Isotope Effects in the Study of Phosphoryl and Sulfuryl Transfer Reactions

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ABSTRACT
Phosphoryl and sulfuryl transfer reactions are essential biological processes. Multiple kinetic isotope effects have provided significant insights into the transition states of these reactions. The data are reviewed for the uncatalyzed reactions of phosphate and sulfate monoesters and for a number of enzymatic phosphoryl transfer reactions. Uncatalyzed phosphoryl and sulfuryl hydrolysis reactions are found to have very similar transition states. The phosphoryl transfer reaction catalyzed by protein-tyrosine phosphatases proceeds by a transition state very similar to that of the uncatalyzed reaction, but isotope effect data reveal an interesting interplay between the conserved arginine and enzyme dynamics involving general acid catalysis.

Introduction

Phosphoryl Transfer. Kinases and phosphatases have been called the Yin and Yang of signaling because of the central role played by phosphoryl transfer in the regulation of cellular processes. Protein kinases convert a tyrosine, serine, or threonine side chain to a phosphate monoester. Phosphatases catalyze the reverse of this process, the net hydrolysis of a phosphate monoester. This phosphorylation/dephosphorylation process functions as a switch between active and inactive states for many proteins. Our research has focused on studies of the details of the chemical mechanism of the phosphoryl transfer reaction catalyzed by phosphatases. These enzymes are of interest because of both their biomedical relevance and their catalytic proficiency. Phosphate esters are rather unreactive under physiological conditions. Phosphatases are extremely efficient enzymes, with k_{cat} values 10 or more orders of magnitude greater than the rate constants for the corresponding uncatalyzed hydrolysis reactions. While there is general agreement about the mechanisms operative in the uncatalyzed reactions of phosphate monoesters, there has been controversy over whether enzymatic phosphoryl transfer follows a similar mechanism.

Three distinct reaction mechanisms have been observed for phosphate esters, shown in Figure 1. Mechanism A is an S_N1-type mechanism ($D_N + A_N$ in the IUPAC nomenclature) in which a metaphosphate intermediate formed in the rate-determining step is subsequently attacked by a nucleophile. Phosphate monoesters have been observed to react by this mechanism only in the gas phase, or in solution with the hindered phosphoryl acceptor *tert*-butyl alcohol. More typically the dianions of phosphate monoesters react via a concerted S_N2-type mechanism (A_ND_N) with no intermediate. The transition state is characterized by a metaphosphate-like phosphoryl group, extensive bond cleavage to the leaving group, and minimal bond formation to the nucleophile (Figure 1B) (the evidence has been reviewed [1,2]). Mechanism C is an addition−elimination process ($A_N + D_N$), with a pentacoordinate phosphorane intermediate. The primary factor influencing which mechanism is followed is the alkylation state of the phosphate. Diesters and triesters follow successively more associative mechanisms, either concerted ones if the leaving group is good (i.e., an aryloxy group)[3,4] but with more nucleophilic participation in the transition state than in monoester reactions, or fully associative mechanisms via phosphorane intermediates under certain circumstances.[2]

The monoanion form of phosphate monoesters reacts by a mechanism that also involves little nucleophilic participation. The proton transfer to the leaving group may, in principle, occur either in a preequilibrium step or simultaneously with P−O bond cleavage, depending upon the basicity of the ester group (Figure 2).[5]

It has been proposed that phosphatases might utilize Lewis acid catalysis to change the transition state for phosphoryl transfer to a more associative one, or even utilize a two-step associative mechanism with a phosphorane intermediate. Isotope effects are ideally suited to answer such questions because one is able to track changes in bonding to both the leaving group and the phosphoryl group.

Sulfuryl Transfer. Sulfate ester formation has a crucial biological role in detoxification, and sulfate monoesters are found among many of the classes of natural products. Despite its biological importance, sulfate ester chemistry has been the subject of comparatively few studies. From what is known to date, the chemistry of sulfate esters is mechanistically similar to that of phosphate esters. In the broad pH-independent region between pH 4 and 12, aryl sulfate ester anions undergo sulfuryl transfer by a mechanism similar to that of aryl phosphate dianions, with a loose transition state in which the sulfuryl group resembles SO_3 (Figure 3). Linear free energy relationships are similar to those for reactions of phosphate monoester dianions. However, the significant negative entropy of activation of −18.5 eu for the pH-independent hydrolysis of *p*-nitrophenyl sulfate anion has raised the question of whether this reaction might proceed with significantly more nucleophilic participation than the corresponding

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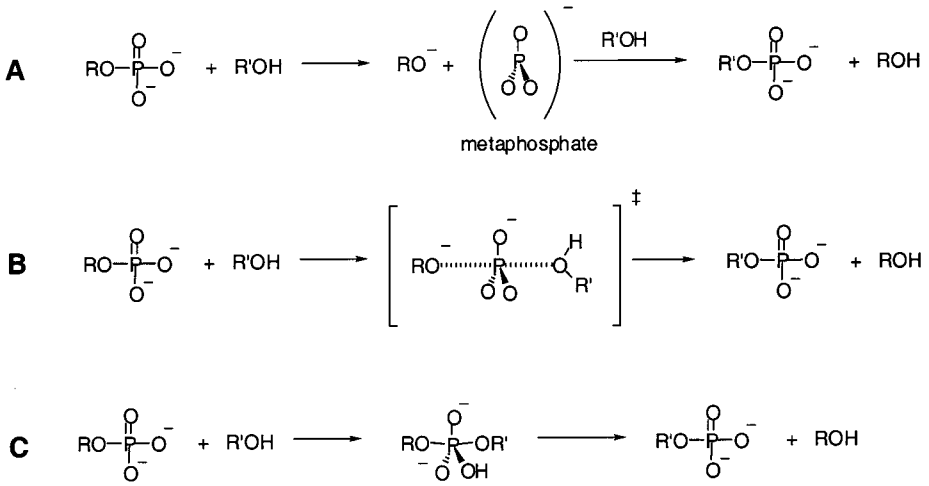


FIGURE 1. Dissociative (A) and associative (C) mechanistic extremes, and the concerted pathway (B) for phosphoryl transfer. The concerted pathway is drawn to indicate the loose transition state typical of phosphate monoester reactions, but in principle this transition state could lie at any point between the mechanistic extremes. In a loose transition state the sum of the bond orders to the nucleophile and leaving group is less than 1; in a tight (or associative) transition state this sum is greater than 1.

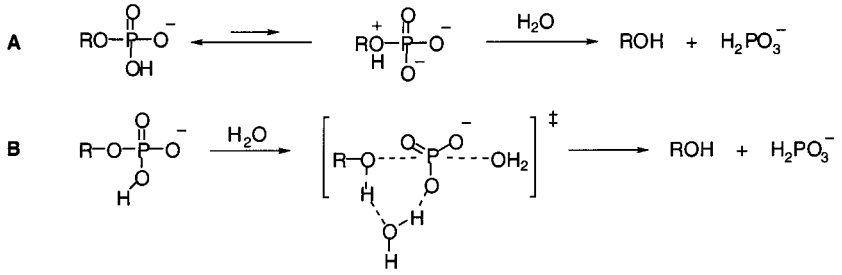


FIGURE 2. Mechanistic possibilities for phosphoryl transfer reactions of the monoanion of a phosphate monoester. (A) Stepwise transfer of the proton to the bridge oxygen atom in a preequilibrium step followed by hydrolysis. (B) Proton-transfer concerted with P−O bond cleavage.

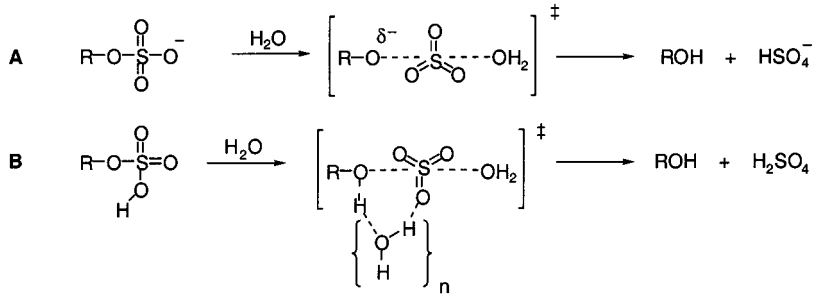


FIGURE 3. Mechanisms for the hydrolysis of a sulfate monoester as (A) the anion and (B) the neutral species. More than one water molecule may be involved in facilitating the proton transfer in mechanism B.

hydrolysis of pNPP, which exhibits an entropy of activation of +3.5 eu.

Under acidic conditions, the hydrolysis of sulfate esters is much faster, and the reactive species is the neutral ester (Figure 3B). Again, analogously with phosphate monoesters, this reaction is believed to proceed by transfer of the proton from the sulfuryl group to the leaving group.

Isotope effects can answer crucial questions about the nature of sulfuryl transfer. In addition, measurement of the isotope effects in positions corresponding to those in phosphate esters allows for direct comparisons between the mechanisms of the two types of compounds. This Account summarizes our work on the study of uncatalyzed reactions of phosphate and sulfate monoesters and in enzymatic phosphoryl transfer. Some of our phosphate diester and triester work has been reviewed elsewhere.[6]

Isotope Effect Terminology

Isotope effects are classified as *primary* if a bond to the labeled atom is made or broken during the reaction. Isotope effects measured in other positions are called *secondary* isotope effects. A kinetic isotope effect (KIE) is the ratio of the rate constant of the light isotope divided by that of the heavy isotope. Similarly, an equilibrium isotope effect (EIE) is a ratio of the corresponding equilibrium constants. A common notation for isotope effects uses a leading superscript of the heavier isotope to

indicate the isotope effect on the following kinetic quantity; for example, ^{15}k denotes k_{14}/k_{15}, the nitrogen-15 isotope effect on the rate constant k.[7] An isotope effect is termed *normal* if it is greater than 1, i.e., if the lighter isotopically labeled compound reacts at a faster rate. The isotope effect is *inverse* if this ratio is less than 1. An isotope effect of unity implies the absence of an isotope effect.

A kinetic isotope effect reflects differences in bonding to the labeled atom in the ground state compared to the transition state of the rate-limiting step. A primary kinetic isotope effect at an atom undergoing bond cleavage will be normal, due to the preference of the heavier isotope for the lower energy (more tightly bonded) position. Secondary kinetic isotope effects are normal if the labeled atom becomes more loosely bonded in the transition state, or inverse if bonding becomes tighter. Similarly, EIEs are determined by differences in ground state bonding, and both inverse and normal EIEs are common.[8] A normal EIE in a forward reaction is accompanied by an inverse EIE in the reverse direction, and vice versa. A fuller description of the contributions to isotope effects appeared in a recent *Accounts of Chemical Research* paper.[9]

Measurement of Isotope Effects

The rate difference, or isotope effect, resulting from isotopic substitution at carbon, nitrogen, or oxygen is never more than about 8%. Hence, the competitive method is usually the method of choice for measurement of heavy-atom isotope effects (atoms heavier than hydrogen). In this method, a mixture of the light and heavy isotopic isomers is allowed to react competitively, and the isotope effect is measured from the change in isotopic composition over the course of the reaction. For example, to measure ^{15}k for a reaction, a mixture of ^{15}N- and ^{14}N-labeled reactants is used. The reaction is stopped at some measured fraction f of reaction, and the ^{15}N/^{14}N ratio of the product (R_p) and that of the remaining starting material (R_s) are measured. If we know the isotope ratio in the original mixture (R_o), the isotope effect can be calculated using eq 1 or 2.[10]

$$\text{isotope effect} = \log(1 - f)/\log((1 - f)(R_s/R_o)) \quad (1)$$

$$\text{isotope effect} = \log(1 - f)/\log(1 - f(R_p/R_o)) \quad (2)$$

A high degree of precision is required in the measurement of the isotope ratios. The most precise method involves the use of an isotope ratio mass spectrometer. The major drawback to the use of this instrument is that it measures only small-molecule gases that do not undergo fragmentation in the mass spec (since such fragmentation will have isotope effects!). Thus, one must convert the atom of interest into a form the instrument is built to handle, which includes H_2, N_2, CO, CO_2, or SO_2. Such conversion must be made cleanly and quantitatively to ensure that there is no isotopic fractionation. This can present a formidable barrier; however, the use of the remote label technique can alleviate this problem. In this method a substrate is prepared that is isotopically labeled in two positions. One label is incorporated into the position of interest, with a second label at a position that is easily accessible. In our work we use a nitrogen atom as our remote label, as a reporter for the O-18 isotope ratios. Most isotope ratio mass spectrometers incorporate a combustion system which converts nitrogen to N_2 by reduction of the intermediate nitrogen oxides. Thus, if the reactant molecule contains a single nitrogen atom, it is easily analyzed. The limitation in this case is that the atom must lend itself to isotopic incorporation from available highly enriched starting materials, and it must be inert to the reaction conditions. Since the nonenzymatic reactions of phosphate and sulfate esters are slow and require fairly harsh conditions, we have used the nitro group in this capacity.

Phosphoryl Transfer Reactions

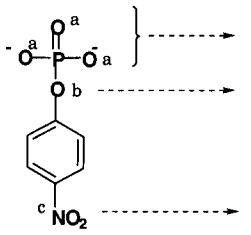
Figure 4 shows the position at which isotope effects have been measured in reactions with the substrate *p*-nitrophenyl phosphate (pNPP). The primary isotope effect, $^{18}k_{bridge}$, gives a measure of the degree of cleavage of the P−O bond in the transition state.

The secondary isotope effect ^{15}k measures the negative charge delocalized into the nitro group. The *p*-nitrophenolate anion has contributions from a quinonoid resonance form, shown in Figure 5. Because N−O bonds are stiffer in terms of vibrational frequencies than N−C bonds, the nitrogen atom is more tightly bonded in neutral *p*-nitrophenol than in the phenolate anion. The ^{15}K EIE for deprotonation of *p*-nitrophenol is thus normal, 1.0023 ± 0.0002.[11] The ^{15}k isotope effect thus gives information as to whether the leaving group departs as the anion, or if protonation of the leaving group has neutralized all or part of the negative charge resulting from P−O bond fission.

One might expect that $^{18}k_{bridge}$ and ^{15}k should always correlate. While we have typically found this to be true, it will not necessarily always be the case. The negative charge arising from P−O bond fission measured by $^{18}k_{bridge}$ may not be fully delocalized in the transition state. A number of instances have been documented in which charge delocalization lags behind charge development in the transition state, one of the best known examples being the "nitroalkane anomaly" discussed by Bernasconi.[12]

Figure 4 also summarizes the expected maximum values of $^{18}k_{bridge}$ and ^{15}k when the P−O bond is essentially fully broken in the transition state and the resulting charge is delocalized. An earlier transition state with a smaller degree of P−O bond fission (and hence less negative charge on the leaving group) will exhibit smaller values for both $^{18}k_{bridge}$ and ^{15}k. Protonation of the leaving group in the transition state will also reduce the magnitudes of both $^{18}k_{bridge}$ and ^{15}k.

The secondary isotope effect, $^{18}k_{nonbridge}$, reveals whether the phosphoryl group resembles metaphosphate in a loose transition state, or if it has a phosphorane-like structure in an associative mechanism. Isotope effects are deter-



a) The nonbridge phosphoryl oxygen atoms ($^{18}k_{nonbridge}$)

b) The bridge oxygen atom, the position of bond cleavage ($^{18}k_{bridge}$)

c) The nitrogen atom in the leaving group (^{15}k).

Expected values for these isotope effects as a function of transition state

Leaving group KIEs	Loose TS – extensive bond fission to leaving group, no charge neutralization	$^{15}k \approx 1.003$ $^{18}k_{bridge} \approx 1.03$
	Tight TS - phosphoryl group resembles pentacoordinate phosphorane, no bond fission to leaving group	$^{15}k \approx 1.000$ $^{18}k_{bridge} \approx 1.000$
Nonbridge KIEs	Loose TS – extensive bond fission to leaving group, metaphosphate-like phosphoryl group	inverse $^{18}k_{nonbridge} \approx 0.995$
	Tight TS - phosphoryl group resembles pentacoordinate phosphorane	normal $^{18}k_{nonbridge} \approx 1.025$

FIGURE 4. Diagram of *p*-nitrophenyl phosphate, showing the positions where isotope effects are measured and their expected values.

FIGURE 5. Resonance contributors of *p*-nitrophenolate ion, showing involvement of the nitro group in charge delocalization that gives rise to the N-15 isotope effect.

mined not only by bond order considerations but also by bending and torsional vibrational modes, and the latter effects can be dominant for secondary isotope effects at an atom bonded to a site undergoing a hybridization change. The bond order changes of the nonbridge oxygen atoms in the mechanisms in Figure 1 would lead one to expect $^{18}k_{nonbridge}$ to be inverse for dissociative transition states and normal for associative mechanisms. The bending modes should be in the opposite direction, however. For example, α-secondary deuterium isotope effects are normal for hybridization changes of the type sp^3 to sp^2 or sp^2 to sp. From the trends in the data, evidently bond order changes are the dominant contributors to $^{18}k_{nonbridge}$, since this isotope effect is normal for diester (with a single exception, which may be anomalous) and triester reactions and inverse (though very small) for dissociative (bond energy-bond order-vibrational) reactions.[13−17] This follows the trend predicted from calculations that predict normal $^{18}k_{nonbridge}$ isotope effects for associative transition states and inverse values for dissociative ones.[18] More recent ab initio calculations we have carried out reaffirm these earlier predictions. Figure 4 shows the expected value of $^{18}k_{nonbridge}$ in a dissociative (metaphosphate-like) transition state, and in a tight transition state in which the phosphoryl group resembles a pentacoordinate phosphorane.

As Table 1 shows, the leaving group isotope effects also show differences in reactions of diesters and triesters where *p*-nitrophenol is the leaving group, compared with the more dissociative monoester reactions. Linear free energy relationships indicate that, in diesters and triesters with good leaving groups, phosphoryl transfer reactions are concerted with no phosphorane intermediate, but that the transition states become more associative with less bond cleavage to the leaving group in aryl diesters and triesters than in the monoester reactions.[3,4] This trend is borne out by the reduced magnitudes of the isotope effects in the leaving group, ^{15}k and $^{18}k_{bridge}$.

Table 1 shows the KIEs for uncatalyzed reactions of both the pNPP dianion and monoanion. In reactions of the dianion, the leaving group departs as an anion, and the transition state is very loose. In the hydrolysis of the pNPP monoanion, the leaving group is protonated in the transition state. Thus, the magnitude of $^{18}k_{bridge}$ is significantly lower than that for the dianion reaction, as loss of the P−O bond is partially compensated for by formation of the O−H bond. The negligible value for ^{15}k indicates that the leaving group remains essentially neutral in the transition state. The value for $^{18}k_{nonbridge}$ in this reaction

Table 1. Range of Isotope Effects Measured for Reactions of Phosphate Esters[a]

transition state	reaction	^{15}k	$^{18}k_{bridge}$	$^{18}k_{nonbridge}$
loose	pNPP dianion, H_2O, 95 °C	1.0028(2)	1.0189(5)	0.9994(5)
↓	pNPP dianion, *tert*-butyl alcohol, 30 °C	1.0039(3)	1.0202(8)	0.9997(16)
	diesters	1.0007−1.0016	1.0042−1.0063	1.0028−1.0056
tight	triesters	1.0007	1.0063[b]	1.0063−1.0250
	pNPP Monoanion, 35 °C	1.0005(1)	1.0094(3)	1.0199(3)

[a] The diester and triester KIEs are those for alkaline hydrolysis. In this and all tables, standard errors in the last decimal place(s) are shown in parentheses. [b] Data from ref 17.

Table 2. Kinetic Isotope Effects for Reactions of Members of the PTPase Superfamily[a]

	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$
YOP, PTP1, VHR	0.9999−1.0001	1.0118−1.0152	0.9998−1.0003
Stp1	1.0007	1.0171	1.0007
D to N mutants: YOP, PTP1, VHR, Stp1	1.0024−1.0030	1.0275−1.0297	1.0019−1.0024

[a] Standard errors are in the range of 0.0001−0.0008.

reflects the known normal isotope effect for deprotonation of a phosphoryl group. [19]

Protein Tyrosine Phosphatases (PTPases)

The PTPase superfamily includes two additional groups of enzymes, the VH1-like dual specificity phosphatases and the low-molecular-weight tyrosine phosphatases.[20] The PTPases hydrolyze only phosphotyrosine residues of polypeptide substrates, while the dual-specific enzymes also accept as substrates phosphoserine and -threonine residues. Despite these differences, these enzymes share virtually identical catalytic sites characterized by a Cys nucleophile, an Arg residue which interacts with the phosphoryl group of the substrate, and an Asp general acid that protonates the leaving group. This Asp residue resides on a flexible loop and lies 8−10 Å away from the active site in the resting enzymes but is brought into position for catalysis upon binding of substrate.[21] The catalytic mechanism proceeds through formation of a phosphocysteine intermediate that undergoes subsequent hydrolysis.

We have measured the isotope effects for the reaction of pNPP catalyzed by the PTPase superfamily members YOP from *Yersinia*, PTP1 from mouse, human VHR, and Stp1 from yeast.[22−26] Since the isotope effects are measured by the competitive method, they are effects on k_{cat}/K_M (customarily referred to as V/K), which includes the portion of the overall mechanism up to and including the first irreversible step. This means that the KIEs are those for the phosphoryl transfer from the substrate to the Cys nucleophile. Results from kinetic studies and the invariance of the isotope effects with pH indicate that the chemical step is rate-limiting in each of these enzymes studied, and thus the intrinsic isotope effects for the phosphoryl transfer step are observed.

The isotope effects from these studies are summarized in Table 2. the results for YOP, VHR, and PTP1 are very similar and are collected in row 1; the data from Stp1 differ slightly but systematically and are shown in row 2. The results from the native enzymes are indicative of a transition state that is loose in nature, with minimal

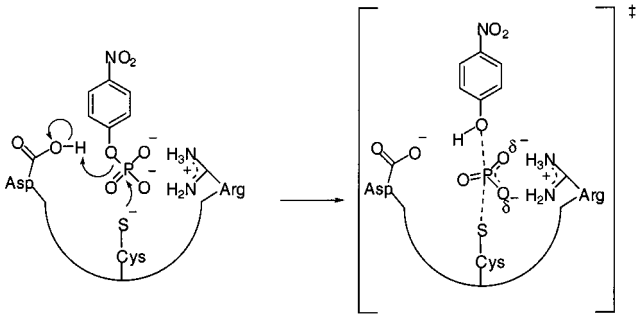


FIGURE 6. Transition state for the PTPase-catalyzed reaction inferred from the KIE data. The dianion is the substrate. The phosphoryl group resembles metaphosphate, and the leaving group is neutralized by protonation.

nucleophilic participation and in which the phosphoryl group resembles metaphosphate. The KIEs in the leaving group are consistent with extensive bond cleavage to the leaving group, with the leaving group fully neutralized by protonation in the transition state (Figure 6). In the Stp1 reaction, protonation of the leaving group seems to lag a bit behind P−O bond cleavage, and the leaving group bears some negative charge. The KIE results for all of the PTPases are also consistent with other data indicating that the dianion form of the phosphate monoester is the substrate.

For all four members of this phosphatase family, when the general acid Asp is mutated to Asn (Table 2, row 3), the values for $^{18}(V/K)_{nonbridge}$ become normal, revealing that the transition state assumes a somewhat larger degree of nucleophilic participation. The magnitudes of $^{18}(V/K)_{bridge}$ and $^{15}(V/K)$ increase by an amount very close to the EIEs for deprotonation of *p*-nitrophenol, indicating extensive bond cleavage to the leaving group, which now is unprotonated and bears essentially a full negative charge.[25]

This suggests that interactions between the phosphoryl group and the arginine do not fundamentally alter the nature of the transition state from that in uncatalyzed reactions. To further probe the role of this conserved residue, we examined the isotope effects for the R409K and R409A mutants of the PTPase YOP. The data revealed an interesting interplay between the arginine residue and the functioning of the general acid. X-ray structural data show that when an oxyanion is bound, [21] Arg 409 rotates to form bidentate hydrogen bonds. This reorientation is accompanied by movement of the loop bearing the general acid, bringing it into position for catalysis. The values for k_{cat} are lower by 4 orders of magnitude for both mutants, but the rate for R409A is about 2.5 times that for R409K.

Table 3. Kinetic Isotope Effects for Reactions of Arginine Mutants of the PTPase from *Yersinia*

	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$
R409K	1.0020(5)	1.0273(3)	1.0049(7)
R409A	1.0012(3)	1.0200(5)	0.9990(7)
R409K/D356N	1.0022(1)	1.0317(3)	1.0045(2)
R409A/D356N	1.0024(4)	1.0340(11)	1.0027(5)

The KIEs for the reaction of pNPP catalyzed by the R409K and R409A mutants of the PTPase YOP are shown in Table 3. The isotope effects for the reaction catalyzed by R409K resemble those for the general acid mutant D356N; thus, the general acid has been rendered nonfunctional. By contrast, in R409A the isotope effects are intermediate between those of the wild type and general acid mutant.[25] This indicates that the general acid is still operative in R409A, although the degree of proton transfer to the leaving group is less complete; the data suggest about 50% charge neutralization. These results emphasize the danger of interpreting changes in rate to a particular effect without doing a mechanistic analysis. One might have reasonably attributed the lowered catalytic rate to a loss of transition state stabilization afforded by Arg 409, when in fact most of the effect results from disabling the general acid.

Since the effect of the arginine residue on the nature of the transition state cannot be disentangled from general acid catalysis, we measured the KIEs for the reaction catalyzed by double mutants in which the arginine residue was either lysine or alanine, and the general acid was mutated to asparagine. A comparison of the data from these double mutants (Table 3) with data from the general acid mutant (Table 2 bottom row) allows an evaluation of the proposal that the arginine residue renders the phosphoryl transfer mechanism more associative. The results show that mutation of the conserved Arg to either Lys or to Ala does not significantly alter the transition state. The small increases in $^{18}(V/K)_{nonbridge}$ are in the opposite direction to that expected if Arg144 imparts associative character to the transition state, which becomes more dissociative upon mutation. Thus, the Arg residue stabilizes the loose transition state but does not alter it.

Metallophosphatases

A second large class of phosphatases utilizes a binuclear metal center. The best known member of this class is alkaline phosphatase, which contains two Zn^{2+} ions that play a role in catalysis. The AP reaction proceeds via an intermediate in which a serine residue (Ser 102 in *Escherichia coli*) is phosphorylated. In this respect, AP differs from the serine/threonine protein phosphatases, which also contain binuclear metal centers but which catalyze phosphoryl transfer directly to a metal-bound water.[27] Our attempts to characterize the transition state of the AP-catalyzed reaction were foiled, as the isotope effects are all near unity,[15] suggesting that a nonchemical step such as binding or an associated conformational change is rate-limiting for k_{cat}/K_M.

Table 4. Kinetic Isotope Effects for Lambda-PP Reactions with PNPP

enzyme form	$^{15}(V/K)$	$^{18}(V/K)_{bridge}$	$^{18}(V/K)_{nonbridge}$
native, Mn^{2+}	1.0006(3)	1.0133(6)	0.9976(3)
native, Ca^{2+}	1.0007(1)	1.0130(4)	0.9984(2)
H76N, Mn^{2+}	1.0016(3)	1.0183(9)	0.9976(1)

We had better success with the bacteriophage lambda Ser/Thr phosphatase, which exhibits significant isotope effects (Table 4) that do not vary when the rate is considerably reduced, whether by varying the pH or by substituting a different divalent metal ion (Ca^{2+} for Mn^{2+}).[28] Thus, we have confidence that we are observing the intrinsic KIEs on the chemical step. The $^{18}(V/K)_{nonbridge}$ isotope effect is consistent with a transition state having minimal nucleophilic involvement. The leaving group isotope effects indicate that only a small negative charge resides on the leaving group in the transition state.

The X-ray structure of the lambda protein phosphatase[29] reveals that, like other Ser/Thr phosphatases, lambda has a conserved His residue that has been proposed to protonate the leaving group. The isotope effect data from the H76N mutant do not provide a definitive answer to this question. The magnitudes of $^{15}(V/K)$ and $^{18}(V/K)_{bridge}$ are indeed elevated, as expected if the leaving group now bears additional negative charge. However, the magnitudes are significantly smaller than those seen in reactions of PTPases in which the general acid has been mutated (compare last rows of Table 4 and Table 2). It is possible that in lambda neutralization of the leaving group is assisted by coordination to one of the metal ions or by protonation by a metal-coordinated water molecule. Alternatively, the transition state for metallophosphatase-assisted phosphoryl transfer may differ from that of the PTPases. The isotope effect data from the lambda-catalyzed reaction are also consistent with a transition state having less bond cleavage to the leaving group, although there is no evidence for greater nucleophilic participation in the magnitude of $^{18}(V/K)_{nonbridge}$. Perhaps metallophosphatases utilize a hybrid transition state that is less dissociative with respect to leaving group departure but that still exhibits the minimal nucleophilic participation typical of uncatalyzed monoester reactions. Further work is needed to answer these questions.

Sulfuryl Transfer

Recently we have extended our use of isotope effects to the study of sulfuryl transfer reactions.[30] The sulfate ester used in these studies is *p*-nitrophenyl sulfate (pNPS), which allows us to use the remote label method to follow the O-18 isotope effects analogous to those in the phosphoryl transfer studies described above. Thus far we have measured the KIEs for the pH-independent hydrolysis of the pNPS anion at pH 9.0, as well as for the hydrolysis of the neutral ester under acidic conditions (Table 5). The similar magnitudes of $^{18}k_{bridge}$ and ^{15}k for the hydrolysis reactions of the pNPS anion and the pNPP dianion indicate that the sulfuryl and phosphoryl transfer reactions have very similar transition states, with a large extent of

Table 5. Kinetic Isotope Effects for the Aqueous Hydrolysis of PNPS

pNPS KIEs	^{15}k	$^{18}k_{bridge}$	$^{18}k_{nonbridge}$
pH 9.0, 85 °C	1.0026(1)	1.0210(10)	0.9951(3)
1 N HCl, 65 °C	1.0002(1)	1.0069(2)	1.0067(1)
1 N HCl, 21 °C	1.0002(1)	1.0097(2)	1.0083(2)

leaving group bond fission and similar amount of charge on the leaving group.

An interpretation of $^{18}k_{nonbridge}$ may be considered in part by analogy with the considerable body of data for phosphoryl transfer. The changes in hybridization and in bonding to the nonbridge oxygen atoms in sulfuryl and phosphoryl transfer reactions are directly analogous. This suggests that a similar trend might be expected for the analogous sulfuryl transfer reaction. This has been confirmed by calculations we have recently carried out at the 6-31++G** level of the expected equilibrium isotope effect between pNPS and sulfur trioxide. The experimental value of 0.9951 is consistent with expectations from calculations, and from KIEs from analogous phosphoryl transfer reactions, for a transition state in which the sulfuryl group resembles SO_3.

Isotope effects were also measured for the reaction of pNPS in acidic conditions for comparison with the reaction of the anion. The reaction is 10^5-fold faster at 35 °C in 1.0 N HCl compared to the rate in the pH-independent region at pH 9.0. KIEs are sensitive to the reaction temperature, but due to this extreme difference in reactivity, these two reactions could not be investigated at the same temperature. A compromise was reached, with the pH 9 reaction studied at 85 °C and the 1.0 N HCl reaction at 65 °C. The acid reaction of pNPS was also examined at a lower temperature for better comparison with data from the monoanion of pNPP.

The magnitude of ^{15}k is essentially unity in the acid reactions, indicating that no significant negative charge is developed on the nitrophenyl group in the transition state. The value for $^{18}k_{bridge}$ is lower than that in the reaction of the anion by about the value of the equilibrium ^{18}K for protonation of *p*-nitrophenol. [31] Thus, the observed kinetic isotope effect is consistent with a transition state in which the O−S bond is largely broken and the leaving group is protonated. Both ^{15}k and $^{18}k_{bridge}$ (Table 5) are very similar to the values from the reaction of the pNPP monoanion (Table 1). The magnitude of $^{18}k_{nonbridge}$ will be influenced by the protonation state of the sulfuryl group. In the mechanistically analogous reaction of the pNPP monoanion, the isotope effects were measured at a pH (3.5) at which the phosphoryl group (pK_a 5.1) was essentially completely monoprotonated. In this case, there is negligible isotopic fractionation in the species present in the correct protonation state for reaction. However, the pK_a of pNPS is too low to obtain a large proportion of the neutral species. Even in 1 N HCl, less than 1% of the compound will be in the neutral form, and therefore the observed KIE should be corrected by the full EIE for protonation. Doing so yields a corrected isotope effect that is close to the value measured for the hydrolysis of the monoanion of pNPP, supporting the hypothesis of a similar mechanism and transition state for the two reactions. The fact that $^{18}k_{nonbridge}$ is normal indicates that deprotonation of the sulfuryl group takes place in the transition state for hydrolysis, with proton transfer (probably via one or more water molecules) to the leaving group (Figure 3B). If a preequilibrium proton transfer occurs (analogous to Figure 2A), then the EIE for protonation and that for the subsequent deprotonation would effectively cancel and $^{18}k_{nonbridge}$ would be inverse, as for the reaction of the anion.[30]

Summary and Future Directions

The multiple KIE experiments on phosphoryl and sulfuryl transfer reactions indicate that the two processes proceed with strikingly similar transition state structures. The tremendous catalytic power of PTPases does not derive from a significant change in the phosphoryl transfer mechanism; these reactions retain the loose transition state characteristic of uncatalyzed reactions. The conserved arginine has a role not just in stabilizing this transition state but also in the accompanying protein dynamics that enables general acid catalysis to occur.

We are presently pursuing work in both phosphoryl and sulfuryl transfer using substrates with a less labile leaving group, *m*-nitrobenzyl phosphate and sulfate. We are beginning to measure KIEs for enzymatic sulfuryl transfer reactions. Enzymatic sulfuryl transfer is relatively unexplored, and due to the relative unreactiveness of alkyl phosphates and sulfates, most of what is assumed about the uncatalyzed reactions of these esters comes from studies with aryl esters. We are also using computational methods to determine the expected magnitudes of the KIEs of the central atoms (phosphorus and sulfur) in these reactions, to see if they may be used to further assist in diagnosing the different mechanistic possibilities. Thus, there remains much fertile ground for application of these techniques in the future.

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