charge from the γ- to the β-phosphoryl group, a charge distribution that is in the direction of the GDP product.

The same technique was applied to the Ras(GAP)-catalyzed reaction, where it was found that phosphate release is the overall rate-limiting step.[172] The complete GTPase reaction pathway was observed, with a time resolution of milliseconds. The shift of negative charge from the γ- to the β-phosphate found in the Ras study was shown to be enhanced by GAP binding. Evidence for an intermediate species was observed in the reaction pathway, after the bond between β- and γ-phosphate is cleaved. Surprisingly, the putative intermediate shows bands expected of a phosphorylated enzyme intermediate. However, the existence of such an intermediate is at odds with the stereochemical outcome of inversion.

3.3. Phosphoglucomutases

The α- and β-phosphoglucomutases (PGMs) catalyze the interconversion of D-glucose-1-phosphate (G1P) and glucose-6-phosphate (G6P) (Figure 10). These enzymes are named for the anomer of G1P on which they act; thus, α-PGM acts on the α-C(1) anomer of G1P, and β-PGM on the β-C(1) anomer. Both employ Mg^{2+} and α- or β-glucose 1,6-diphosphate (G1,6-diP) as cofactors.

Stereochemical analysis of the products using [^{16}O, ^{17}O, ^{18}O]phosphate ester methods shows that the PGM reaction proceeds with overall retention of configuration.[173] Both α- and β-PGM form a phosphoenzyme intermediate, by the reaction of an active-site nucleophile, which is a Ser in α-PGM and an Asp in β-PGM. The phosphorylated PGM binds either G1P or G6P, and transfers the phosphoryl group to the C(6)OH or C(1)OH, respectively (Figure 10).

Reorientation of glucose-1,6-diphosphate within the active site occurs at a faster rate than dissociation with PGM from rabbit muscle and from rat.[174] In contrast, dissociation is more rapid with the PGMs from *Bacillus cereus* and *Micrococcus lysodekticus*, and as a result, the latter enzymes have an absolute requirement for glucose-1,6-diphosphate as a cofactor to rephosphorylate the enzyme.

An X-ray structure of the β-phosphoglucomutase from *Lactococcus lactis* reveals two neighboring Asp residues, Asp-8 and Asp-10. It was proposed that one might function as the phosphoryl acceptor and the other as the donor, thus eliminating the need for dissociation and rebinding or for reorientation of the glucose 1,6-diphosphate intermediate within the active site.[175]

On the basis of spectroscopic studies using the transition-state analogue glucose 1-phosphate 6-vanadate, the transition state for phosphoryl transfer was concluded to be S_N2-like in character.[176] Recently, a 1.2 Å resolution X-ray structure of β-PGM from *Lactococcus lactis* obtained at cryogenic temperature revealed an unusual species (Figure 11)[7] that has been the subject of some controversy.[177−181] The original publication describes it as a pentacovalent phosphorane intermediate,[7] but the unusually long apical P−O bond lengths of 2.0−2.1 Å in the X-ray structure, using Pauling's rule,[182] correspond to very low bond orders of between 0.2 and 0.3. Such a structure might be better described as a stabilized metaphosphate.

In a computational study that used the X-ray structure as a starting point, a concerted reaction pathway with no phosphorane intermediate was favored, with proton transfer from the hydroxyl group of glucose to Asp10 in the same step as phosphoryl transfer.[180] Further experimental data are

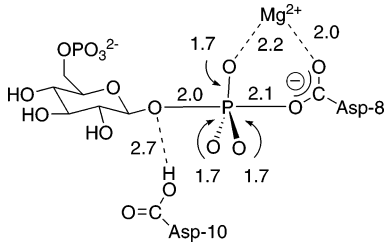

Figure 11. Elongated pentacoordinate phosphorus species observed in the X-ray structure of β-PGM from *Lactococcus lactis*. Distances shown are in angstroms. In addition to the interactions shown, the PO_3 moiety also makes hydrogen bonding contacts with two backbone amide N−H groups and the hydroxyl of Ser-114.[7]

needed to determine whether Asp-10 functions as an acid−base catalyst, as suggested by the calculations,[180] or as an alternate phosphoryl donor, as proposed on structural grounds.[175]

3.4. Kinases

Kinases are enzymes that transfer the γ-PO_3 unit of MgATP to an acceptor. The reactions with alcohols have favorable equilibrium constants and thus permit phosphorylation of sugars and other small molecules so that they can more easily be bound and oriented properly in the active sites of metabolic enzymes. The phosphorylation of serine, threonine, and tyrosine hydroxyls on proteins is catalyzed by protein kinases, which are a major part of the genome of any organism.

In contrast to the phosphorylation of hydroxyl groups, the reaction of MgATP with an amino or guanidinium nitrogen is an unfavorable reaction. Thus, the phosphorylation of creatine to creatine phosphate which takes place in muscle has an equilibrium constant of 0.012 at pH 7.4 with 1 mM free Mg^{2+}.[183] But the high MgATP/MgADP ratio in resting muscle allows build up of a creatine phosphate/creatine ratio of ~2, so that when MgADP is generated by muscle movement, it can be rapidly regenerated to MgATP by the action of creatine kinase.

The phosphorylation of carboxylic acids is even more unfavorable, and these kinases catalyze the reverse reactions in cells. Thus, 3-phosphoglycerate kinase regenerates MgATP from MgADP and 1,3-bis-phosphoglycerate during glycolysis, and acetate kinase uses acetyl phosphate to do the same. Pyruvate kinase, which catalyzes reaction of phosphoenolpyruvate with MgADP, also has a high equilibrium constant, but as the result of having the enolpyruvate product revert to the keto form.

Adenylate kinase uses MgATP to phosphorylate AMP, while nucleoside diphosphate kinase uses MgATP to convert other nucleoside diphosphates to their triphosphate form. While most kinases catalyze direct transfer of the PO_3 unit to the acceptor after both reactants are bound and thus have sequential kinetic mechanisms, nucleoside diphosphate kinase has a ping-pong mechanism in which a nucleoside triphosphate phosphorylates a histidine, and the phospho-histidine form of the enzyme then phosphorylates a nucleoside diphosphate.

We will illustrate the catalysis involved in kinase activity by focusing on several well studied cases.

3.4.1. Creatine Kinase

Creatine kinase catalyzes the reversible phosphorylation of creatine by MgATP. Under physiological conditions (pH 7.4, 1 mM free Mg^{2+}), the equilibrium constant favors the reverse reaction so that in muscle a MgATP/MgADP ratio of 10 is maintained by a creatine-P/creatine ratio of 0.12.[183] Thus, creatine-P provides the initial resynthesis of MgATP during muscle movement. The equilibrium constant for phosphorylation of creatine rises a factor of 10 per pH unit as the pH increases, so that it reaches unity above pH 9.3.

The kinetic mechanism is sequential and at pH 8 is rapid equilibrium random.[184] At pH 7 or below, however, creatine does not form a binary complex and the mechanism in the forward direction becomes equilibrium ordered with MgATP binding first.[185] The reverse reaction is still random, but no longer rapid equilibrium, as creatine-P reacts to give products 4−6 times faster than it dissociates from the ternary complex with MgADP.[186]

The pH profiles of $V/K_{creatine}$ and $V/K_{creatine-P}$ show that a group with a p$K \sim 7$ has to be unprotonated in the forward direction but protonated in the reverse reaction.[186] This group is presumably the general base in the forward direction and was thought to be a histidine on the basis of changes in the pK with the addition of 25% dimethylformamide. But the decrease was 1.2 pH units in a neutral acid buffer and 0.8 pH units in a cationic acid buffer. Since the latter change is 0.4 pH units less, one could argue that the group is a neutral acid and that the 1.2 pH unit decrease is a nonspecific effect of DMF.

Creatine kinase forms a tight dead end complex of enzyme, MgADP, creatine, and small planar molecules such as nitrate, nitrite, or formate. In the presence of MgADP, nitrate binds with a K_i of 125 mM in the absence of creatine and 2 mM in its presence, so the binding is highly synergistic. Binding of nitrate is pH independent, while the binding of creatine decreases below a pK of 6.[186]

It took a long time for crystal structures of creatine kinase to appear, but the structure of the enzyme−MgADP−creatine−nitrate complex has made it possible to deduce the arrangement of catalytic groups in the active site and the mode of binding of reactants.[187] The general base is a glutamate (232 in human muscle enzyme) which is hydrogen bonded to both terminal nitrogens of the guanidinium group of creatine. Changing it to aspartate decreases the V/K values for creatine by 900 in the forward reaction and those for creatine-P by 1300 in the reverse direction, while a glutamine mutant loses $\sim 10^5$ in activity and an alanine mutant is inactive.[188] These data are consistent with the expected behavior of an acid−base catalytic group.

The active site contains a cysteine (282 in human muscle enzyme) that is not essential but contributes to binding of creatine. Its pK is ~5.4, and mutation to serine decreases $V/K_{creatine}$ by a factor of 1000.[189] The pH profile of $V/K_{creatine}$ for the serine mutant lacks this pK, suggesting that it may have been the group responsible for the decrease in binding of creatine in the presence of MgADP and the presence or absence of nitrate.[189] This cysteine is close enough to interact with the nitrogen of the guanidinium group of creatine that is not involved in phosphorylation. The negative charge on the thiolate will attract the positive charge of the guanidinium group to this nitrogen, leaving the other terminal nitrogen as a better nucleophile to attack MgATP.

In the structure of the enzyme−MgADP−creatine−nitrate complex, the distances from the nitrate nitrogen to the nitrogen of creatine and the oxygen of MgADP are both 2.9 Å, which is consistent with a loose transition state as expected for a phosphoryl transfer.[187] The distances may be

Table 1. Synergism between MgATP and Substrates of Yeast Hexokinase[a]

substrate	K_{sugar} (mM)	K_{MgATP} (mM)	relative V_{max}
D-mannose	0.09	0.028	42
D-fructose	1.8	0.036	136
D-glucose	0.1	0.063	(100)
2-deoxy-D-glucose	0.3	0.087	96
D-glucosamine	1.5	0.10	70
2,5-anhydro-D-mannose	0.56	0.24	120
2,5-anhydro-D-mannitol	4.7	0.3	170
1,5-anhydro-D-glucitol	3.0	0.42	3
1,5-anhydro-D-mannitol	8.2	1.5	10
1-deoxy-D-fructose	150	2.2	1.8
2,5-anhydro-D-glucitol	500	2.2	22
D-arabinose	9.6[b]	2.3	21
water (ATPase)		4−7	0.026

[a] From ref 188. [b] Actual K_m, 475 mM; the value quoted is for β-furanose, which is 2% of the sugar in solution and is the actual substrate.[193]

somewhat shorter in the transition state of the reaction, but the nitrate would likely not bind as well as it does if the transition state were much tighter than this.

^{18}O isotope effects have the potential of providing further evidence on the transition state of the creatine kinase reaction. With ATP labeled in all four oxygens of the γ-phosphate, the ^{18}O isotope effects were 1.007 with creatine and 1.034 with cyclocreatine ($k_{cat}/K_m \sim 20\%$ that for creatine).[190] This isotope effect is a combined primary and secondary one, but the large value with cyclocreatine (a methylene group joins the *N*-methyl group and the nitrogen that is phosphorylated) argues that the chemistry is rate limiting and this is largely a primary isotope effect. Once the secondary isotope effect is determined with ATP labeled only in the nonbridge oxygens of the γ-phosphate, the transition-state structure will be revealed.

The current state of our knowledge of creatine kinase is covered in some detail in an excellent review.[191]

3.4.2. Hexokinase

Yeast hexokinase phosphorylates the 6-hydroxyl of sugars such as glucose, mannose, and fructose. The kinetic mechanism is random; however, there is a strong preference for the sugar to bind first, and the presence of MgATP drastically decreases the off rate constant for the sugar. The binding of MgATP and sugar is highly synergistic for good substrates but less so for poor ones. Table 1 shows a range of K_m values for MgATP varying from 28 μM with mannose to 4−7 mM in the absence of a sugar.[192]

The X-ray structures of free yeast enzyme and the E-glucose complex show that binding of the sugar causes the active site to close down and create the binding site for MgATP.[194] A structure of brain hexokinase containing MgADP and glucose shows the closed ternary complex structure.[195] This synergism is evident in the effects of sugar analogues as inhibitors. Xylose and lyxose, analogues of glucose or mannose lacking C-6, induce partial substrate inhibition by MgATP that is competitive vs glucose.[196,197] These sugars induce MgATP to bind with a K_d of 100 μM and reduce the off rate constant for inhibitory sugar release sufficiently to cause the substrate inhibition. The inhibition is partial, since MgATP binding cannot prevent sugar release but only slows it down, and competitive, since glucose prevents binding of the inhibitory sugar.[197]

In the presence of xylose or lyxose and MgATP, there is a slow ATPase reaction which is faster than that in the absence of these sugars, and in addition, the enzyme slowly becomes inactivated by phosphorylation in the presence of xylose.[198] The ATPase reaction results from phosphorylation of a water molecule occupying the space usually corresponding to C-6 of the sugar substrate, but if this water is missing, MgATP phosphorylates a neighboring serine. Incubation with xylose and MgADP leads to reactivation of the enzyme, so the phosphorylation is reversible.[199]

Synergistic binding of MgADP and sugar phosphates is evident in the reverse reaction, which is slower than the forward one. The K_d of MgADP from free enzyme (from product inhibition of the slow ATPase) was 6 mM, while its K_m with sugar phosphates was 230 μM with glucose-6-P, 120 μM with fructose-6-P, and <100 μM with mannose-6-P.[192]

The phosphorylation of a hydroxyl group requires general base catalysis, and the group providing it in yeast hexokinase is an aspartate.[194,195] Its importance is illustrated by the use of amine and thiol analogues of glucose as substrate. 6-Aminoglucose is a slow substrate with a V_{max} 0.012% that of glucose and a K_m of 1.3 mM at pH 10.[200] V/K for the sugar decreases below the pK of the amino group at 9, so the neutral amine is the substrate. When used as a competitive inhibitor vs glucose, the 6-amino analogue showed a K_i increasing below a pH of 9 but leveling off at pH 7.2. Thus, protonation decreases affinity by 2.4 kcal/mol. Binding of 6-aminoglucose and MgATP is synergistic, with a K_m of MgATP of 83 μM at pH 8.7.

The low V_{max} with 6-aminoglucose is expected, since the pK for ionization of the neutral amine is higher than that for ionization of the 6-hydroxyl group of glucose. With 6-mercaptoglucose, where the pK is 9.5, one might expect a higher V_{max}, but in fact, the rate is 5 orders of magnitude less than that with glucose.[201] This presumably results from the failure of the SH group to hydrogen bond to the catalytic aspartate with resultant loss of general base catalysis. In the reverse direction, the protonated aspartate does not hydrogen bond to the bridge sulfur, so that there is no general acid catalysis. 6-Mercaptoglucose-6-phosphate is in fact more stable toward hydrolysis on the enzyme than in solution.

Secondary ^{18}O isotope effects in the γ-nonbridge oxygens of ATP provide evidence on the transition state structure in the hexokinase reaction. These were measured by the remote label method using the exocyclic amino group of adenine as the remote label. With glucose as substrate, the value per ^{18}O was 0.9987 at pH 8.2 and 0.9965 at pH 5.3.[202] With 1,5-anhydro-D-glucitol, a slow substrate, the value was 0.9976 at pH 8.2. The chemistry is likely to be rate limiting with 1,5-anhydro-D-glucitol and with glucose at pH 5.3, which is below the pK of 6.15 in the V/K_{MgATP} pH profile. The inverse value for the former and the more inverse value with glucose at low pH are consistent with a loose transition state similar to that seen in nonenzymatic reactions of phosphate monoesters.

3.4.3. Glycerokinase

This enzyme provides a clear example of how random mechanisms can resemble ordered ones, with the apparent order depending on the substrate used. With glycerol as the substrate, the product is L-glycerol-3-P and the mechanism appears ordered, with glycerol adding first.[203] CrATP and MgAMPPCP are competitive vs MgATP but uncompetitive

vs glycerol, suggesting that they bind only to the E-glycerol complex. However, there is a slow ATPase with a V_{max} 0.005% that with glycerol and a K_m for MgATP of 540 μM at pH 8.2 vs 15 μM for K_{MgATP} in the presence of glycerol.[204] Thus, there is clearly some randomness present, although the binding of the substrates is highly synergistic.

The synergism is shown clearly by the initial velocity patterns with aminopropanediols.[204] The *R* isomer was phosphorylated on oxygen (V_{max} 0.7% that with glycerol), while the *S* isomer was phosphorylated on nitrogen (V_{max} 0.4% that with glycerol). The orientation of the 2-hydroxyl thus determines the product. But the initial velocity patterns at pH 9.4 with the amino analogues are equilibrium ordered, with MgATP binding first with a dissociation constant of 2 mM (same as the K_m of the ATPase at this pH). The K_m's of the aminopropanediols were 0.28 mM (*S*) and 8 mM (*R*). The binding of MgATP and these slow substrates is clearly synergistic, with the amino analogues binding so poorly to free enzyme that they appear to bind only to E-MgATP. The equilibrium ordered pattern reflects the slow turnover number, while the normal intersecting pattern with glycerol as a substrate is the result of glycerol dissociating from its binary complex more slowly than the turnover number.

In contrast with the amino analogues, mercaptopropanediols do not show synergism in binding with MgATP and give normal intersecting initial velocity patterns.[204] The *S* isomer is phosphorylated on oxygen (V_{max} 3.5% that with glycerol) while the *R* enantiomer is phosphorylated on sulfur (V_{max} 0.001% that of glycerol). Again, the orientation of the 2-hydroxyl determines the product (L configuration at C-2, phosphate at C-3). The principle is shown by the reaction of glyceraldehyde as a substrate.[203] L-Glyceraldehyde is phosphorylated at C-3, while the hydrate of D-glyceraldehyde is phosphorylated at C-1, with subsequent breakdown to give phosphate and D-glyceraldehyde accompanied by transfer of ^{18}O from the hydrate to phosphate.[205]

The X-ray structure of glycerokinase in complex with ADP and glycerol shows that Asp-245 is the general base for the reaction.[206]

3.4.4. L-Ribulokinase

This enzyme from *E. coli* is similar to yeast hexokinase in that the mechanism is really random but the high synergism in binding of substrates makes it look ordered, with sugar binding first.[207] The enzyme catalyzes phosphorylation of both L- and D-ribulose, as well as L- and D-xylulose, L-arabitol, and ribitol, with nearly equal k_{cat} values. The K_m's are 0.14 and 0.39 mM for L- and D-ribulose, 3.4 and 16 mM for L- and D-xylulose, and 4 and 5.5 mM for L-arabitol and ribitol. All substrates are phosphorylated at C-5, with ribitol giving D-ribitol-5-P. The K_m of MgATP shows a similar trend, being 0.02 and 0.027 mM with L- and D-ribulose, 0.028 and 0.46 mM for L- and D-xylulose, and 0.34 and 0.48 mM for L-arabitol and ribitol. There is also a slow ATPase (k_{cat} 1% that of the sugars) with a K_m of 7 mM. Thus, all substrates show synergistic binding with MgATP, although to different degrees.

MgAMPPNP is competitive vs MgATP and uncompetitive vs L-ribulose, while L-erythrulose (L-ribulose without C-5) is competitive vs L-ribulose but induces substrate inhibition by MgATP.[207] The inhibition is partial, as was the case with lyxose and yeast hexokinase, with the rate of release of L-erythrulose slowed by MgATP by more than a factor of 20. This pattern is characteristic of a random mechanism with a highly preferred order of addition of substrates and high synergism in binding.

3.4.5. Galactokinase

Galactokinase is unique in phosphorylating the anomeric hydroxyl at C-1 of galactose, instead of the terminal hydroxymethyl group of the sugar, as is the case with other sugar kinases. Kinetic studies of the *E. coli* enzyme showed that the kinetic mechanism was random, but isotope exchange between MgATP and MgADP was 2.5 times faster than exchange between galactose and galactose-1-P.[208] Less complete kinetic studies of enzymes from other species have suggested ordered mechanisms with either MgATP or galactose adding first, but it is likely that all of these enzymes have random mechanisms.

X-ray structures are available for enzymes from several bacteria as well as from humans.[209−211] They show a highly conserved aspartate in position to act as a general base in the reaction. A similar aspartate is found in the structure of mevalonate kinase, another member of the GHMP superfamily of small molecule kinases.[212] A third member of the family, homoserine kinase, has Asn-141 (*M. jannaschii* numbering) in this position, and it has been proposed that the general base is the γ-phosphate of the ATP.[213] This mechanism requires an intervening water or alcohol, as direct proton transfer is not geometrically possible. It also requires that the product be the monoprotonated homoserine phosphate, which should be apparent from the pH profile of the back-reaction. What is surprising is that Asp-140 in homoserine kinase is the amino acid in the sequence that is conserved in all kinases of the GHMP family. This raises questions about the assignments of the groups in the active site of homoserine kinase, or whether an alternate arrangement of reactants in the active site could place Asp-140 next to the hydroxyl of homoserine.

3.4.6. Pyruvate Kinase

This enzyme catalyzes the phosphorylation of MgADP by phosphoenolpyruvate (PEP). A second Mg^{2+} ion and a K^+ or NH_4^+ ion are also required. The product of the transphosphorylation is the Mg-bound enolate of pyruvate, which is then protonated on the *si* face to give the keto form before release.[214] Oxalyl phosphate is an alternate slow substrate,[215] and the X-ray structure of the rabbit muscle enzyme with MgATP, oxalate, a second Mg^{2+}, and K^+ at 2.1 Å resolution clearly shows the arrangement of groups in the active site.[216] One Mg^{2+} is coordinated to all three phosphates of ATP (plus three water molecules), while the other Mg^{2+} coordinates two oxygens of oxalate, one of the γ-phosphate oxygens of ATP, Glu-271, Asp-295, and a water molecule. The K^+ ion is coordinated to four enzyme residues (only one charged—Asp-112), the remaining γ-phosphate oxygen of ATP, and a water molecule.

It is clear that, in the physiological reaction, phosphoryl transfer from PEP to MgADP leads to a Mg-bound enolate of pyruvate. What is *not* clear is how the pyruvate enolate is protonated and released from the enzyme. The pH profiles of the reaction suggest that protonation yields at first an inner sphere complex of the carbonyl oxygen but that this is replaced by a water molecule prior to release from the enzyme.[217] The enzyme catalyzes exchange of tritium from pyruvate to the solvent, and this requires the presence of ATP, CrATP, $Co(NH_3)_4ATP$, or just phosphate or a phosphate ester, plus Mg^{2+}.[218] There appears to be a group with

a pK of ~8.2 (with Mg^{2+}) that has to be ionized for the tritium exchange and for the reaction of MgATP and pyruvate.[217] This group must be protonated for the forward reaction of MgADP and PEP, although the pK is displaced to 9.0 by the stickiness of PEP. This group is thus the acid−base catalyst for the reaction.

Interestingly, the group with a pK of 8.2 is not important in other reactions catalyzed by the enzyme. The alkoxides of glycolate and the carbamate of hydroxylamine displace water from the enzyme-bound Mg^{2+} and are then phosphorylated as analogues of enolpyruvate.[219] They cannot replace hydroxide, so their V/K profiles decrease below a pK of 9. The V/K for fluoride decreases *above* the pK of 9, which appears to be the pK of water bound to the enzyme-bound Mg^{2+}. Thus, fluoride is phosphorylated to fluorophosphate after it replaces water (but not hydroxide) in the inner coordination sphere of the Mg^{2+}.[219] Glyoxylate induces an ATPase which appears to result from reaction of MgATP with hydroxide bound to the second Mg^{2+}, or with water on this Mg^{2+} at a rate 2 orders of magnitude slower. There is no ^{18}O transfer from glyoxylate to phosphate during this reaction.[220] The enzyme also decarboxylates oxaloacetate. The pH profile shows that oxaloacetate must form an inner sphere complex with the enzyme-bound Mg^{2+} by displacing water prior to decarboxylation, but cannot replace hydroxide (pK 9.2 for Mg^{2+}-bound water).[219] This reaction does not require ATP or phosphate.

The X-ray structure of the MgATP−oxalate−Mg^{2+}−K^+ complex does not show an obvious candidate for the acid−base catalyst with a pK of ~8.2.[216] There are, however, several waters in the structure and an apparent path of hydrogen bonding to Glu-363 via these water molecules and perhaps Thr-327 and Ser-361. Water coordinated to the Mg^{2+} of MgATP may play a role as well, but the fact that Co$(NH_3)_4$ATP promotes tritium exchange from pyruvate at a rate only 3 times slower than MgATP tends to rule this out[221] (the NH_3 group cannot be part of a proton relay, since there are no free lone pairs on the nitrogen).

Mutation of Glu-363 to glutamine causes a drastic increase in the K_m's of MgADP (from 0.3 to 46 mM) and PEP (from 37 μM to 14 mM), although k_{cat} is only reduced by a factor of 11.[222] The rate of tritium exchange from pyruvate in the presence of Co$(NH_3)_4$ATP, Mg^{2+}, and K^+ is reduced by a factor of 100 at pH 7.7, which is below the pK of ~8.2. It appears that Glu-363 is the group that protonates the enolate of pyruvate to permit it to leave the enzyme, using a proton relay via water molecules. Mutation to glutamine makes the active site more positive and may also result in other conformation changes to loosen the binding of the substrates. The binding of oxalate is also decreased by a factor of 500 by the mutation. The lesser effect of the mutation on k_{cat} of the reaction between MgADP and PEP may reflect the opening of the active site and release of enolpyruvate itself from the enzyme if a proton is not available. The decreased affinity for reactants in the active site suggests that the active site has less tendency to stay closed in the mutant.

3.4.7. Nucleoside Diphosphate (NDP) Kinase

This enzyme differs from other kinases in having a ping-pong mechanism with a phosphohistidine intermediate. This histidine is hydrogen bonded to a glutamate, which is in turn hydrogen bonded to a serine.[223,224] A Glu to Gln mutant retains only 0.5% activity, while mutation of the serine has little effect.[225] This glutamate stabilizes the positive charge on the histidine when it is phosphorylated, so that it does not lose the proton, as it would at neutral pH in solution. The histidine attacks the γ-phosphate of MgATP (or other nucleoside triphosphate) to yield MgADP.

An X-ray structure of a complex of enzyme and MgADP with trigonal AlF_3 mimics the geometry of the transition state, while a similar complex with tetrahedral BeF_3^- mimics either E-MgATP or EP-MgADP.[226] The Al is 2.5 Å from an oxygen of ADP and from the Nδ of histidine, while the Be is 2.1 Å from one group and 3.0 Å from the other. Mg^{2+} coordinates both α- and β-phosphate oxygens, as well as one fluorine in both structures, while the other fluorines are hydrogen bonded to lysine and tyrosine or to arginine and an amide nitrogen. Mutation of the tyrosine slows catalysis by a factor of 50,[227] but a larger effect results from a short (2.6 Å) hydrogen bond from the 3′-hydroxyl of ribose to the β-phosphate oxygen of ADP, which faces the Al or Be and is the leaving group during phosphorylation of histidine. Dideoxy-ATP is 10^4 less active than ATP, showing the importance of this hydrogen bond in maintaining the proper geometry for the reaction.[228] Since the pK of this oxygen in MgADP is ~6, a hydrogen bond to it from a hydroxyl group with a pK ~ 15 will not be a terribly strong one, but maintaining proper geometry for phosphoryl transfer is very important, and this is certainly the major role of this hydrogen bond.

The normal substrates for NDP kinase are the usual four nucleoside tri- and diphosphates. It was a surprise when adenosine-5′-sulfatopyrophosphate ($ADPSO_3$), formed by pyruvate kinase from sulfoenolpyruvate,[229] was found to be a substrate for NDP kinase and with GDP formed GDP-SO_3.[230] It was found that $ADPSO_3$ is also a substrate for adenylate kinase (but at a rate 10^5- to 10^6-fold slower than with MgATP), but not for hexokinase, phosphofructokinase, acetate kinase, or pyruvate phosphate dikinase. The lack of reactivity with other kinases is not due to a failure to bind to the active site, as $ADPSO_3$ is a competitive inhibitor against ATP, with dissociation constants that are close to the K_m values for ATP.

Curiously, while the reaction with ATP has a strict requirement for a divalent metal ion, like all known kinase reactions, the reaction with $ADPSO_3$ has no such requirement, though Mg^{2+} does increase the rate. With NDP kinase, the sulfation of the enzyme showed a k_{cat} of 20 min^{-1} in the presence of Mg^{2+}, and 4 min^{-1} in its absence, which is 3 orders of magnitude slower than the reaction with MgATP.[230] The equilibrium constant for formation of the sulfoenzyme was 0.12 in the absence of Mg^{2+} but 12 in its presence. The difference comes from the much tighter binding of Mg^{2+} to ADP than to $ADPSO_3$. With MgATP, the equilibrium constant for phosphoenzyme formation was also 0.12.

The fact that reaction of $ADPSO_3$ does not require a metal ion raises interesting questions about the role of Mg^{2+} in phosphoryl transfer reactions. Is Mg^{2+} needed for charge neutralization, withdrawal of electrons from phosphorus, proper orientation, or what? Clearly, sulfuryl transfer does not require this effect, whatever it is.

3.5. Enzymes with a Carboxyphosphate Intermediate

A number of enzymes phosphorylate bicarbonate by either MgATP or PEP to produce carboxyphosphate. This very labile intermediate (estimated lifetime in water less than 1 s[231]) then either reacts with ammonia to give carbamate or

reacts to carboxylate an acceptor. We will discuss several well studied enzymes of this type.

3.5.1. PEP Carboxylase

This enzyme reacts PEP with bicarbonate to give phosphate and oxaloacetate.[232] In grasses such as corn, the oxaloacetate is reduced to malate and transported into special cells where it is decarboxylated to build up CO_2 levels for fixation by ribulose-bis-P carboxylase (Rubisco). While this costs ATP (to regenerate PEP), it overcomes the competition by oxygen for Rubisco, which is a problem for plants which do not use this method for sequestering CO_2.

The kinetic mechanism of PEP carboxylase is random, but with a high synergism in the binding of substrates so that it appears to be ordered.[233] Mg^{2+} adds first with equilibrium ordered kinetics, and then PEP binding precedes addition of bicarbonate. The synergism is seen in the effect of phosphoglycolate, which is competitive vs PEP but induces substrate inhibition by bicarbonate which is partial, with the release of phosphoglycolate reduced by a factor of at least 170 by the presence of bicarbonate. This is similar to the effect of lyxose with hexokinase or the effect of erythrulose-4-P with L-ribulokinase discussed above.

Double inhibition studies showed high synergism between oxalate as an enolpyruvate mimic and the carboxyphosphate mimic carbamoyl-P (770-fold), with lower effects with phosphonoacetamide (12-fold), acetyl-P (8-fold), and phosphonoacetate (2-fold).[233] Phosphonoformate and oxalate were actually antisynergistic by a factor of nearly 3. Clearly, carbamoyl-P is the best analogue of carboxyphosphate and analogues with three negative charges are not bound well. This is consistent with the fact that carboxyphosphate when initially formed will have only two negative charges.

The mechanism appears to involve phosphoryl transfer from PEP to bicarbonate to give a Mg-bound enolate of pyruvate and carboxyphosphate. The latter then decarboxylates to HPO_4^{2-} and CO_2, and the CO_2 reacts with the enolate of pyruvate to give oxaloacetate. Evidence supporting this mechanism includes transfer of ^{18}O from bicarbonate to phosphate.[234] With *E*- or *Z*-fluoro-PEP, in fact, more than one ^{18}O is incorporated into phosphate and ^{18}O is found in residual fluoro-PEP.[235] This can only happen if carboxyphosphate is decarboxylated to CO_2 reversibly in the active site and the reaction with the enolate of fluoropyruvate is also reversible (this is basically a positional isotopic exchange or PIX experiment).

The enzyme accepts a number of PEP analogues as substrates, but most of these are hydrolyzed, rather than carboxylated. Exceptions are *Z*-chloro-PEP (25% carboxylation), *Z*-fluoro-PEP (3% carboxylation), and *E*-fluoro-PEP (86% carboxylation).[236] The ^{13}C isotope effect in CO_2 for formation of oxaloacetate is small with PEP (1.002), as the result of a high forward commitment, but the value was 1.049 for the *Z* isomer of fluoro-PEP and 1.009 for the *E* isomer.[235] The 1.049 value is close to the intrinsic isotope effect for carboxylation to give oxaloacetate because the high ratio of hydrolysis to carboxylation for this isomer allows full expression of the isotope effect at the branch point in the mechanism. The isotope effect should be the same on carboxylation and decarboxylation, since the equilibrium isotope effect is close to unity, and values of 4−5% are typical for decarboxylations. With the *E* isomer, which gives a very low ratio of hydrolysis to carboxylation, the intrinsic isotope effect is suppressed.

PEP carboxylase also accepts formate as an alternate substrate for bicarbonate, producing pyruvate and formyl-P (which is unstable) at a rate 100 times less than that with bicarbonate.[237] ^{18}O is transferred from formate to phosphate during the reaction, showing that phosphoryl transfer does actually take place. No formylation of enolpyruvate occurs during the reaction, which supports the idea that carboxyphosphate cannot react directly with enolpyruvate but must decarboxylate to CO_2, which then reacts.

3.5.2. Biotin-Containing Carboxylases

A number of biotin-containing carboxylases carboxylate biotin using bicarbonate and MgATP. These reactions appear to involve carboxyphosphate as an intermediate, since there is ^{18}O transfer from bicarbonate to phosphate during the reaction.[238,239] There is some uncertainty about how carboxyphosphate carboxylates biotin, but it is thought that it decarboxylates to HPO_4^{2-} and CO_2 and the CO_2 then reacts with the enolate of biotin.[239] But since the reactions are reversible, CO_2 and phosphate must be able to combine to form carboxyphosphate in the active site. The PEP carboxylase reaction discussed above is precedent for this, but no biotin-containing carboxylase shows PIX, even in the slow bicarbonate-induced ATPase reaction of biotin carboxylase seen in the absence of biotin, so proof is lacking.[239] Suitable ^{18}O isotope effects to establish CO_2 as an intermediate have not been carried out.

3.5.3. Carbamoyl Phosphate Synthetase

This enzyme contains three active sites connected by a 96 Å long tunnel.[240] Glutamine is hydrolyzed at the first site to give ammonia, which diffuses through the tunnel to the second site. There, it is thought to react with carboxyphosphate formed from MgATP and bicarbonate. This reaction yields carbamate, which diffuses to the third site, where it is phosphorylated by MgATP to carbamoyl-P.

One might think, by analogy with the PEP carboxylase reaction, that carboxyphosphate would decarboxylate to CO_2, which would react with ammonia to give carbamate. However, in the bicarbonate-induced ATPase reaction catalyzed by CPS, which presumably involves carboxyphosphate formation followed by its breakdown, it appears that the product of carboxyphosphate breakdown is bicarbonate rather than CO_2. Proton release during the ATPase reaction is linear without a lag and has the same rate as ADP formation.[241] The reaction of MgATP and bicarbonate to give carboxyphosphate dianion (protonated carboxyl) does not involve proton release or uptake, nor does breakdown of carboxyphosphate dianion to CO_2 and HPO_4^{2-}. But reaction of carboxyphosphate dianion with water to give bicarbonate and HPO_4^{2-} *does* produce a proton, as observed. What is not clear is whether in the ATPase reaction the dianion of carboxyphosphate reacts with water while in the active site (in analogy with its putative reaction with ammonia) or whether it is released first and decomposes in solution with an expected rate of 10 s^{-1}.[231]

It is surprising that carboxyphosphate decomposition appears to produce bicarbonate rather than CO_2; this presumably means that the carboxyl group is protonated, as it would be upon initial formation. Since ionization of carboxyphosphate to the trianion should be fast ($>10^5$ s^{-1}), and trianion breakdown would certainly produce CO_2, this suggests that the observed decomposition to bicarbonate takes place on the enzyme before the proton is lost. These data suggest that

carboxyphosphate dianion reacts with ammonia on the enzyme without intermediate formation of CO_2, and the data were interpreted this way by Raushel and co-workers.[241]

At the third site, the kinase reaction appears normal, and in the presence of carbamoyl-P and MgADP, there is formation of MgATP and carbamate accompanied by PIX, scrambling the bridge and nonbridge oxygens of carbamoyl-P.[241,242]

It is clear that much more needs to be done to establish the properties and behavior of carboxyphosphate, which appears to play an important role as an intermediate in a number of enzymatic reactions.

3.6. Transcarbamoylases

3.6.1. Aspartate Transcarbamoylase

This enzyme catalyzes carbamoyl transfer from carbamoyl-P to aspartate to give carbamoylaspartate. The enzyme exists as an R form in which all six active sites are active and a T form in which they are not. It is subject to allosteric control, with MgCTP being an inhibitor binding to the T form and MgATP an activator binding to R. The resting holoenzyme is largely in the T form, but aspartate binds only to R. This leads to a sigmoid velocity vs aspartate curve which is converted to normal hyperbolic kinetics by MgATP but is made more sigmoid by MgCTP.[243]

When ^{13}C isotope effects in carbamoyl-P were determined, the value was 1.022 at low aspartate concentration, but unity at high aspartate concentration, showing that the kinetic mechanism was ordered, with carbamoyl-P adding first.[244] The same dependence of the ^{13}C isotope effect on aspartate concentration was obtained with activating levels of MgATP or inhibitory levels of MgCTP, showing that the properties of the R form were independent of the presence of allosteric modifiers. When the enzyme was dissociated to free trimers containing only the active site, all allosteric properties were lost,[245] but now the ^{13}C isotope effect decreased from 1.024 at low aspartate to 1.0039 at an infinite level.[244] Thus, the kinetic mechanism becomes partly random in these catalytic trimers. With the slow and poorly bound aspartate analogue cysteine sulfinate, the ^{13}C isotope effect was 1.039 for both holoenzyme and catalytic trimers and did not vary with the level of cysteine sulfinate.[244] Thus, the mechanism was now fully random. A similar isotope effect of 1.041 was seen with a His134Ala mutant with ~5% activity, and again the level of aspartate did not change the isotope effect.[246] The change from an ordered mechanism with the holoenzyme to fully random ones in the H134A mutant or with cysteine sulfinate as the substrate results from failure to reduce the off rate constant of carbamoyl-P to a level well less than the turnover number, which appears limited by the chemistry in these cases.

The actual chemistry of the reaction was established by use of ^{15}N isotope effects in carbamoyl-P.[247] While it has been assumed that the reaction involved attack of aspartate on carbamoyl-P to give a tetrahedral intermediate which broke down by release of phosphate, the spontaneous breakdown of the dianion of carbamoyl-P occurs by C−O cleavage to give cyanic acid and HPO_4^{2-}.[248] In this reaction, in which the phosphate group acts as a general base, the ^{15}N isotope effect was 1.0115.[247] This is a primary isotope effect, but most of the motion in the transition state for N−H cleavage is hydrogen motion, so the ^{15}N isotope effect is not as large as it would be for a C−N cleavage. By contrast, the ^{13}C isotope effect is 1.058,[249] since the carbon has to move into a collinear arrangement with the O and N of the cyanic acid product and, thus, is in motion in the transition state.

The ^{15}N isotope effect for breakdown of the monoanion of carbamoyl-P, which involves P−O cleavage,[248] was 1.0028, a secondary isotope effect probably resulting from a change of the ^{15}N-sensitive N−C−O−P torsional vibration to a ^{15}N-insensitive N−C−O−H one.[247]

The aspartate transcarbamoylase reaction proceeds by C−O cleavage, but the ^{15}N isotope effects with wild type, H134A, or cysteine sulfinate as substrate were 1.0014, 1.0027, and 1.0024.[247] Since the ~4% ^{13}C isotope effects for the latter two cases suggest that the chemistry is fully rate limiting, these values of ~1.0025 are too small for a primary ^{15}N isotope effect and presumably reflect a secondary isotope effect resulting from loss of C−N double bond character from amide resonance in forming the tetrahedral intermediate. Thus, the ^{15}N isotope effects are consistent with the reaction involving a tetrahedral intermediate but not with one involving cyanic acid.

3.6.2. Ornithine Transcarbamoylase

The reaction catalyzed by this enzyme is similar to that with aspartate transcarbamoylase, except that the transfer is to the δ-amino rather than to the α-amino group. The ^{13}C isotope effect in carbamoyl-P was 1.0095 at low ornithine concentration but unity at high levels, showing that the kinetic mechanism was ordered.[250] With the slow substrate lysine (activity ~4% that with ornithine), however, the ^{13}C isotope effect was 1.076, which is certainly the intrinsic isotope effect. Thus, the value of 1.0095 with ornithine shows that the chemistry is not rate limiting and there is a commitment of 3.2−7, depending on whether one assumes an intrinsic isotope effect of 1.04 (as seen with aspartate transcarbamoylase) or 1.076.

Ornithine transcarbamoylase is strongly inhibited by a mimic of the tetrahedral intermediate which is a toxin produced by the *Pseudomonas* strain that causes halo blight in French beans (K_i = 1.6 pM).[251] This inhibitor consists of ornithine with an (*R*)-sulfodiaminophosphinyl residue [-(PONH$_2$)−N−(SO$_3$)] attached to the δ-amino group. The X-ray structure of the enzyme complexed with this inhibitor shows that the SO_3 group mimics the PO_3 group of the tetrahedral intermediate and that the phosphorus with O, NH_2, and bridging N to the SO_3 group mimics the tetrahedral carbon coming from carbamoyl-P.[251] Thus, the inhibitor is a near perfect mimic of the tetrahedral intermediate, but with S replacing P and P replacing C. The geometry and the atoms that contact the enzyme are the same.

The organism that makes this inhibitor has an ornithine transcarbamoylase that is not inhibited by the inhibitor. This enzyme has similar affinity for the substrates, but a k_{cat} 70-fold slower.[252] The ^{13}C isotope effect in carbamoyl-P was 1.034 at low ornithine but 1.012 at high concentrations, so that the mechanism is partly random. Thus, ornithine slows down but fails to prevent carbamoyl-P release from the enzyme. This probably results from the 70-fold decrease in k_{cat}, while the same off rate with the higher k_{cat} of the normal enzyme would make it appear that carbamoyl-P cannot leave while ornithine is present.

3.7. ATPases and ATP Synthesis

3.7.1. Myosin

A number of enzymes cause motion, using the energy released by hydrolysis of MgATP. Myosin, which is responsible for muscle movement, is one of the best studied examples of this. ATP is a stable molecule in solution, since water molecules hydrogen bond to the nonbridge oxygens of the γ-phosphate and, thus, are not in the correct position to attack the γ-phosphorus. The problem for an ATPase such as myosin is thus to position a water molecule so that it *is* in a position to attack the γ-phosphorus. This requires steric restraints as well as organized hydrogen bonding networks. And more specifically, there must be a path for one proton of the attacking water molecule to reach a suitable acceptor. ATPases appear not to use general bases such as the aspartates usually found in kinase active sites. Rather they arrange a hydrogen bonding path for proton transfer to a nonbridge oxygen of the γ-phosphate. This proton cannot transfer directly, as the geometry does not permit such a four center reaction.

The 1.9 Å structure of myosin complexed with MgADP and vanadate shows that the hydroxyl of Ser-236 is 2.9 Å from the apical oxygen of vanadate that mimics the attacking water molecule and 2.8 Å from a nonbridge equatorial oxygen of vanadate.[253] Vanadate mimics the transition state of the reaction, with three equatorial oxygens and an oxygen from ADP as the other apical ligand. The apical bonds of vanadium are 2.3 Å from the oxygen mimicking the attacking water and 2.1 Å from the ADP oxygen. The apical oxygen mimicking water is also hydrogen bonded to another immobilized water molecule (2.6 Å) and the hydroxyl of Ser 237 (2.7 Å). The attacking water molecule is thus held in place by these three hydrogen bonds, and a path for proton transfer to the γ-phosphate nonbridge oxygen is provided via the hydroxyl of Ser-236. The products of the reaction are thus MgADP and $H_2PO_4^-$.

Myosin only hydrolyzes MgATP at a specific point in the overall path for muscle movement, and thus, the configuration shown in the MgADP−vanadate complex is only attained when hydrolysis is required. The secret to phosphoryl transfer is orientation, and the ability of an ATPase to control the orientation of the attacking water molecule provides the mechanism to turn hydrolysis on or off.

A recent review on myosin and related ATPases discusses the structures and mechanisms of these enzymes in more detail.[254]

3.7.2. ATP Synthase

One of the most fascinating enzymes that catalyze phosphoryl transfer is the ATP synthase of mitochondria and chloroplasts, which synthesize MgATP from MgADP and phosphate, using a gradient of protons, or in some cases Na^+, to provide the energy for the synthesis (for an excellent review, see ref 255). The enzyme has three catalytic sites, which undergo sequential conformation changes as a rotating "stalk" is driven by proton transfer across the membrane in which the enzyme is embedded.

Several X-ray structures are available of the portion of the enzyme that includes the active sites and shows ATPase activity. The first structure reported contained MgADP in one site, MgAMPPNP in the second, and nothing in the third active site.[256] The first two sites were closed, while the third was open. A more recent structure shows MgADP−AlF_4^- in two closed sites and MgADP and sulfate in the third "half-closed" site.[257] Binding studies show that MgATP binds to the three active sites with affinities of ~1 nM, 1 μM, and 30 μM.[258] Structural and binding studies thus suggest that each active site can assume several different conformations during the rotation of the "stalk."

It is clear that each active site goes through the following changes. First, in an open configuration, MgADP and phosphate are bound. As the active site closes, the phosphate has to be lined up properly to attack MgADP, and the appropriate hydrogen bonding patterns have to be established to the nonbridge oxygens and to the oxygen that is to become water. At this point, phosphoryl transfer to give MgATP and water comes to equilibrium. The subsequent conformation change *must be one that allows the water to move, but not the phosphate groups*; this will lock the equilibrium into MgATP and water. The final conformation change opens the active site to release MgATP and permit binding of MgADP and phosphate in its place.

Because the ratio of MgATP/MgADP can exceed 100 in resting muscle, it is necessary for the open form of the catalytic site to have a high affinity for MgADP and phosphate, and a low one for MgATP. This is postulated to result from insertion of Arg-376 into the active site at this point in the cycle to expel MgATP.[257] Removal of Arg-376 from the active site is proposed to help freeze the MgADP + phosphate − MgATP equilibrium in favor of MgATP later in the cycle.

In the transition state for the synthesis of MgATP, the Mg^{2+} of MgATP has moved to become bidently coordinated to the β- and γ-phosphates of ATP. The nonbridge oxygens of the PO_3 unit are hydrogen bonded to Arg-376, Arg-182, and Lys-155, while the oxygen that becomes water is hydrogen bonded to Glu-181. It appears from the geometry that Glu-181 may act as a general acid during the reaction, which is a different mechanism than that seen with myosin ATPase.

3.8. Phosphodiesterases

3.8.1. Staphylococcal Nuclease

Staphylococcal nuclease (SNase) possesses one of the greatest rate accelerations known relative to the corresponding uncatalyzed reaction, as much as 10^{16}.[259] This phosphodiesterase utilizes a Ca^{2+} ion for catalysis to hydrolyze the linkages in DNA and RNA, cleaving the 5′O−P nucleotide bond to yield a free 5′-hydroxyl group. The active site has two arginine residues in a position to interact with the phosphoryl group, and a glutamate. X-ray structures[260−263] of SNase have been solved for the wild type and mutants, but the exact roles of active site residues are still uncertain.

Stereochemical experiments show that the reaction proceeds with inversion of configuration, consistent with a single in-line nucleophilic attack by water.[264] The first X-ray structure[260] led to the proposal that Glu-43 is a general base. The E43D mutant has a 200-fold lower k_{cat} at pH 9.5;[265] however, the E43D mutant was subsequently found to be significantly structurally altered.[262−266] A later study of the pH−rate dependency of the reaction led to the conclusion that Glu-43 does not act as a general base.[267] An investigation of the roles of Arg-35 and Arg-87 by mutation to lysine led to the discovery that while these mutations result in large reductions in catalytic activity, they also result in structural alterations.

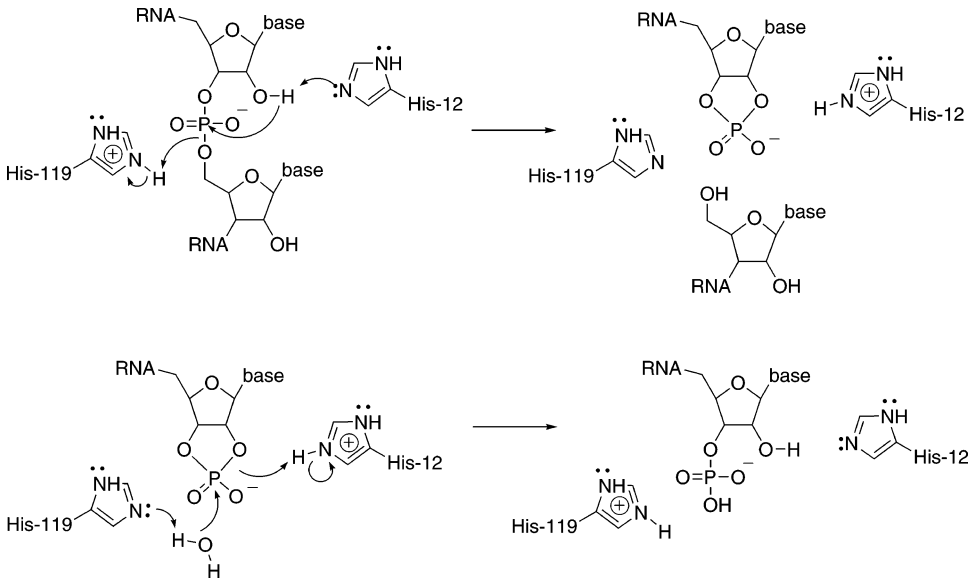

Figure 12. Two steps of the RNase A-catalyzed reaction. Despite the fact that, after the initial formation of the 2′,3′ cyclic phosphate, His-12 and His-119 are in the correct protonation states to immediately catalyze its hydrolysis by water, the cyclic phosphate is released by the enzyme faster than it is hydrolyzed.

The rate-limiting step for the hydrolysis of single-stranded DNA by SNase was subsequently shown to be the physical process of product dissociation, possibly involving residues in a protein loop near the active site; substrate binding is diffusion-controlled when the pH is >7.3.[267] The pH optimum is between 8.6 and 10.3, and the enzyme is routinely assayed at pH 9.5. Thus, under these conditions, kinetic studies cannot reveal the effect of mutations on the chemical step.

3.8.2. Snake Venom Phosphodiesterase

Snake venom exonuclease (phosphodiesterase I) successively hydrolyzes 5′-mononucleotides from 3′-hydroxy-terminated ribo- and deoxyribo-oligonucleotides. It is nonspecific with respect to the identity of base or sugar moieties of nucleotides, and a variety of synthetic substrates are hydrolyzed. The enzyme has been widely utilized as a tool for structural and sequence studies of nucleic acids. The stereochemical outcome of the reaction has been determined a number times, and the reaction has been found to proceed with retention of configuration.[268−271] A threonine residue was identified as the enzymatic nucleophile,[272] and atomic absorption spectroscopy on the purified enzyme indicates the presence of a single zinc ion.[273] The catalytic reaction exhibits a bell-shaped pH−rate profile, with a maximum ranging from ∼8.5 to 9.5, depending on the substrate.[274,275] The bovine intestinal phosphodiesterase has also been shown to produce a phosphothreonine intermediate during catalysis.[276]

Alcohols will react with the phosphoenzyme intermediate, and the enzyme will convert ATP to AMP-O-alkyl esters when alcohols are present in solution. Such esters are themselves substrates for the enzyme but are hydrolyzed at lower rates than substrates such as ATP, resulting in the accumulation of transesterification products.[277] Kinetic isotope effects measured with the substrate 3,3-dimethylbutyl *p*-nitrophenyl phosphate revealed a significant inverse value for $^{18}(V/K)_{nonbridge}$ of 0.9842 ± 0.0006,[23] which is the same as the equilibrium isotope effect for protonation of the phosphoryl group (0.984).[278] This suggests the enzyme may protonate the phosphoryl group during catalysis, though, given the alkaline pH optimum, another source for the inverse KIE, such as interactions with a metal ion in the transition state, cannot be ruled out. No structural information of consequence yet exists for either the snake venom or the bovine intestinal diesterases.

3.8.3. Ribonuclease

Ribonucleases catalyze the hydrolysis of the phosphodiester bonds of RNA. The initial reaction is attack of the 2′-hydroxyl group to form a 2′,3′ cyclic phosphate (Figure 12). Hydrolysis of this intermediate results in a 3′-phosphomonoester. It has been shown by ^{31}P NMR[279] and by chromatographic means[280−282] that RNase releases most of the cyclic intermediate during the catalytic cycle. In the alkaline reaction, by contrast, the cyclic phosphate hydrolyzes faster than it forms.[279]

Ribonuclease A (RNase A) is the paradigm of the ribonuclease family and has been the subject of a number of reviews.[283−286] Figure 12 shows the primary catalytic groups involved in catalysis, using the numbering for RNaseA. The enzyme also has a lysine (Lys-41 in RNase A) that is within hydrogen bonding distance of the phosphoryl group. Both the transphosphorylation[287,288] and the hydrolysis of the cyclic intermediate[289] proceed with inversion, suggesting a concerted, in-line phosphoryl transfer process. The catalytic reaction has a bell-shaped pH−rate dependency, indicative of general acid−base catalysis, and His-12 and His-119 are assigned to these roles. The pH−rate profile for k_{cat} is shifted to more acidic pH in a semisynthesized form of RNaseA in which both histidines are replaced by 4-fluorohistidine (pK_a of 3.5 compared to 6.8 for histidine).[290]

The role of lysine-41 is commonly attributed to stabilization of negative charge on the phosphoryl group in the transition state. It has generally been assumed that this is an electrostatic interaction, but formation of a short, strong (low-barrier) hydrogen bond has also been proposed.[291] A detailed analysis of the interactions involving Lys-41[292] suggested that the role of Lys-41 is not merely electrostatic but that the donation of a single hydrogen bond is important. This role seems to be the same both for the initial transphospho-

rylation reaction and for the subsequent RNase-catalyzed hydrolysis of the 2′,3′-cyclic phosphate.[285]

The kinetic isotope effects with uridine-3′-*m*-nitrobenzyl phosphate, which has a leaving group pK_a very similar to that of the natural substrate, are 1.005 ± 0.001 for $^{18}(V/K)_{nonbridge}$ and 1.016 ± 0.001 for $^{18}(V/K)_{bridge}$, consistent with a concerted mechanism with the moderately tight transition state expected of a diester.[293] The mechanism in Figure 12 is consistent with all of the data collected for the enzymatic reaction, which point to a concerted process with deprotonation of the nucleophilic 2′-hydroxide by His-12 accompanied by protonation of the leaving group by His-119.

Based on results obtained in imidazole buffers, an alternative mechanism was proposed for RNaseA.[294] The arguments in favor and against this controversial proposal have been reviewed.[31,295] In this alternative proposal, His-119 is purported to first protonate the phosphoryl group; the 2′-OH group then attacks to form a phosphorane intermediate; His-119 then transfers this proton from the phosphorane to the leaving group. No evidence obtained on the enzymatic reaction favors this mechanism, and whether this mechanism is even followed in imidazole buffer has been the subject of debate. In the RNase active site, the substrate is ideally positioned for simultaneous acid−base catalysis to facilitate a concerted mechanism. Such a serendipitous arrangement in imidazole buffer would be highly disfavored on entropic grounds.

3.9. Nucleotidyltransferases

3.9.1. Kanamycin Nucleotidyltransferase

A number of enzymes catalyze reaction of MgATP with an acceptor to give an adenylylated acceptor and Mg-pyrophosphate. The acceptor thus attacks the α-phosphate, rather than the γ-phosphate as in kinase-catalyzed reactions. The enzyme that adenylylates kanamycin A and similar antibiotics has been studied using *m*-nitrobenzyl triphosphate to replace MgATP.[296,297] The K_m for this substrate is similar to that of MgATP, but the k_{cat} is 130-fold less than that with MgATP, thus ensuring that the chemistry will be rate limiting.[297] With MgATP as substrate, the rate-limiting step is release of the adenylylated kanamycin, so it is not practical to use ^{18}O isotope effects to determine transition-state structure.[298] The *m*-nitrobenzyl phosphoryl group is transferred to the same 4′-hydroxyl of kanamycin A as the adenyl group in the reaction with MgATP, so the attacking nucleophile and the leaving Mg−PP complex are the same.[297]

^{18}O isotope effects were determined with ^{18}O in all four oxygens attached to the α-phosphorus, and with just the nonbridge and benzyl-P bridge positions containing ^{18}O.[296] The former substitution causes combined α−β bridge primary and nonbridge secondary isotope effects, while the latter gives only the nonbridge secondary isotope effect. It was assumed that ^{18}O in the bond between the α-phosphate and the benzyl group would not give an isotope effect, as its bonding should not change during the reaction.

The primary ^{18}O isotope effect in the α−β bridge oxygen was 1.6%, while the nonbridge secondary isotope effect was 0.3%.[296] The size of the primary isotope effect indicates that the chemistry is rate limiting, while the secondary isotope effect suggests a concerted S_N2 reaction with a transition state that is slightly associative. From the X-ray structure with Mg-AMPCPP and kanamycin bound, it appears that Glu-145 is the general base that removes the proton from the attacking 4′-hydroxyl of kanamycin and that Lys-149 stabilizes the negative charge on the α-nonbridge oxygens.[299] The reaction is very similar to that catalyzed by ribonuclease A, except that a general acid is not needed to protonate the Mg-PP leaving group.[300]

3.9.2. Glucose-1-phosphate Cytidylyltransferase

Enzymes that synthesize nucleoside diphosphate sugars catalyze attack of a sugar-1-P on the α-phosphate of a nucleoside triphosphate, releasing Mg-pyrophosphate. X-ray structures of glucose-1-P cytidylyltransferase show clearly how this reaction occurs. Structures with CDP-glucose[301] or with MgCTP bound[302] both have CMP in identical positions. However, the glucose-1-P portion and the MgPP portion of these bound ligands occupy positions 180° apart with respect to the phosphate of CMP.[302] Thus, it is clear that the reaction involves a direct in-line displacement of MgPP as glucose-1-P attacks the α-phosphate of MgCTP. No acid−base chemistry is involved in the reaction, but Lys-25 and Arg-15 and a second Mg^{2+} ion may help orient the reactants.[302]

3.9.3. Hexose-1-phosphate Uridylyltransferase

In contrast to the enzymes that synthesize nucleoside diphosphate sugars from nucleoside triphosphates and sugar-1-phosphates, this enzyme catalyzes transfer of UMP from one sugar-1-P to another. Thus, UDPglucose plus galactose-1-P give UDPgalactose plus glucose-1-P. The kinetic mechanism is ping-pong, with UMP forming an adduct with His-166.[303] X-ray structures are available of the wild-type enzyme with UDP bound[304] or with UMP covalently attached to His-166.[305] Structures of the H166G mutant with either UDPG or UDPGal bound define the binding site of the sugar-1-P's.[306] These structures also have a bound K^+ ion replacing the imidazole ring of His-166, suggesting that the imidazole ring is protonated in the covalent UMP intermediate and the K^+ is replacing this positive charge.[306] The geometry shows that His-166 and the sugar-1-P are on opposite sides of the phosphate of UMP, so that the reaction involves a direct in-line displacement. Interestingly, His-166 is hydrogen bonded to the carbonyl oxygen of His-164, while in NDP kinase the histidine is hydrogen bonded to a glutamate.[223,224] In both cases, however, this prevents loss of the proton, which would make the histidine a much poorer leaving group.

The H166G mutant is inactive in reacting UDPG with galactose-1-P or in reacting UDPGal with glucose-1-P. It does, however, catalyze reaction of imidazole with UDPG or UDPGal to give a sugar-1-P and UMP-imidazolate with a k_{cat} 80−200-fold slower than those for the reactions of wild-type enzyme.[307] The reaction is reversible, and UMP-imidazolate reacts with glucose-1-P to give UDPG with a k_{cat} 3.5-fold slower than that for the reverse reaction. While slow, this mutant is now a new enzyme (UDP-hexose synthase) with a sequential mechanism rather than a ping-pong one. It may prove useful for synthesis of UDP-sugars from sugar-1-P's without the necessity of converting UDP to UTP.

3.10. Phosphotriesterase

Phosphotriesterases catalyze the hydrolysis of triesters to yield diesters. Phosphotriesters are not naturally occurring compounds but have been introduced into the environment as pesticides and insecticides, since the middle of the twentieth century. Despite the limited time available for the

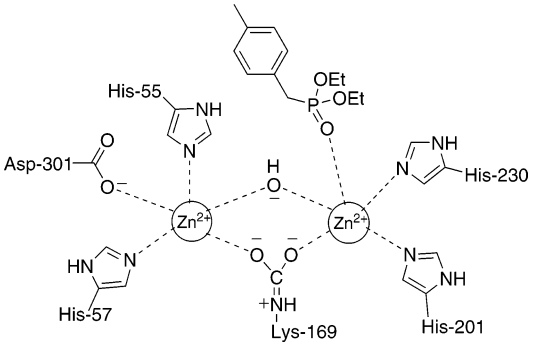

Figure 13. Structure of the active site of the phosphotriesterase with the competitive inhibitor diethyl-4-methylbenzyl phosphonate.

evolution of enzymatic activity for their hydrolysis, several phosphotriesterases have been identified. These enzymes were discovered by means of the resistance of some insects to insecticides and the ability of microbes to degrade pesticides.[308,309] The phosphotriesterase from the soil bacterium *Pseudomonas diminuta* has been the best characterized. This enzyme hydrolyzes a large number of phosphotriester substrates, but the best substrate is paraoxon (diethyl *p*-nitrophenyl phosphate), which is hydrolyzed at a rate $\sim 10^{12}$ faster than the uncatalyzed rates.[310] This phosphotriesterase does not catalyze the hydrolysis of monoesters, and diesters are hydrolyzed only poorly.[311]

The active site has two Zn^{2+} ions, and an X-ray structure[312] with the competitive inhibitor diethyl-4-methylbenzyl phosphonate (Figure 13) shows a binding mode with only minimal direct electrostatic interactions between the inhibitor and the active site, consistent with the ability of the enzyme to hydrolyze a wide range of substrates. The zinc ions are bridged by a hydroxide and by a carbamate moiety formed from the ϵ-amino group of Lys-169.[312]

The phosphotriesterase reaction proceeds with inversion of configuration at phosphorus,[313] indicative of direct phosphoryl transfer to an activated water molecule. Starting from the X-ray structure with the inhibitor, the modeling of a substrate into the active site led to the conclusion that the leaving group is positioned opposite the bridging hydroxide, suggesting it as the nucleophile.[312]

The native enzyme contains two Zn^{2+} ions, but the apoenzyme can be activated by a number of other divalent ion pairs including Co, Ni, Cd, and Mn, as well as mixed Zn/Cd. These metal substitutions yield enzyme forms that exhibit catalytic activities as high as or higher than that of the native enzyme.[314,315]

The enzymatic reaction depends on a group that must be deprotonated, with a pK_a of 6.1.[316] This pK_a depends on the identities of the metals, consistent with the notion that this pK_a is that of a metal-coordinated water nucleophile. A break is observed in the Brønsted β_{lg}, suggesting that for good leaving groups (pK_a < 7) the phosphoryl transfer step is only partially rate-limiting; it is common for nonchemical step(s) in enzymatic mechanisms to be rate-limiting, or partially so. In the phosphotriesterase reaction, this is confirmed by primary and secondary kinetic isotope effects on the enzymatic reaction with paraoxon and diethyl 4-carbamoylphenyl phosphate, and by the LFER data. For poorer leaving groups, a very large and negative β_{lg} of -1.8 is found, indicative of fully rate-limiting phosphoryl transfer.[317] By comparison, the uncatalyzed hydrolysis of the same compounds yields a smaller $\beta_{lg} = -0.44$, and for the alkaline hydrolysis of diethyl 4-carbamoylphenyl phosphate, a smaller $^{18}k_{bridge} = 1.025$ is given, compared to 1.036 for the enzymatic reaction with this substrate.[318] The cumulative results support a mechanism involving attack by the bridging hydroxide, with a transition state in which bond fission to the leaving group is far advanced and the leaving group bears a substantial negative charge, without significant leaving group stabilization provided by the enzyme.

The residue Asp-301, which is hydrogen bonded to His-254, has been proposed to deprotonate the bridging hydroxide nucleophile.[319] Mutation of His-245 to alanine or asparagine reduces the rate of hydrolysis of paraoxon by 1−2 orders of magnitude. In contrast, the hydrolysis for the slower substrate diethyl *p*-chlorophenyl phosphate increases 2- to 33-fold.[319] This suggests that, for the more activated substrate, the rate-limiting step involves proton transfer that is assisted by the His-254/Asp-301 pair. For the Zn/Zn form of the enzyme, the solvent isotope effects on k_{cat}/K_m are very small for both substrates. With the faster substrate paraoxon, $k_{catH_2O}/k_{catD_2O} = 2.0$ while, with the slower substrate, diethyl *p*-chlorophenyl phosphate, $k_{catH_2O}/k_{catD_2O} = 1.3$. Similar trends were seen with the Cd/Cd and the Zn/Cd forms of the triesterase. The results support the proposal that, in the hydrolysis of paraoxon, k_{cat} is limited by a step involving proton transfer that follows P−O bond fission.[319]

4. Sulfate Transfer

4.1. Types of Sulfate Esters

Sulfuric acid may be esterified at one or two positions, to form a monoester or a diester. Diesters are highly reactive and have not been found to occur naturally. Monoesters have a number of important biochemical roles, but sulfate ester chemistry has been the subject of much less study than its phosphate counterpart. Sulfate ester formation has a crucial biological role in detoxification, and sulfate monoesters are found among many of the classes of natural products.

The same mechanistic possibilities exist for sulfuryl transfer as for phosphoryl transfer. If a monoester were to follow a fully dissociative, $D_N + A_N$ mechanism, a sulfur trioxide intermediate would form, analogous to the metaphosphate in the phosphoryl system. An addition−elimination ($A_N + D_N$) mechanism would form a pentacoordinate sulfurane intermediate.

4.2. Uncatalyzed Reactions of Sulfomonoesters

From what is known to date, the chemistry of sulfate monoesters is mechanistically similar to that of phosphate monoesters. Hydrolysis is faster under strongly acidic or basic conditions, with a broad pH-independent region between pH 4 and 12.[320,321] ^{18}O tracer studies have shown that aryl sulfates undergo hydrolysis by S−O bond fission in the pH-independent region.[320]

In the pH-independent region, the reactions of aryl sulfate proceed by mechanisms that are similar to those of aryl phosphate dianions, with a loose transition state in which the sulfuryl group resembles SO_3. This is supported by linear free energy relationships[320−324] and kinetic isotope effects in the sulfuryl group and in the leaving group,[325] data that are similar to those for reactions of phosphate monoester dianions. Sulfur isotope effects on the hydrolysis of *p*-nitrophenyl sulfate are also consistent with such a mechanism.[326] Stereochemical studies of sulfuryl transfer from

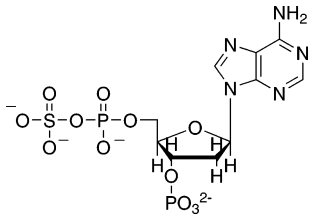

Figure 14. 3′-Phosphoadenosine-5′-phosphosulfate (PAPS), the most common biological sulfuryl donor.

phenyl [(R)-^{16}O,^{17}O,^{18}O] sulfate to an acceptor alcohol rule out the intermediacy of a free SO_3 intermediate.[327]

In very basic solutions (pH ≥ 13), where the rate of hydrolysis increases with [HO^-], both aryl and alkyl sulfate esters undergo hydrolysis via C−O bond cleavage.[328,329]

Under acidic conditions, the reactive species is the neutral ester. This reaction is believed to proceed by transfer of the proton from the sulfuryl group to the leaving group,[330] as in reactions of the monoanion of phosphate monoesters. A pre-equilibrium proton transfer is often assumed, but proton transfer in the same step as S−O bond fission is also consistent with the available data. A reduced value for β_{lg} [321] and a solvent isotope effect of 2.43[331] are consistent with proton transfer to the leaving group. Experiments with alkyl sulfates in ^{18}O-enriched water show that the reaction proceeds by S−O bond fission.[332] The intermediacy of free SO_3 in the acid hydrolysis is sometimes assumed but has never been proven.

4.3. Uncatalyzed Reactions of Sulfate Diesters

Sulfate diesters are not naturally occurring compounds. Dialkyl sulfates have found use in the laboratory as effective alkylating agents, and both these and alkyl aryl sulfate diesters react with nucleophiles via alkyl-O bond fission.[333,334] The hydrolysis of diaryl sulfate esters proceeds by nucleophilic attack at sulfur.[335,336]

4.4. Enzymatic Sulfuryl Transfer from PAPS

In 1876, Baumann demonstrated that phenol administered to a patient was excreted as phenyl sulfate.[337] It was not until some 80 years later that the most common biological sulfate donor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (Figure 14), was identified.[338]

Sulfotransferases (SULTs) catalyze the transfer of the sulfuryl group from a donor, which is most often PAPS, to hydroxyl or amine nucleophiles. The reverse reaction, the hydrolysis of sulfate esters, is catalyzed by sulfatases. Thus, these enzymes have a complementary relationship analogous to that of kinases and phosphatases.

The activation of an inorganic sulfate via its conversion to PAPS occurs in two steps, the first catalyzed by ATP sulfurylase. This enzyme catalyzes two reactions, the reaction between sulfate and ATP to form adenosine-5′-phosphosulfate (APS) and pyrophosphate, and the hydrolysis of GTP to GDP and inorganic phosphate. The GTPase activity is allosterically regulated by ATP and by sulfate. The binding of GTP and ATP are near equilibrium, and the rate-determining step in the hydrolysis of GTP is fission of the β,γ bond, in both the presence and the absence of ATP.[339] The energetics of the two reactions are linked, with GTP hydrolysis providing the energy to drive the less favorable reaction of activated sulfate synthesis. Upon binding of the three substrates ATP, sulfate, and GTP, a rate-limiting isomerization is followed by rapid GTP hydrolysis, which is in turn followed by the attack of sulfate at the α-phosphoryl group of ATP.[340] Product release is ordered, with inorganic phosphate departing before either GDP or pyrophosphate.

The synthesis of PAPS is completed by APS kinase, which uses ATP to phosphorylate the 3′-hydroxyl group of APS.

4.4.1. Sulfotransferases

These enzymes catalyze the formation of sulfate monoesters from an activated sulfuryl donor (often, but not always, PAPS) and amine or alcohol nucleophiles. Several sulfotransferases that do not use PAPS as their source of activated sulfur have been studied. The aryl sulfotransferases from *Aspergillus oryzae*, *Enterobacter amnigenus*, and *Eubacterium A-44* use a labile aryl sulfate such as *p*-nitrophenyl sulfate as a source of activated sulfur *in vitro*. These enzymes have been shown to follow ping-pong, bi−bi kinetic mechanisms.[341] Studies with radioactive ^{35}S-*p*NPS show that a tyrosine residue is sulfated,[342] and subsequent stereochemical investigation showed that a chiral sulfate group was transferred with net retention of configuration.[343] These enzymes are inactivated by the histidine-modifying agent diethyl pyrocarbamate, which led to a proposal that histidine acted as a nucleophilic sulfate carrier from *p*NPS to the enzymatic tyrosine, before transfer to the ultimate sulfuryl acceptor. This was ruled out by the stereochemical result, since such a sequence of three sulfuryl transfers should result in inversion. It is more likely that this histidine residue serves as a general base.

Among sulfotransferases that utilize PAPS, a number of structural and mechanistic investigations have been reported. Structures have been solved for catecholamine ST,[344] dopamine ST,[345] and hydroxysteroid ST.[346] The X-ray structures have been solved for estrogen sulfotransferase (EST), with PAP and estradiol bound,[347] and for the EST−PAP−vanadate complex.[348] The biological activities and methods of inhibition of sulfotransferases are summarized in recent reviews.[349,350]

Estrogen Sulfotransferase (EST). Estrogen sulfotransferase (EST) catalyzes sulfuryl transfer between PAPS and the phenolic oxygen atom of β-estradiol. The sulfation prevents β-estradiol from binding to and activating the estrogen receptor. EST catalyzes both the forward and the reverse reactions, and *p*-nitrophenol as well as *p*-nitrophenyl sulfate (*p*NPS) are alternate substrates. At the pH optimum of 6.3, EST catalyzes the sulfuryl transfer from PAPS to β-estradiol with an equilibrium constant of 4.5×10^3.[351] Kinetic studies reveal that the enzyme binds two β-estradiol molecules, with the second one providing allosteric inhibition ($K_i = 80$ nM).[351]

The most informative set of X-ray structures from the sulfotransferase family are those for EST. Two crucial residues are His-108 and Lys 106, which are each ∼3 Å from the oxygen atom of estradiol, respectively. Mutation of either of these residues has been found to significantly reduce EST activity.[352] Lys-106 is the only residue in a position to interact with the nonbridging oxygens of the transferring SO_3 moiety, and it probably functions in substrate positioning and transition-state stabilization. These structures led to the proposal of a concerted, in-line mechanism, and the absence of positively charged residues around the sulfuryl moiety argues against an associative mechanism that would result in an increased in negative charge on the sulfuryl group.

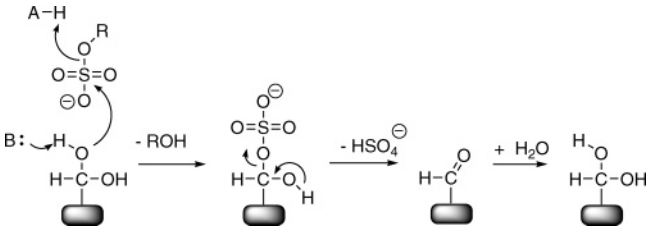

Figure 15. Mechanism consistent with the data for sulfate ester hydrolysis catalyzed by members of the aryl sulfatase A family. In the first step, nucleophilic attack by an aldehyde hydrate oxygen on the substrate forms a sulfoenzyme intermediate. In the second step, elimination of inorganic sulfate leaves an aldehyde moiety, which is hydrated by water to regenerate the active form of the enzyme.

β-Arylsulfotransferase IV. β-Arylsulfotransferase IV catalyzes the sulfation of a variety of substituted phenols, using PAPS as the sulfuryl donor. A linear free energy investigation carried out on the sulfuryl transfer from PAPS to a series of phenols, and the reverse reaction, found modest Brønsted β_{nuc} and β_{lg} values of +0.33 and −0.45, respectively.[353] These values were interpreted to indicate a loose, sulfur trioxide-like transition state. However, the influence of proton transfers to the leaving group and from the nucleophile on the Brønsted parameters is uncertain. His-104 is a likely candidate to deprotonate the nucleophilic phenol in the forward reaction and thus protonate the phenolate leaving group in the reverse process, but pH−rate data do not provide clear support for this role.[353]

An investigation of the uncatalyzed hydrolysis of PAPS found evidence for a very loose, sulfur trioxide-like transition state with weak involvement of the nucleophilic water molecule.[354] No evidence yet presented gives reason to believe that sulfotransferases significantly alter this transition state.

4.4.2. Sulfatases

Aryl sulfatases catalyze the hydrolysis of aryl sulfate monoesters to yield inorganic sulfate and the free phenol. The aryl sulfatases are the best characterized sulfatases, and the X-ray structure of the human ASA has been solved.[355] These enzymes utilize an unusual mechanism involving a conserved cysteine residue that is oxidized to an aldehyde, yielding a formylglycine. Without this post-translational modification, sulfatases are catalytically inactive, as revealed by a lysosomal storage disorder known as multiple sulfatase deficiency. Surrounding the hydrated aldehyde moiety are a number of polar residues, tightly interconnected by a network of hydrogen bonds, most of which are highly conserved among the eukaryotic and prokaryotic members of the sulfatase family.[356] One of the geminal oxygen atoms of the aldehyde hydrate is coordinated to a metal ion, believed to be either magnesium or calcium *in vivo*. Labeling experiments show that sulfuryl transfer proceeds via S−O bond fission, and ^{18}O is incorporated from water into inorganic sulfate.[357,358] Both kinetic studies and the stereochemical result with chiral sulfate indicate a ping-pong mechanism.[341,359] Bell-shaped pH−rate profiles of V_{max} for the hydrolysis of *p*-nitrocatecholsulfate by human ASA[356,360] and of V_{max}/K_m for the hydrolysis of *p*NPS by the ASAs from *Aerobacter aerogenes* and from *Helix pomatia*[361] indicate the utilization of acid−base catalysis. A mechanism consistent with these data is shown in Figure 15. The identities of the putative general acid and base residues are uncertain. A site-directed mutagenesis study of nine active site residues found that, in all cases, V_{max} was decreased to 1−26% of wild-type activity, failing to identify specific roles for any of these residues.[356]

4.4.3. Sulfate–Phosphate Chimeras

In some instances, sulfate has been substituted for phosphate in biological molecules and the mechanisms of the enzymatic reactions of these species investigated. Sulfoenolpyruvate, the sulfuryl analogue of phosphoenolpyruvate (PEP), has been shown to be a substrate for pyruvate kinase, producing pyruvate and adenosine 5′-phosphosulfate.[229] The sulfuryl-transfer reaction from sulfoenolpyruvate is 250−600-fold slower than the natural reaction of phosphoryl transfer from PEP under identical conditions. The metal ion dependencies are quite different for the two reactions; with PEP as the substrate, the relative catalytic efficiencies of the metal ions are $Mg^{2+} = Co^{2+} > Mn^{2+} > Ni^{2+}$, while the sulfuryl transfer is best catalyzed by Mn^{2+}.

Adenosine 5′-diphosphosulfate ($ADPSO_3$) is a substrate for nucleoside diphosphate kinase, as discussed above in section 3.4.7.[230] The reaction follows a ping-pong mechanism that is analogous to the natural reaction with ATP, though slower by 2−3 orders of magnitude.

5. Conclusion

This review has dealt with enzymes where structures and kinetic (particularly the isotope effect) studies have provided information on chemical mechanism and transition-state structure. As more X-ray structures are determined and more isotope effect and pH studies are carried out on other enzymes that catalyze transfer of phosphate and sulfate groups, our knowledge will clearly expand.

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