Sulfur as a mechanistic probe in enzymatic and non-enzymatic substitution at phosphorus†

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Sulfur continues to be a valuable mechanistic probe for nucleophilic substitution at phosphorus. The stereochemical courses of many enzymatic phosphoryl- and nucleotidyltransferases have been elucidated by the use of P-chiral phosphorothio-analogs of biological substrates. The results have clarified the issue of single displacement *versus* double displacement mechanisms in enzyme catalysis. The principle of economy in the evolution of binding sites appears to govern whether an enzymatic phosphotransfer proceeds by a double displacement mechanism or a single displacement mechanism. The weakness of the P–S bond has allowed evidence for the transient formation of monomeric metaphosphate to be obtained in the hydrolysis of *sym*-μ-monothiopyrophosphate.

Introduction

Phosphotransfer reactions play essential roles in most biological processes, including energy metabolism, intermediary metabolism, nucleic acid biosynthesis, protein biosynthesis, complex carbohydrate biosynthesis, natural product biosynthesis, vitamin biosynthesis, and signaling and control in biochemical processes. Westheimer discussed the essential properties of the phosphate group that makes it uniquely suited to its functions in living cells.[1] The biological phosphates are all products of enzymatic phosphotransfer. Biological phosphotransfers include phosphoryl group transfer, nucleotidyl transfer, phosphate ester hydrolysis, phosphoanhydride hydrolysis, and nucleotide hydrolysis among others. Enzymes grouped as phosphotransferases, phosphoisomerases, nucleotidyltransferases, phosphohydrolases, and nucleotidases catalyze these reactions. The functions of these enzymes, and the chemical mechanisms by which they act, are continuing themes in modern biochemistry.

In the period from the 1950s through the 1970s, the chemical mechanisms in the actions of phosphoryl- and nucleotidyltransferases were highly controversial. One central issue was the question of whether phosphoryl or nucleotidyl transfer proceeded in single enzymatic steps between donors and acceptors within non-covalent Michaelis complexes, as in eqn (1a)–(1c) for a phosphotransferase.

$$R_1OPO_3 + R_2OH + \mathbf{E} \rightleftharpoons R_1OPO_3\cdot\mathbf{E}\cdot HOR_2 \quad (1a)$$

$$R_1OPO_3\cdot\mathbf{E}\cdot HOR_2 \rightleftharpoons R_1OH\cdot\mathbf{E}\cdot O_3POR_2 \quad (1b)$$

$$R_1OH\cdot\mathbf{E}\cdot O_3POR_2 \rightleftharpoons R_1OH + R_2OPO_3 + \mathbf{E} \quad (1c)$$

Alternatively, the reactions might take place in two chemical steps *via* covalent phosphoenzyme intermediates, such as the **E**–X–PO_3 in eqn (2a) and (2b) where X = O, N or S in enzymes catalyzing phosphoryl group transfer.

$$R_1OPO_3 + \mathbf{E}\text{–}XH \rightleftharpoons \mathbf{E}\text{–}XH\cdot R_1OPO_3 \rightleftharpoons \mathbf{E}\text{–}X\text{–}PO_3 + R_1OH \quad (2a)$$

$$\mathbf{E}\text{–}X\text{–}PO_3 + R_2OH \rightleftharpoons \mathbf{E}\text{–}X\text{–}PO_3\cdot R_2OH \rightleftharpoons \mathbf{E}\text{–}XH + R_2OPO_3 \quad (2b)$$

The enzymatic nucleophiles O, N and S in enzymes are found in serine, threonine, aspartate, glutamate, tyrosine, histidine, lysine and cysteine.

In principle, the two mechanisms could be distinguished kinetically and biochemically. Steady-state analysis would show ternary enzyme–substrate complex formation in the

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† The author is pleased to dedicate this article to Professor W. J. Stec, a good friend and a dominant force in the chemistry and biochemistry of phosphates, on the occasion of his 70th birthday. This article is part of a themed issue on Biophosphates.

Perry A. Frey

Perry Frey's research on enzymatic phosphate metabolism led him to discover the stereochemical courses of phosphotransfer by many enzymes. His research on enzymatic free radical reactions led to the discovery of more than a dozen organic free radicals in enzymatic sites. His research on serine proteases led to the identification of a low barrier or short-strong hydrogen bond in the active site of chymotrypsin. He was elected to the U. S. National Academy of Sciences in 1998.

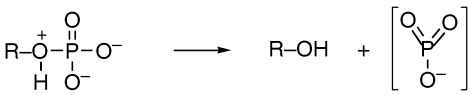
direct transfer mechanism of eqn (1a)–(1c). Alternatively, a kinetic analysis might show strictly binary enzyme–substrate complexes and a chemically altered enzyme species in the double displacement mechanism of eqn (2a) and (2b), where the covalent phosphoryl-enzyme **E**–X–PO_3 is a compulsory intermediate. The issue could be addressed biochemically in experiments designed to isolate and characterize covalent phosphoenzymes.

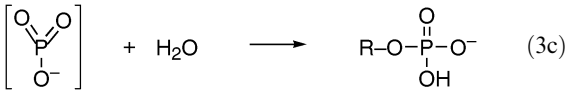
In practice, the traditional kinetic and biochemical methods failed in definitively distinguishing single and double displacement mechanisms. This was in part because steady-state kinetics could not rule out covalent intermediates intervening between non-covalent Michaelis complexes such as R_1OPO_3·**E**·HOR_2 and R_1OH·**E**·O_3POR_2 in eqn (1). In principle, transient kinetics coupled with chemical trapping might enable the detection of a covalent phosphoenzyme, but the absence of such a species would constitute negative evidence upon which no conclusion could be based. A further complication was that many phosphotransferases reacted with phosphodonor substrates to form covalent phosphoenzymes in side reactions that were kinetically incompetent and not part of the actual catalytic mechanism.

Stereochemical analysis of phosphoryl- and nucleotidyl-transfer by enzymes overcame the ambiguities and complications. Substrates could be synthesized with chiral phosphorus centers fashioned by stereospecific placement of ^{18}O, ^{17}O, or/and S about the phosphorus undergoing nucleophilic substitution. Then, if the P-chiral group in the product was inverted in configuration, an uneven number of transfers, generally one, had occurred in the mechanism. If the configuration of the phosphorus was retained in the product, an even number of transfers, generally two, had occurred in the mechanism. The stereochemical information was always positive evidence and allowed definitive mechanistic conclusions. Professor W. J. Stec was an early and major figure in the field of stereochemical analysis in enzymatic substitution at phosphorus. In particular, he developed valuable synthetic methods for the preparation of P-chiral substrates for enzymes, and he employed these P-chiral molecules in research on biological reactions.[2] These studies became enduring contributions to the science of enzymatic phosphoryl- and nucleotidyl-transfer.

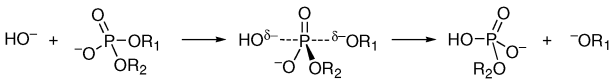
Another issue during the period of the 1960s through the 1990s was the question of whether enzymatic transition states were loose, characterized largely by bond cleavage to phosphorus, or tight and characterized by bond formation to phosphorus. The extreme case in phosphoryl group transfer was whether metaphosphate monoanion [PO_3^-] was an intermediate. Early physical organic chemical analysis of non-enzymatic hydrolysis of phosphomonoester monoanions supported the intermediacy of [PO_3^-] as a chemical intermediate in a classical S_N1 mechanism according to eqn (3a)–(3c).[3]


(3a)


(3b)


(3c)

In the cases of phosphoryl-ester transfer in general, physical organic analysis of non-enzymatic hydrolyses of phosphodiesters and phosphotriesters clearly implicated a classical S_N2 mechanism, in which the reacting nucleophile displaced the leaving group in a single, tight transition state, as in eqn (4).[4] The transition states in such reactions are characterized by increased bonding to phosphorus, in contrast to the decrease in bonding to phosphorus in the hydrolysis of phosphomonoester monoanions.


(4)

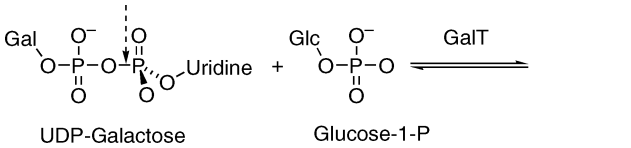
Stereochemical analysis gave valuable information about the transition states for both classes of reaction. The main evidence in resolving these issues came from structure/reactivity studies.[5] Detailed structure–function kinetic analysis of phosphoryl group transfer showed that in general the transition state includes the phosphoryl-acceptor in a very loose association, with little bonding to either the phosphoryl donor or acceptor. The rates only slightly depended on the basicity of the acceptor but were strongly dependent on the basicity of the leaving group in the donor.[5] Thus, free [PO_3^-] could not be sustained as an intermediate in most phosphoryl transfer reactions in aqueous solutions, but the transition state was concluded to be very loose, with little bonding to the leaving group and little bonding to the acceptor.[5]

Stereochemistry of enzymatic substitution at phosphorus

The work of this laboratory in the field of phosphotransfer has been directed mainly toward the question of whether the mechanisms involved single displacement or double displacement at phosphorus. We adopted the stereochemical approach. It had been proposed that all group-transferring enzymes functioned by covalent catalysis, wherein the group was first transferred from the donor substrate to an enzymatic nucleophile to form a covalent enzyme–substrate intermediate.[6] The specific examples put forward were phosphotransferases and the issue of single *versus* double displacement mechanisms. We addressed this hypothesis first in the cases of uridylyltransferases and then in the cases of phosphoryl group transferases. We found that the hypothesis of a unified double displacement mechanism failed for both uridylyltransferases and phosphotransferases. Both single and double displacement mechanisms were found by stereochemical, kinetic and biochemical methods. A rationale for the differences put forward from this laboratory could be stated as the "principle of economy in the evolution of binding sites".[7]

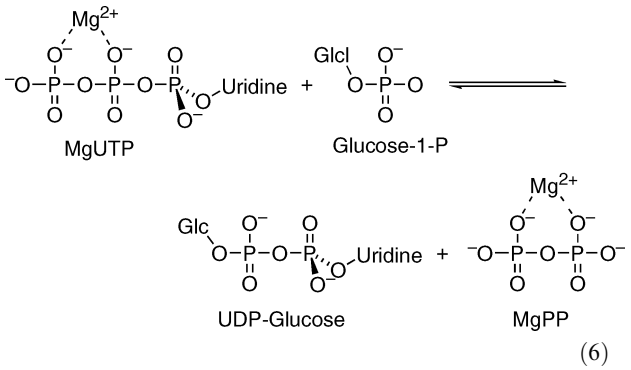
Uridylyltransferases

Eqn (5) shows the reaction catalyzed by hexose-1-P uridylyltransferase (GalT in *Escherichia coli*).



(5)

This uridine-5′-phosphoryl (uridylyl) interchanges between galactose-1-P and glucose-1-P. GalT is required for the metabolism of galactose in all cells. A different enzyme, UDP–glucose pyrophosphorylase, catalyzes uridylyl interchange between glucose-1-P and magnesium pyrophosphate (MgPP), according to eqn (6). UDP–glucose pyrophosphorylase is required for the production of UDP–glucose in the biosynthesis of complex carbohydrates.

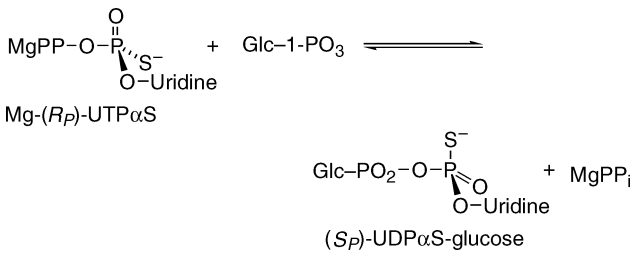


(6)

GalT and UDP–glucose pyrophosphorylase catalyze chemically similar reactions, the interchange of uridylyl groups between phosphate acceptors. However, the kinetic and biochemical evidence suggested different reaction mechanisms. In the case of GalT, the steady state and transient kinetics supported a double displacement mechanism proceeding through a covalent uridylyl–GalT intermediate (**E**–X–UMP in eqn (2a) and (2b)).[8] Evidence from biochemical, mutagenic and chemical rescue experiments implicated H166 in the amino acid sequence as the active site nucleophile.[8]

In contrast, the steady-state kinetics of UDP–glucose pyrophosphorylase implicated a mechanism such as that of eqn (1a)–(1c), involving ternary complex formation, with substrates binding in compulsory order, uridine nucleotide first, and no chemical reaction until both substrates were bound.[9] A report of a uridylyl–pyrophosphorylase complex proved to be due to the very tight, non-covalent binding of substrates to this enzyme.[9] Nevertheless, a covalent uridylyl–enzyme intermediate intervening between the ternary complexes in eqn (1a)–(1c) could not be excluded by kinetic and biochemical evidence.

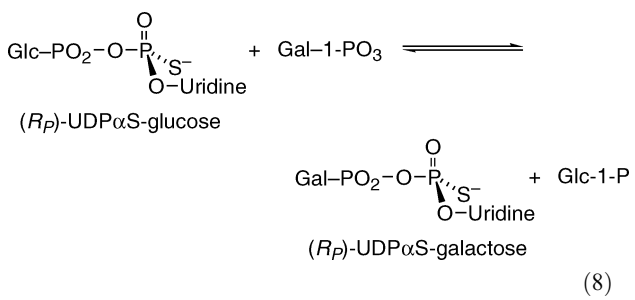
The stereochemical test excluded the double displacement mechanism for UDP–glucose pyrophosphorylase. The α-thio analogs of UTP, UDP, and UDP–glucose could be synthesized by phosphoanhydride coupling between uridine-5′-phosphorothioate and PP_i, P_i, or glucose-1-P, and the epimeric mixtures of α-thio-nucleotides could be separated by chromatography and distinguished spectroscopically by ^{31}P-NMR chemical shifts. UDP–glucose pyrophosphorylase accepted (R_P)-UTPαS as a substrate, and it catalyzed the reaction of this α-thio epimer with glucose-1-P to produce (S_P)-UDPαS-glucose, that is, with inversion of configuration according to eqn (7).[10]



(7)

If the enzymatic reaction had proceeded by a double displacement mechanism, the product of retention of configuration should have been produced.

To verify that retention of configuration does indeed occur in a double displacement at phosphorus, the stereochemical test was applied to GalT. Enzymes catalyzing nucleotidyl transfer with α-thio-nucleotides display a high degree of epimeric specificity at the α-P. Whereas, UDP–glucose pyrophosphorylase acted specifically on (S_P)-UDPαS-glucose, GalT functioned best on the (R_P)-epimers of substrates. When GalT was presented with (R_P)-UDPαS-glucose and galactose-1-P, it produced (R_P)-UDPαS-galactose, the product of retention, as the nucleotide product according to eqn (8).[11]



(8)

Based on the principle of economy in the evolution of binding sites, the GalT-mechanism implied the presence of a single glycosyl-phosphate binding site in GalT, which could bind either galactose-1-P or glucose-1-P, or either glycosyl-P moieties of UDP–galactose or UDP–glucose. A molecular structure of GalT obtained by X-ray crystallography opened the way for detailed investigation of this issue. The ribbon diagram in Fig. 1 showed GalT to have chain fold that was unique at the time.[12] The crystal structure of the isolated uridylyl–GalT, shown in Fig. 2, further confirmed the assignment of H166 as the catalytic nucleophile.[13] That galactosyl and glucosyl moieties bind at the same site was confirmed by the crystal structures of H166G-GalT with either UDP–galactose or UDP–glucose bound at the active site.[14]

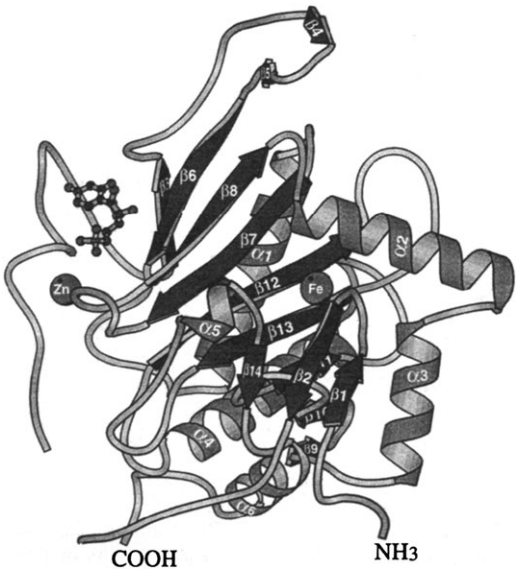


Fig. 1 Ribbon diagram of the molecular structure of GalT. Each subunit of dimeric GalT comprises an 8-stranded antiparallel β-sheet bounded by 6 α-helices. The ball and stick model is UMP bound to the active site. An essential Zn^{2+} is ligated to H164 of the active site motif $_{164}$HPHGQ$_{168}$. The essential nucleophile, H166, is shown in Fig. 2. Adapted from Fig. 4 of ref. 12 with permission from the publisher, the American Chemical Society.

A need for a second acceptor binding site in the structure is obviated by the presence of the nucleophilic catalyst, H166, in the active site. H166 accepts the uridylyl-group from the donor substrate, which allows one hexose-1-P to dissociate and the other to bind, and then the uridylyl-group can be transferred to form the product. Thus, in eqn (2a) and (2b) for GalT, **E**–XH is **E**–H166, and **E**–X–PO_3 is **E**–H166–UMP. The reaction proceeds in two steps with inversion of configuration at phosphorus in each step, and overall retention.

In the case of UDP–glucose pyrophosphorylase, the acceptor substrates in the forward and reverse directions, $MgPP_i$ and glucose-1-P, are too different structurally and electrostatically to bind to a single site. Consequently, the enzyme had to evolve with separate binding sites for each acceptor substrate. These sites corresponded to the subsites for the MgPP- and glucosyl-1-P-moieties of the uridylyl-donor (nucleotide) substrates. Thus, both the donor and acceptor substrates could bind and occupy both sites in ternary complexes. With both uridylyl-donor and acceptor substrates present in the ternary complexes, there is no need for a catalytic nucleophile or covalent intermediate to preserve bond energy during an interchange between uridylyl-group acceptors, and the reaction proceeds with direct transfer of the uridylyl-moiety from donor to acceptor substrate and inversion of configuration at phosphorus.

Phosphotransferases

The question of single *versus* double displacements in enzymatic phosphoryl transfer was similarly resolved. The P-chiral γ-thio-γ-^{18}O-analog of ATP could be synthesized as in Scheme 1. Phosphorylation of AMPS,$^{18}O_2$ by ATP proceeds exclusively at the pro-S_P ^{18}O in the presence of adenylate kinase. Coupling of the resultant (S_P)-ADPαS,$^{18}O_2$ with 2′,3′-methoxymethylidene-AMP gives the half-protected dinucleoside triphosphate. Removal of the unprotected adenosyl moiety by periodate

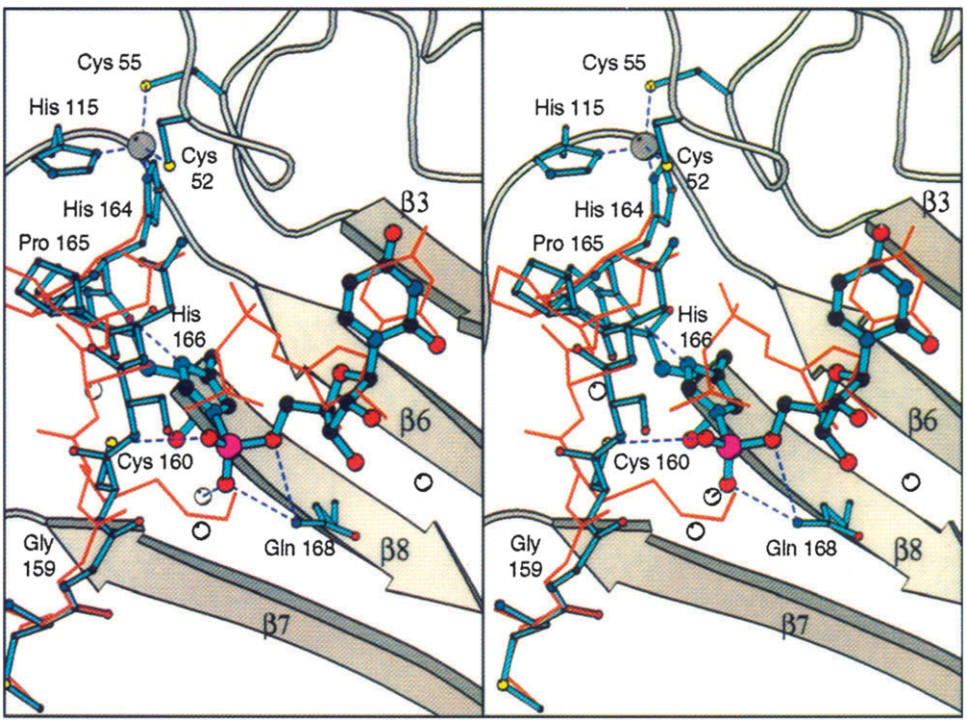
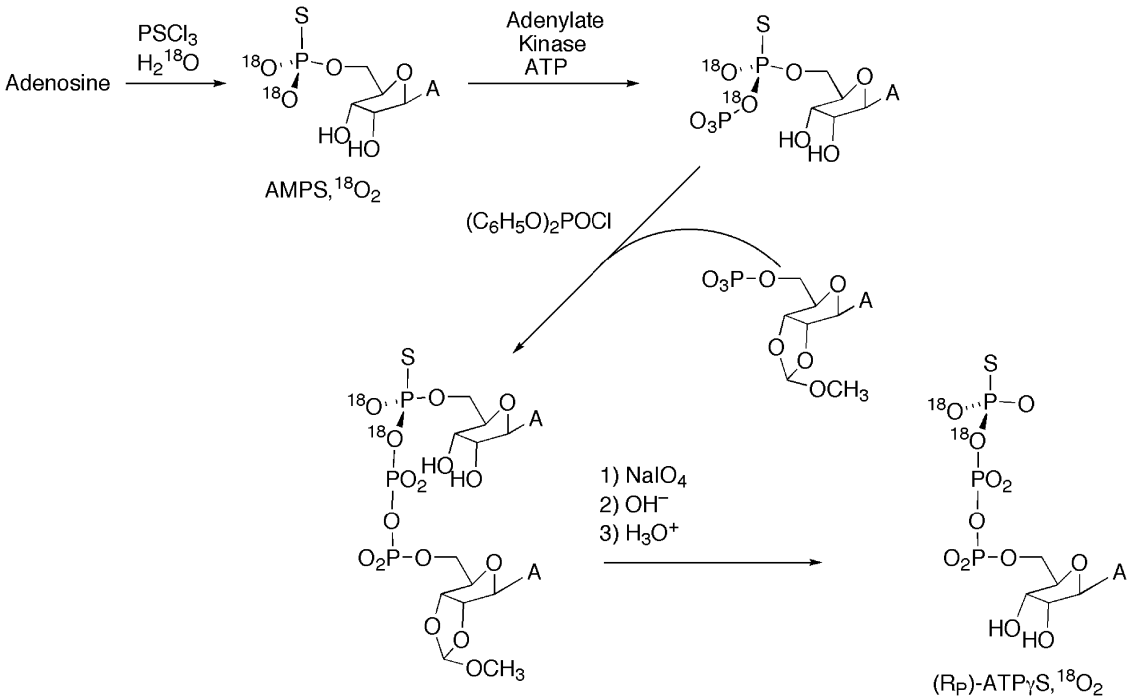


Fig. 2 The nucleotide binding site of uridylyl–GalT. Shown are the left and right images of a stereodiagram of a ball and stick model of the active site motif in uridylyl–GalT embedded in the protein structure. H164 is shown ligated to Zn^{2+}, and H166 is covalently bonded to the uridylyl-group. Adapted from Fig. 6 of ref. 13 with permission from the publisher, the American Chemical Society.



Scheme 1 Synthesis of (R_p)-ATPγS,$^{18}O_2$.

cleavage and alkaline elimination, followed by deprotection, gives (R_P)-ATPγS,$^{18}O_2$.[15]

(R_P)-ATPγS,$^{18}O_2$ serves as a thiophosphoryl-donor substrate for adenylate kinase and nucleoside diphosphate kinase, which catalyze the reactions of eqn (9) and (10)

$$\mathrm{ATP} + \mathrm{AMP} \overset{\text{Adenylate kinase}}{\rightleftharpoons} \mathrm{ADP} + \mathrm{ADP} \qquad (9)$$

$$\mathrm{ATP} + \mathrm{GDP} \overset{\text{Nucleoside diphosphate kinase}}{\rightleftharpoons} \mathrm{ADP} + \mathrm{GTP} \qquad (10)$$

Adenylate kinase replenishes the adenine nucleotide pools by keeping them phosphorylated. Nucleoside diphosphate kinase also serves to keep nucleotide pools highly phosphorylated. It is shown in eqn (10) phosphorylating guanosine-5′-diphosphate, but it accepts all nucleoside-5′-diphosphates and so can act in concert with adenylate kinase to transform AMP into ATP. Nucleoside diphosphate kinase also maintains GTP levels, in that way supporting the role of GTP in biological signaling.

The two reactions in eqn (9) and (10) are chemically similar. However, the phosphoryl-acceptor substrates in the forward and reverse directions for adenylate kinase, AMP and ADP, are structurally and electrostatically dissimilar in their phospho-substituents. They could not be expected to bind to the same subsite of an enzyme. The steady-state kinetics implicates a ternary complex mechanism such as that in eqn (1a)–(1c), with the donor and acceptor substrates binding randomly.[16] No evidence of a covalent phosphoryl-enzyme has been reported. In contrast, the acceptor substrates for nucleoside diphosphate kinase in forward and reverse directions are structurally and electrostatically similar. They could bind to a single subsite in the enzyme. In this case, the steady-state kinetic mechanism implicates the mechanism of eqn (2a) and (2b), the double displacement or ping pong mechanism, and the covalent phosphoryl-enzyme has been isolated and characterized as a phosphohistidyl enzyme.[17]

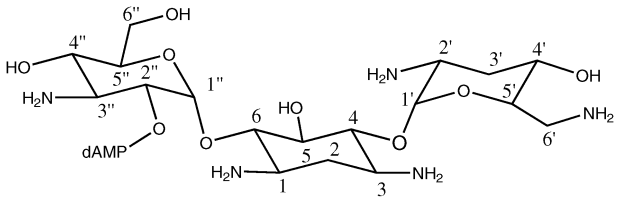
Employing the P-chiral substrate (R_P)-ATPγS,^{18}O, adenylate kinase catalyzed thiophosphoryl transfer with inversion of configuration at phosphorus, and the reaction of nucleoside diphosphate kinase proceeded with retention of configuration.[11,18] The chemically similar reactions occur by distinct mechanisms, single displacement within ternary complexes for adenylate kinase, and double displacement *via* binary complexes and a phosphoryl-enzyme intermediate for nucleoside diphosphate kinase. Analogous to the uridylyltransferases, the evolution of the two phosphotransferases appears to have been guided by the "principle of economy in the evolution of binding sites".

Adenylyltransferases

Aminoglycosides are rendered biologically inactive by aminoglycoside adenylyltransferases, which catalyze adenylylation of the C2″-OH groups in the antibiotics. These enzymes accept ATP or dATP as adenylylating substrates and release PP_i upon adenylyl transfer.[19] Chart 1 shows the structure of the adenylylated 2″-dAMP-tobramycin.[19]

The adenylyl-acceptor substrates in forward and reverse reactions are chemically, sterically and electrostatically dissimilar, and the reaction kinetics indicates a sequential binding mechanism proceeding through ternary complexes.[19]

Adenylyl-group transfer by gentamicin adenylyltransferase proceeds with overall inversion of configuration at P_α of (S_P)-dATPα^{17}O.[20] Scheme 2 outlines the synthesis of (S_P)-dATPα^{17}O. Activation of dAMPS with $(PhO)_2POCl$ followed by coupling with AMP gives (R_PS_P)-P^1-5′-deoxyadenosine-P^2-5′-adenosine-[1-thiodiphosphate]. After separation of the P-epimers



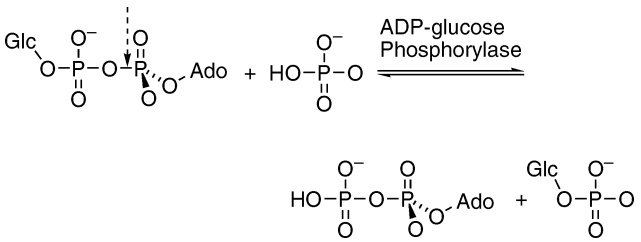
2''-dAMP-tobramycin

Chart 1

by HPLC, the (R_P)-epimer treated with BrCN in $H_2{}^{17}O$ displaces S with ^{17}O (inversion) to produce (S_P)-P^1-5′-deoxyadenosine-P^2-5′-adenosine-[1-^{17}O]diphosphate. Periodate cleavage followed by alkaline elimination of the adenosyl moiety gives (S_P)-[α-^{17}O]ADP, which is phosphorylated by P-enolpyruvate and pyruvate kinase to (S_P)-[α-^{17}O]ATP.

After adenylylation of gentamicin by the adenylyltransferase employing (S_P)-[α-^{17}O]ATP, hydrolytic degradation of the product with $H_2{}^{18}O$ and snake venom phosphodiesterase (retention) led to (R_P)-adenosine-5′-[^{17}O,^{18}O]phosphate, the product of inversion in the adenylylation of gentamicin. This stereochemistry ruled out a double displacement mechanism, in accordance with expectations, owing to the structural differences between pyrophosphate and gentamicin, and the anticipated need for separate binding sites.

The ADP–glucose phosphorylase recently identified in *Arabidopsis thaliana* catalyzes adenylyl group transfer from ADP–glucose to P_i according to eqn (11).[21]



(11)

The reaction is chemically reminiscent of that catalyzed by GalT (see eqn (5)). The chemical and kinetic mechanisms are indeed strictly analogous. All species of galactose-1-P uridylyltransferase include the sequence motif **HXHXQ**; in GalT the sequence is $_{164}$HPHGQ$_{168}$, and the catalytic nucleophile is H166. ADP–glucose phosphorylase contains the motif $_{184}$HSHSQ$_{188}$, and the crystal structure is very similar to that of GalT.[21] The kinetic mechanism is the same as that of GalT, ping pong bi bi, consistent with the double displacement mechanism. The structure of the enzyme soaked with ADP–glucose shows the adenylyl

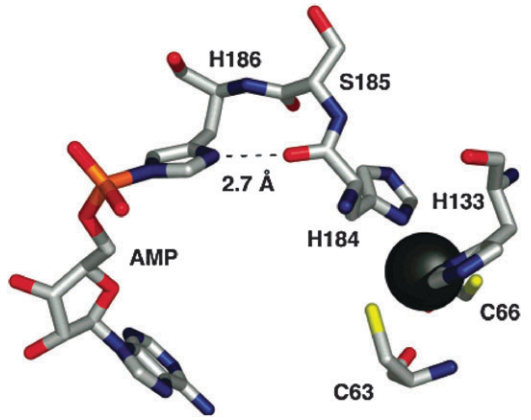


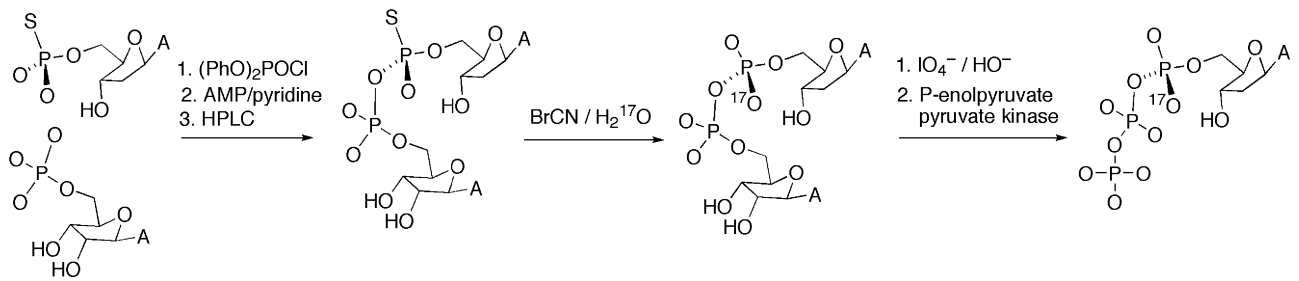
Fig. 3 Active site of adenylyl-ADP–glucose phosphorylase. The adenylyl-group of the covalent intermediate was found bonded to H186 of the active site peptide $_{184}$HSHSQ$_{188}$ in the crystal structure of the adenylyl-intermediate. This view also shows how H184 is ligated to Zn^{2+}, together with C63, C66, and H133. Reproduced from Fig. 6 of ref. 21 with permission from the publisher, the American Chemical Society.

group bonded to H186, the catalytic nucleophile, as shown in Fig. 3.[21]

As in the cases of GalT and nucleoside diphosphate kinase, the acceptor substrates for ADP–glucose phosphorylase are phosphates with similar electrostatic requirements. Although phosphate is sterically smaller than glucose-1-P, the two could well alternately occupy the same binding site. Moreover, ADP–glucose phosphorylase accepts galactose-1-P and mannose-1-P as substrates, as well as glucose-1-P.[21] Like the other enzymes displaying double displacement mechanisms, ADP–glucose phosphorylase appears to be an example of the "principle of economy in the evolution of binding sites", wherein the acceptor substrates in the forward and reverse directions alternately occupy the same binding site.

The histidine triad (HIT) superfamily

A family of nucleotide binding proteins known as the HIT superfamily is characterized by the sequence motif **HXHXH**. This motif is analogous to the **HXHXQ** of the hexose-1-P uridylyltransferases such as GalT. The steric requirement and hydrogen bonding patterns of glutamine (Q) are similar to those of histidine (H), and the replacement of one by the other is regarded as structurally, but not chemically, conservative. One member of the HIT superfamily, Fhit for fragile histidine triad in humans, catalyzes the hydrolysis of P^1,P^3-diadenosine

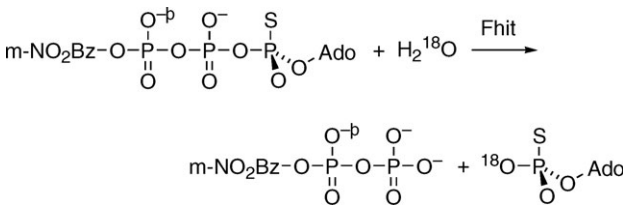


Scheme 2 Synthesis of (S_p)-2′-deoxyadenosine-5′-[1-^{17}O]triphosphate.

triphosphate (Ap_3A) to AMP and ADP. Fhit is often associated with human cancers. Another member, Hint, was originally identified as an AMP-binding protein, but is now known to be a nucleoside-5′-phosphoramidase.

The crystal structure of the dimeric Hint protein with AMP bound proved to be similar in chain fold and overall molecular morphology with GalT. An interesting difference was that the molecular weight of Hint was about half that of GalT. Nevertheless, the chain fold of the Hint-dimer nicely overlaid that of the GalT monomer.[22]

The analogies between the characteristic sequence motifs in the GalT and HIT families, together with the structural similarities between GalT and Hint, suggested a commonality in reaction mechanism. Because of the hydrolytic specificity of Fhit, steady-state kinetics was not a promising method to test for a double displacement mechanism. Instead, the stereochemical approach was adopted. It was first necessary to determine the orientation of the hydrolytic addition of water to AP_3A, that is, whether water was added to P_α or P_β to produce AMP and ADP. Fhit-catalyzed hydrolysis of AP_3A in $H_2{}^{18}O$ led exclusively to adenosine-5-[^{18}O]phosphate, proving that water had reacted with P_α of Ap_3A.[23] While Ap_3A was known to be an excellent substrate for Fhit, the enzyme was not highly specific and readily accepted substrates in which one adenosyl moiety was replaced with the *m*-nitrobenzyl substituent. Thus, P^3-*m*-NO_2Bz-ATP proved to be nearly as good a substrate as AP_3A.[23] For the stereochemical analysis, the reactions of either (R_P)- or (S_P)-P^1-5′-adenosyl-P^3-*m*-nitrobenzyl-1-thiotriphosphate proceeded in $H_2{}^{18}O$ with retention of configuration at phosphorus, as illustrated in eqn (12) for the (S_P)-epimer.[23] Both epimers reacted as substrates for Fhit with similar kinetic parameters, and both reacted with stereochemical retention at P_α.



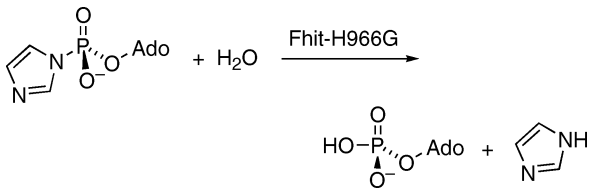
(12)

Retention of P-configuration supported the hypothesis that Fhit acted by a double displacement mechanism through a covalent adenylyl–enzyme intermediate. The isolation of an adenylyl–enzyme was complicated by its rapid hydrolysis in the putative mechanism of eqn (13a) and (13b)

$$\text{Fhit-His96} + AP_3A \rightarrow \text{Fhit-His96-AMP} + \text{ADP} \quad (13a)$$

$$\text{Fhit-His96-AMP} + H_2O \rightleftharpoons \text{Fhit-His96} + \text{AMP} \quad (13b)$$

Therefore, a denatured covalent adenylyl-Fhit was isolated by alkaline denaturation of the enzyme in the presence of [8,8′-^{3}H]Ap_3A.[24] That the central histidine in the triad, H96, was the active site nucleophile was proven by the complete inactivity of the mutated enzyme Fhit-H96G toward Ap_3A. The activity could be rescued by the addition of free imidazole. Moreover, this variant of Fhit efficiently catalyzed the hydrolysis of adenosine-5′-phosphoimidazolide (AMP-Im according to eqn (14), as if it were the intermediate Fhit-His96-AMP).[24]



(14)

This latter experiment served as a kind of chemical rescue of the otherwise inactive Fhit-H96G. Studies of pH-dependence in the action of Fhit indicated that H98 participates in the binding of the substrate to Fhit.[25]

As a further test of the chemical rescue by AMP-Im, and the implication that it serves as a surrogate for the covalent adenylyl-Fhit, Fhit-H96G has been employed as a dinucleoside polyphosphate synthase.[25] The design principle for this application is the reversibility of eqn (13a) and the presumptive surrogacy of AMP-Im. Under this principle, Fhit-H96G should catalyze the reaction of AMP-Im with ADP to produce Ap_3A; high concentrations of ADP should suppress the hydrolytic reaction in favor of Ap_3A formation; and Ap_3A should accumulate as the stable end product owing to the inactivity of Fhit-H96G toward normal substrates. Fhit-H96G does indeed catalyze the reaction of AMP-Im and ADP to produce Ap_3A in very high yield relative to AMP-Im.[25] This process further confirms the mechanism of eqn (13a) and (13b) and the chemical surrogacy of AMP-Im for adenylyl-Fhit.

Because of the low substrate specificity of Fhit, numerous P^1,P^3-dinucleoside triphosphates and P^1,P^4-dinucleoside tetraphosphates could be synthesized in high yields in reactions of nucleoside-5-phosphoimidazolides with nucleoside di- and triphosphates catalyzed by Fhit-H96G.[26] Most dinucleoside triphosphates were produced in good yields by use of nucleoside-5′-diphosphates as nucleotidyl acceptors with a panel of nucleoside-5′-phosphoimidazolides. Magnesium complexes of nucleoside-5′-triphosphates also served as acceptors in the synthesis of dinucleoside tetraphosphates. Fhit has also been shown to catalyze a number of other reactions, including the cleavage of P–F bonds.[27]

Other members of the HIT superfamily catalyze hydrolyses of nucleotide derivatives, for example the hydrolytic cleavage of the P–N bond in adenosine-5′-*O*-[*N*-(tryptophanylamide)]-phosphoramidothioate by Hint has recently been shown by Professor Stec and his associates and collaborators to proceed with retention of configuration at phosphorus.[28]

The similarities in protein structure, active site motifs, and reaction mechanisms between enzymes of the GalT family and those in the HIT superfamily have led to the incorporation of the GalT family as a branch of the HIT superfamily.

Sulfur as a mechanistic probe in phosphoanhydride hydrolysis

In the preceding sections, the use of sulfur as a substituent of phosphorus has been described for the induction of stereochemistry. The P–S bonds were employed either to create

enzymatic substrates with chiral centers at phosphorus as substrates for phospho- or nucleotidyltransferases or as the starting material to create a P-chiral center by displacement of S with ^{17}O. In the following sections the chemical and biochemical properties of *sym*-μ-monothiopyrophosphate monoanion ($HO_3P–S–PO_3^-$) are presented. This molecule allows the chemical consequences of the replacement of oxygen with sulfur in pyrophosphate to be explored.

The P–S (~45 kcal mol^{-1}) bond is much weaker than the P–O bond (~80 kcal mol^{-1}). Therefore, in a transition state in which the cleavage of a bond to phosphorus is the most important feature of the transition state, the P–S bonded molecule should react faster than the P–O bonded species. In a reaction in which bond formation to phosphorus is the dominant feature of the transition state, there should be little difference in the reactivities of P–S and P–O molecules. To exploit this principle, the chemical and biochemical reactivities of *sym*-μ-monothiopyrophosphate (MTP) and of its tetramethylester (Me_4MTP) have been explored.

Me_4MTP was synthesized as described by Michalski and co-workers by a procedure involving the use of electrophilic sulfur and nucleophilic phosphorus to complete the P–S bond formation.[29] Me_4MTP was demethylated by a modification of the procedure of Loewus and Eckstein[30] and stored as its tetralithium salt at −80 °C, conditions under which it was stable.

Hydrolysis of MTP

The pH- and temperature-dependencies for the hydrolysis of MTP yielded estimates of the third and fourth acid dissociation constants and of the enthalpies and entropies of activation.[31] The kinetically estimated value pK_{a_3} was 4.7 at 25 °C, somewhat lower than that of pyrophosphate (6.0) as expected. The kinetically estimated value of pK_{a_4} was 8.4, very similar to that of pyrophosphate. The entropy of activation ($\Delta S^{\ddagger}$) for hydrolysis of the trianion at pH 7 was +0.2 cal deg^{-1} mol^{-1}, and the enthalpy of activation ($\Delta H^{\ddagger}$) was 20 kcal mol^{-1}. The thermodynamic parameters were consistent with a kinetically monomolecular process. Changes in solvent polarity, either by increasing the ionic strength or by addition of dioxane, had very little effect on the rate of hydrolysis of MTP.

The striking differences between the hydrolysis of PP_i and MTP reside in the rates.[31] The ratios of first order rate constants (k_{MTP}/k_{PP_i}) are 5.8×10^7 for the dianions and 1.7×10^7 for the trianions. For the trianions the rate constants at 25 °C are 2×10^4 s^{-1} for MTP and 1×10^{-3} s^{-1} for PP_i. The >10-million-fold rate differences are indicative of transition states characterized by bond cleavage to the leaving group rather than by bond formation to water. Thereby, the weakness of the P–S bond in MTP strongly promotes the hydrolysis reaction.

The effects of added nucleophiles on the hydrolysis of MTP suggest that monomeric metaphosphate monoanion is a discrete intermediate within a solvation envelope. Like other hydrolytic reactions of phosphate compounds, the hydrolysis of MTP is not subject to general base catalysis. Moreover, hydrolysis in the presence of nucleophiles at very high concentrations does not increase the rate of P–S cleavage.[32] Nucleophiles displaying the α-effect in reactivity, including hydrazine, hydroxylamine and methyoxylamine, do not increase the rate, and they are not phosphorylated by MTP. Tris-(hydroxymethyl)methylamine (tris) and ethylenediamine (en) at high concentration (1–3 M) do not increase the rate of P–S cleavage, but at these concentrations they compete with water to capture the phosphoryl group. Thus, MTP phosphorylates tris and en at the same observed rate that it undergoes hydrolysis. The results support the mechanism in Scheme 3 for the reaction of tris.[32]

In this mechanism, as shown on the upper line, MTP^{3-} is normally solvated by water and decomposes with a rate constant k_1 to $HPSO_3^{2-}$ and $[PO_3^-]$ within the solvation envelope. One of the solvating water molecules reacts with $[PO_3^-]$ within the solvation sphere at a much higher rate constant to produce $H_2PO_4^-$. In the presence of a high concentration of tris, the solvation sphere can accommodate a molecule of tris as a solvating species at the expense of some of the solvating water, as shown on the lower line of Scheme 3. The decomposition of MTP^{3-} proceeds with the same rate constant k_1, and the resultant $[PO_3^-]$ is captured nearly randomly by solvating water, a CH_2OH of solvating tris, or the NH_2 of tris to form a mixture of products.[32]

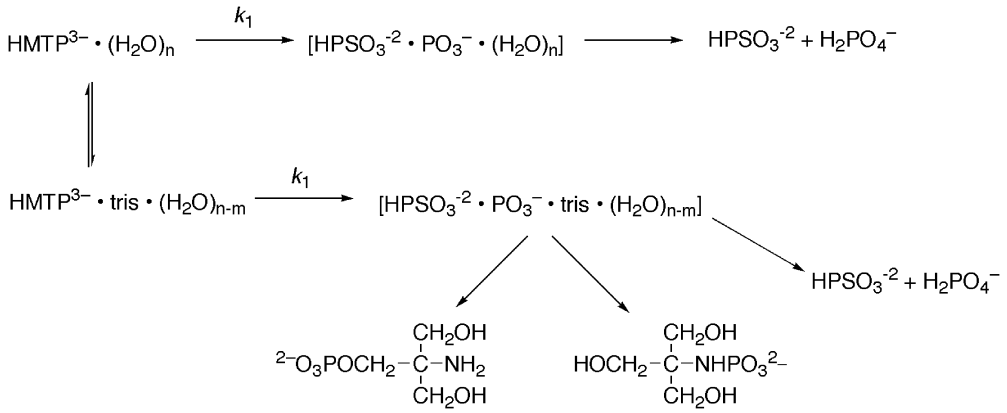
Monomeric metaphosphate monoanion cannot be a discrete intermediate in the hydrolysis of phosphomonoesters or phosphoanhydrides because of observations proving that the phosphoryl group acceptor participates in a loose transition state characterized by P–O bond cleavage to the leaving group and little bond formation to the acceptor.[5] However, when the bonding between phosphorus and the leaving group is weak, as it is in MTP^{3-}, available evidence implicates monomeric metaphosphate monoanion as a discrete intermediate.

Hydrolysis of Me_4MTP

Unlike MTP, the activation entropy ($\Delta S^{\ddagger}$) for the hydrolysis of Me_4MTP is large, −23 cal deg^{-1} mol^{-1} and the enthalpy of activation is smaller, 13 kcal mol^{-1}.[31] The activation parameters are consistent with a rate limiting bimolecular process. Moreover, Me_4MTP undergoes hydrolysis only 48-times faster than tetraethylpyrophosphate, in contrast to the $>10^7$-fold difference for MTP^{3-} *versus* PP^{3-}. All things considered, the hydrolytic mechanism for Me_4MTP appears analogous to that of phosphotriesters, highly associative and characterized by a transition state with increased bonding to phosphorus.

Perspectives

Sulfur continues to be a valuable mechanistic probe for nucleophilic substitution at phosphorus. The stereochemical courses of many enzymatic phosphoryl- and nucleotidyltransferases have been elucidated by the use of P-chiral phosphorothio-analogs of biological substrates. The results have clarified the issue of single displacement *versus* double displacement mechanisms in enzyme catalysis. The weakness of the P–S bond has allowed evidence for the transient formation of monomeric metaphosphate to be obtained in the hydrolysis of *sym*-μ-monothiopyrophosphate. Other applications of sulfur in nucleoside phosphorothioates beyond the scope of this review include the differential metal binding properties of anionic sulfur and



Scheme 3 A mechanism for cleavage of MTP *via* discrete $[PO_3]^-$.

oxygen and variant chemical properties of P–S and P–O bonds.[33] Sulfur analogs of nucleotides are also used in research into the actions of ribozymes and as inhibitors of signaling proteins such as protein kinases. The focus of research on sulfur-containing probes of biological processes is shifting toward mechanisms of biological regulation at all levels of cellular chemistry.

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