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Understanding the Transition States of Phosphodiester Bond Cleavage: Insights from Heavy Atom Isotope Effects

Abstract: *The nucleotides of DNA and RNA are joined by phosphodiester linkages whose synthesis and hydrolysis are catalyzed by numerous essential enzymes. Two prominent mechanisms have been proposed for RNA and protein enzyme catalyzed cleavage of phosphodiester bonds in RNA: (a)* intra*molecular nucleophilic attack by the 2′-hydroxyl group adjacent to the reactive phosphate; and (b)* inter*molecular nucleophilic attack by hydroxide, or other oxyanion. The general features of these two mechanisms have been established by physical organic chemical analyses; however, a more detailed understanding of the transition states of these reactions is emerging from recent kinetic isotope effect (KIE) studies. The recent data show interesting differences between the chemical mechanisms and transition state structures of the inter- and intramolecular reactions, as well as provide information on the impact of metal ion, acid, and base catalysis on these mechanisms. Importantly, recent nonenzymatic model studies show that interactions with divalent metal ions, an important feature of many phosphodiesterase active sites, can influence both the mechanism and transition state structure of nonenzymatic phosphodiester cleavage. Such detailed investigations are important because they mimic catalytic strategies employed by both RNA and protein phosphodiesterases, and so set the stage for explorations of enzyme-catalyzed transition states. Application of KIE analyses for this class of enzymes is just beginning, and several important technical challenges remain to be overcome. Nonetheless, such studies hold great promise since they will provide novel insights into the role of metal ions and other active site interactions.*
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INTRODUCTION

At equilibrium in aqueous solution, the phosphodiester backbones of RNA and DNA would be hydrolyzed to mononucleotides. However, in the absence of a catalyst, phosphodiester bonds in nucleic acids are extraordinarily kinetically stable towards solvolysis with half-lives of ca. 100,000 years.[1] This kinetic

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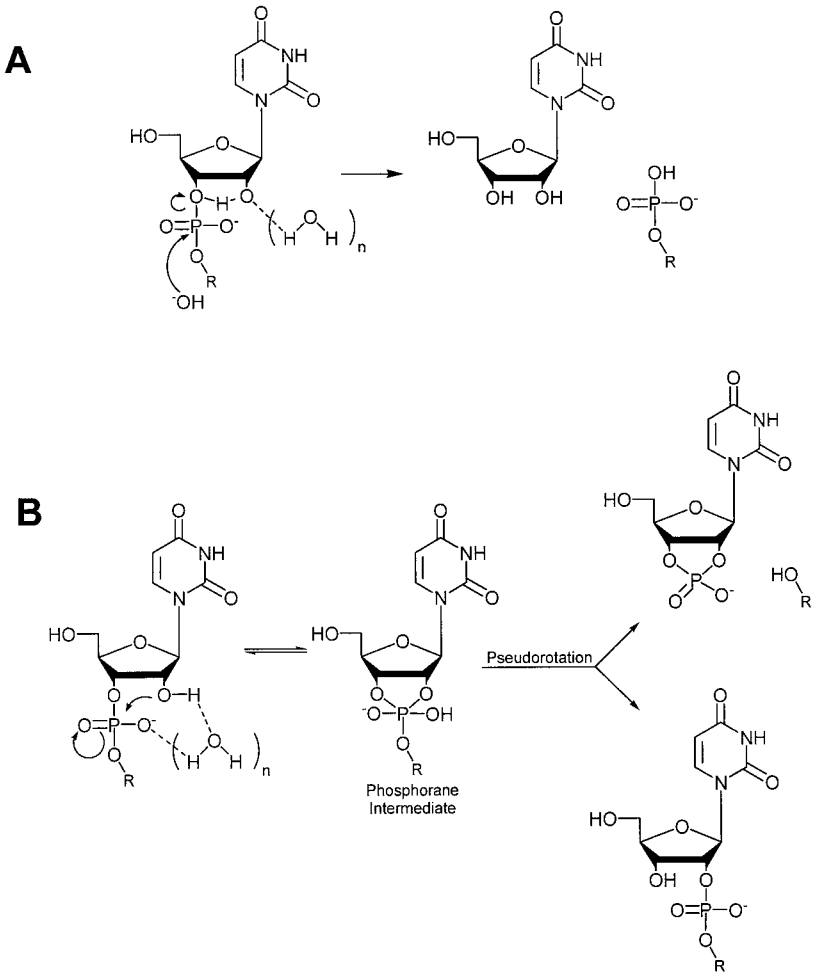

FIGURE 1 Inter- and intramolecular mechanisms of RNA phosphodiester bond cleavage. A: Intermolecular nucleophilic attack. The nucleophile shown in the figure is a solvent hydroxide, however, it can also be the 2′ or 3′ hydroxyl of another ribonucleotide. B: Intramolecular nucleophilic attack by the adjacent 2′ hydroxyl.

stability coupled to the thermodynamic instability permits enzyme catalysts to control biologically important transformations involving phosphodiester bonds. Indeed, reactions involving phosphodiester hydrolysis are integral to a multitude of important cellular functions, and are catalyzed by numerous essential enzymes.[2–6] Additionally, most naturally occurring catalytic RNAs, or ribozymes, catalyze phosphodiester cleavage, and therefore these reactions offer the unique opportunity to directly compare mechanisms of RNA and protein catalysis.[7] For these reasons the chemistry of phosphoryl transfer reactions, and specifically the mechanisms by which enzymes accelerate the rates of these reactions, continues to attract intense interest.

RNA and protein enzymes utilize two distinct mechanisms for achieving phosphodiester bond cleavage (Figure 1A and B). In the first mechanism, the nucleophile is hydroxide, or a ribose 2′ or 3′ oxygen, and the P-O3′ bond is broken resulting in characteristic 5′ phosphate and 3′ hydroxl termini in the resultant hydrolysis products. The second mechanism is specific to RNA cleavage and involves intramolecular attack by the 2′ hydroxyl adjacent to the reactive phosphate. The reaction results in breaking of the P-O5′ bond and formation of a 2′,3′ cyclic phosphate. Extensive analysis of the nonenzymatic hydrolysis of cylic phosphate esters showed that reactions of phosphate proceed through pentavalent transition states or intermediates with trigonal bipyrimadyl geometry.[8] Analysis of the greater rate of reaction of cyclic phosphate esters compared to their acyclic counterparts, and their ability to incorporate oxygen via exchange with solvent lead to the concept of pseudoro-

tation in which substituents in the equatorial and apical positions of the intermediate change place.[9] For example, intramolecular attack by the adjacent 2′OH of in model compounds like uridine 3′ nitrobenzyl phosphate can result in either P—O bond cleavage or isomerization from a 3′ to 2′ linkage, and acidic reaction conditions result in exchange between water and the nonbridging phosphate oxygens.[10–12] These observations indicate this reaction can proceed via a pentavalent phosphorane intermediate with sufficient lifetime to undergo pseudorotation (Figure 1B). In contrast, intermolecular nucleophilic attack on phosphodiesters results in inversion of stereochemical configuration, and an abundance of other physical data including kinetic isotope effect (KIE) studies indicate these reactions are concerted.[13–15] In the concerted reaction, there is no formation of a phosphorane intermediate, and therefore no possibility of pseudorotation. The distinct behaviors of these two general mechanisms clearly indicate that there is significant interplay between nucleophilic attack and displacement of the leaving group. Do the constraints imposed by an intramolecular mechanism result in differences in nucleophile and leaving group bonding relative to the intermolecular reaction? Does the change in mechanism from a two-step to a concerted reaction depend on leaving group and/or nucleophile reactivity? How will the tightly packed and asymmetric chemical environment of an enzyme active site influence, or constrain, the transition state relative to nonenzymatic reactions? A more detailed understanding of transition state charge distribution will be necessary to understand how these mechanistic and environmental differences influence overall bonding in the transition state.

The intermolecular mechanism (Figure 1A), utilized by both catalytic RNAs such as the group I intron and ribonuclease P, and protein enzymes including the DNA restriction enzymes, employs an exogenous nucleophile such as hydroxide, or an alkoxide.[3,5,16] Although mechanistic models for these enzymes often invoke acid–base catalysis, active site metal ion interactions also appear to play critical roles. The precise number of active site metal ions for many of these enzymes, and their influence on the catalytic mechanism and transition state structure, remain the subject of some debate. Additionally, the direct involvement of solvent (water) in the chemical transformation raises the issue of how water is bound by enzyme active sites in order to achieve both positioning and chemical activation. Given the involvement of water as a substrate, what is the pathway of water binding, activation and attack? As with any enzymatic substrate we want to know the reaction steps and equilibria, such as deprotonation and/or metal ion binding, that lie between the free reactant and the active enzyme–substrate complex.

The second mechanism, involving intramolecular attack by the adjacent O2′ (Figure 1B), is also employed by both RNA and protein active sites, including small catalytic RNAs like the hairpin, HDV, and hammerhead ribozymes,[16] and protein ribonucleases such as RNase A and T1.[17,18] Extensive enzymological studies have highlighted the crucial role of acid–base catalysis in these "traditional" protein enzymes.[19–21] Significant progress has also been made in understanding the structure and function of RNA active sites that catalyze intramolecular attack by the adjacent 2′OH. In part, these studies reveal that RNA side chains can also function as acid–base catalysts.[22–25] Active site metal ion interactions appear to be less important for the small self-cleaving ribozymes compared to those that employ the intermolecular mechanism; nonetheless, important questions remain regarding the precise role for metal ions in catalysis. Additionally, questions have arisen concerning the formation of chemical intermediates that are observed for similar nonenzymatic reactions. Thus, a detailed understanding of the transition state structure and the chemical factors that influence it is crucial for determining how proteins and RNAs create the appropriate catalytic environment, and how that environment functions.

A complete understanding of how RNA and protein–phosphodiesterases implement these two mechanisms requires an analysis of the transition states (and their surrounding environments) for the reactions as they occur in aqueous solution and in enzyme active sites. Defining the various reaction steps and equilibria on the pathway from reactants to products, and determining the extent of P—O bonding and proton transfer for each transition state, are both intrinsic to achieving this goal. By definition, the chemical transition states are fleeting entities, and their properties must be probed kinetically. For aqueous reactions, the dependence of the reaction rate on the pK_a of either the leaving group or nucleophile (a linear free-energy relationship or LFER) can provide insight into transition state structure.[26,27] However, strict substrate specificity often precludes using this powerful technique for studying enzymatic transition states. A second approach involves analyzing the effect of isotopic substitution on both the rates and the equilibria of chemical reactions. Isotopic substitution represents the smallest possible chemical perturbation of the system, but can nonetheless have important influences on chemical reactivity.[28,29] Therefore, isotope effects are an especially useful tool for studying

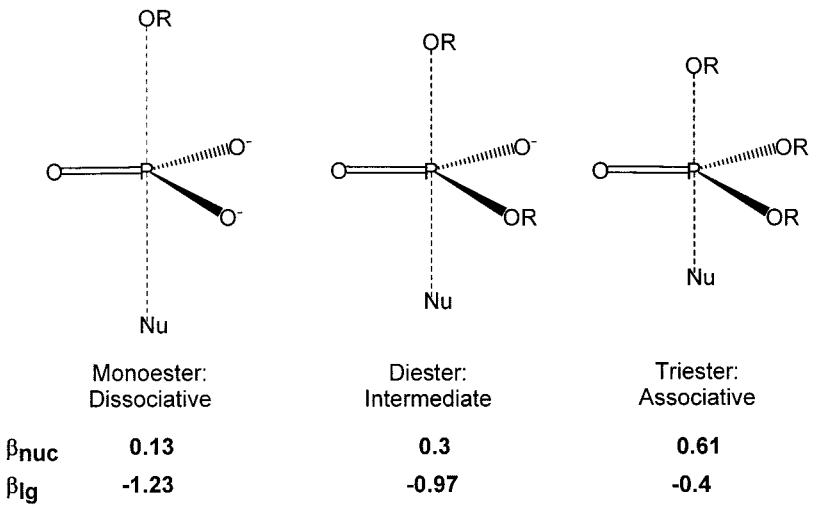

FIGURE 2 Transition state models for phosphoryl transfer from linear free energy relationship studies. Model transition states are shown for phosphomonoester, diester, and triester cleavage. The extent of bonding to the nucleophile and the leaving group are represented by the length and width of dashed line connecting them to the phosphoryl center. Literature values for β_{nuc} and β_{lg} are provided below each model.

enzymes because the effects will reflect mechanism (both kinetic and chemical) as opposed to specificity. However, the magnitude of isotope effects for nonhydrogen atoms are generally very small (a few percent, at most) and so measuring such effects is a significant challenge. Nonetheless, the magnitude and direction of such effects, when they can be measured with sufficient precision, provide unique information about reaction mechanism and place important constraints on models of transition state structure.[30] The purpose of this article is to concisely review recent progress in understanding nonenzymatic phosphodiester cleavage and to highlight the potential for using such classical mechanistic analyses to address outstanding issues in ribozyme and protein phosphodiestereses.

TRANSITION STATES OF PHOSPHORYL TRANSFER REACTIONS

As introduced above, phosphodiester bonds are extraordinarily stable except when either an intramolecular nucleophile in present, such as the adjacent 2′OH in RNA, or the leaving group is very reactive. Thus, compounds with good aryl leaving groups like *p*-nitrophenol ($pK_a = 7.15$) are commonly employed to study nonenzymatic reactions. Intermolecular phosphodiester reactions are generally considered to be concerted, not stepwise, and so involve partial bonding to both the nucleophile and leaving group in the transition state. Brønsted analyses of leaving group reactivity show that diester reactions have relatively large, negative β_{lg} values (e.g., a β_{lg} of -0.97 for hydrolysis of a series of diaryl phosphates[13]), indicating substantial bond breaking between the departing oxygen and phosphorus in the transition state (Figure 2). Similar analysis of attack by both oxyanions and substituted pyridines show a marked dependence on nucleophile pK_a ($\beta_{nuc} = 0.3$ for a 2,4-dinitrophenyl leaving group), with the reactivities of water and hydroxide differing by almost five orders of magnitude. These results demonstrate that phosphodiester cleavage is concerted, involving simultaneous bond formation and bond cleavage.

The data for phosphodiester reactions contrasts with that obtained for both monoesters and triesters (Figure 2). Monoesters display both a *larger* dependence on *leaving group* reactivity ($\beta_{lg} = {}^{-}1.23$ for hydrolysis reactions[31]), and a *smaller* dependence on *nucleophile* pK_a ($\beta_{nuc} = 0.13$ for substituted pyridines attacking 4-nitrophenyl phosphate[31,32]). These data indicate a dissociative transition state for phosphomonoester cleavage, with extensive bond cleavage to the leaving group, but little bond formation to the nucleophile. Phosphotriesters, on the other hand, exhibit *increased* sensitivity to *nucleophile* ($\beta_{nuc} = 0.61$ for substituted pyridines and a 2,4-dinitrophenyl leaving group[33]) and *decreased* sensitivity to the *leaving group* ($\beta_{lg} = {}^{-}0.4$ for a hydroxide nucleophile[33]) compared to phosphodiester cleavage. Here, the data indicate an associative transition state, with bond formation to the nucleophile more advanced than bond

breaking to the leaving group. Interestingly, diesters and triesters display the same dependence on the pK_a of oxyanions nucleophiles ($\beta_{nuc} = 0.3$ for a 2,4-dinitrophenyl leaving group).[13] This result may suggest that phosphodiester cleavage with an oxyanion nucleophile may require a more associative transition state than would be expected in order to overcome electrostatic repulsion (see below) between the nucleophile and the negatively charged phosphoryl center. Such electrostatic repulsion would not exist in reactions involving a neutral phosphotriester. However, the lesser magnitude of β_{lg} for the triester reaction indicates that triester cleavage is still more associative than diester cleavage, even when the nucleophile possesses a negative charge.

Although the reactivities of both nucleophile and leaving group are known to exert important effects on the rates of nonenzymatic reactions involving phosphodiesters, the influence of the second, or "spectator" R group of phosphate diesters has only recently been assessed.[34] Comparison of the rate of methylene monoesters shows that changing this oxygen to carbon has only a small effect on reaction rate. A reexamination of previous physical organic analyses indicates that this group undergoes little charge buildup in the transition state; thus, enzyme interactions with this group are not expected to provide significant catalytic advantage. Similarly, KIE analysis show that there are only slight differences in the transition states of diesters containing alkyl vs aryl "spectator" R groups.[35] Additionally, for reactions involving nucleic acids, this group will always be an alkyl group with a relatively high pKa.

Phosphoryl transfer reactions are sensitive to ionic strength. This sensitivity is due, in part, to stabilization of the negatively charged transition state.[36,37] Additionally, for both monoesters and diesters, comparison of the sensitivities to ionic strength for negatively charged and neutral nucleophiles indicates that there is significant electrostatic repulsion between anionic nucleophiles and the negatively charged phosphoryl center. As might be expected, there is less electrostatic repulsion between anionic nucleophiles and the monoanionic phosphoryl center of phosphodiesters compared to dianionic monoesters. When combined with the greater β_{nuc} for phosphodiester cleavage, this observation suggests the catalytic advantage for deprotonation of the attacking nucleophile will be greater for phosphodiesterases than for phosphatases.

Even for model compounds with good leaving groups, hydrolyses of diesters such as *p-ter*-butylnitrophenyl phosphate are very slow at neutral pH, but display U-shaped pH-rate profiles demonstrating both specific acid and base catalysis.[13] Understanding the mechanism of acid and base catalysis for these reactions is informative with respect to phosphodiesterase function, where acidic and basic active site functional groups clearly contribute to catalysis. For example, both the intermolecular and intramolecular reactions result in breakage of the P—O bond between phosphate and a ribose hydroxyl group. The high pK_a of these hydroxyl groups indicates that the developing negative charge on the leaving group is unstable, making them poor leaving groups and accounting for part of the stability of the phosphodiester backbone. Protonation, or neutralization of developing negative charge is proposed to result in a significant degree of rate enhancement. However, enzyme active sites can potentially utilize additional mechanisms, such as metal ion coordination, to stabilize the developing negative charge on the leaving group. Therefore, it is not clear whether leaving group protonation in the transition state is a prerequisite for enzyme catalyzed phosphodiester hydrolysis. Better experimental approaches to both differentiate between these specific mechanisms of charge stabilization, and to assess the extent to which they contribute to transition state structure would clearly be advantageous.

Similarly, deprotonation of the nucleophile is also invoked as a catalytic strategy in many enzyme active sites. Given the relatively large dependence of diester reaction rates on nucleophile pK_a, deprotonation to yield the oxyanion will greatly accelerate the rate of the reaction. Model phosphodiesters with good leaving groups undergo alkaline hydrolysis, and appear to do so by a specific base mechanism with hydroxide ion acting directly as the nucleophile.[37] Thus, it seems likely that a strong nucleophile-like hydroxide will be required to expel the intrinsically unreactive 3′ or 5′ oxygen. However, other enzyme interactions with the leaving group and nonbridging oxygens may activate the phosphoryl center to such an extent that general base activation of the nucleophile is sufficient for the intermolecular reaction. Additionally, with pre-positioned nucleophiles like that observed for the intramolecular reaction, the nucleophile need not always attack as the anion. Cleavage and isomerization of uridine 3′ *p*-nitrophenyl phosphate and uridylyl 5′ uridine monophosphate is catalyzed by imidazole, which appears to activate the nucleophile by a general base mechanism[10,38] (see below). Enzyme active sites could similarly pre-position an external nucleophile so that it is sufficiently activated by interacting with a general base. Thus, an accurate means of characterizing the nucleophile protonation state in enzyme active sites, and an understanding of the bonding interactions to the nucleophile in the transition state are clearly desirable.

Additionally, protonation of nonbridging oxygen atoms can also contribute to catalysis. Although they are strongly acidic (pK_a = 1.7), protonation is possible under highly acidic conditions, and perhaps in the specialized environment of an enzyme active site as well. Protonation of a phosphodiester nonbridging oxygen neutralizes the negative charge on the phosphoryl center, making it electronically similar to a phosphotriester. Therefore, electrostatic repulsion would then no longer be a significant factor in the reaction, resulting in a greater reaction rate and an associative, triester-like transition state characterized by less bonding to the nonbridging oxygen atoms compared to the ground state. In addition to protonation, enzyme active sites can also interact with the nonbridging oxygen atoms via metal coordination, or other charge–charge interactions. The extent to which these interactions contribute to catalysis and transition state structures compared to protonation remains unknown. A method for assessing the extent of P—O bonding and protonation to the nonbridging oxygen atoms in the transition state is thus essential for addressing these issues.

KIE ANALYSIS OF INTERMOLECULAR NUCLEOPHILIC ATTACK ON PHOSPHODIESTERS

As introduced above, complete understanding of the overall charge distribution for phosphodiester cleavage transition states requires an assessment of the degree of bonding to the attacking, leaving group, and nonbridging oxygens. Given the important influence of acids and bases on reaction rate, it is also important to understand how interactions with these functionalities affect transition state structure and chemical mechanism. Additionally, we know that metal ions accelerate the rates of diester cleavage reactions, and such mechanisms are clearly an important factor in enzyme catalysis. Do metal ions interactions influence the transition state structure in meaningful ways? If they do, then in principle understanding and monitoring these changes could serve as a chemical signature for testing hypothetical active site interactions in enzymes. Fortunately for enzymologists interested in phosphodiesterases, a tremendous amount of groundwork has been laid by Hengge, Cleland, and colleagues on application of KIE analysis to understand the transition states of nonenzymatic reactions involving phosphodiesters. These effects represent an essential backdrop for interpreting effects on enzyme reactions.

General Aspects of Isotope Effect Measurement and Interpretation

Isotopic substitution can affect both the rate and the equilibria of chemical reactions, and such effects are accordingly referred to as "kinetic" and "equilibrium" isotope effects, respectively. These effects are expressed at the ratio of the relevant reaction constants ($^{light}k/^{heavy}k$).[26,30,39] If the isotopically substituted atom is directly involved in bond formation or breaking, the effect is referred to as "primary," and effects on atoms that are not linked to the broken or formed bonds are described as "secondary." Bonding, and thus the sensitivity to isotopic substitution, can change for atoms that are not directly involved in the reaction coordinate; consequently, secondary isotope effects also provide important information on transition state structure and chemical mechanism. The greatest isotope effect is observed when the ratio of the masses of the two isotopes is large (e.g., H vs D). But more often the ratios are much smaller (^{18}O vs ^{16}O in the oxygens of phosphate, for example), and so the differences in the rate and equilibria are correspondingly more subtle (a few percent, or even tenths of a percent in some cases). This fact generally precludes direct comparison of the reaction rates or equilibria, and more specialized methods are required (see below). Nonetheless, because the perturbation to the system is small, the corresponding isotope effects, whose magnitude may seem unimpressive, can provide indispensable mechanistic information.

In general, lighter isotopes will react faster resulting in isotope effects ($^{light}k/^{heavy}k$) that are greater than 1, or "normal." However, in some circumstances, the heavier atom is favored resulting in isotope effects that are "inverse"—that is, less than 1. The magnitude of *kinetic* isotope effects depends on two factors: the temperature independent factor (TIF), which results from differences in the imaginary frequency, or reaction coordinate motion in the transition state and is always normal, and the temperature dependent factor (TDF), which depends on changes in bonding between the ground state and transition state. In general, more strongly bonded environments favor the heavier isotope; thus, for atoms in which bonds are broken—for example, the leaving group oxygen in phosphodiester bond hydrolysis—the TIF and the TDF are both normal.[40,41] On the other hand, for nucleophiles, where bond order increases during the reaction, the contribution from the TDF will be inverse.[42] Although this fact will mitigate the observed isotope effect, most experimentally determined nucleophile effects are normal, likely due to the dominant contribution from reaction coordinate motion for the gen-

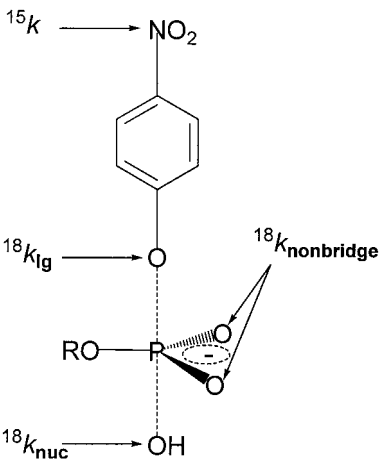

FIGURE 3 ^{18}O and ^{15}15 isotope effects in phosphodiester hydrolysis. The dashed lines represent partial bonding to the nucleophile and leaving group in the reaction coordinate. The $^{18}k_{lg}$ and $^{18}k_{nuc}$ are primary isotope effects; $^{18}k_{nonbridge}$ and ^{15}k are secondary isotope effects.

erally less massive nucleophile. Additionally, inverse nucleophile isotope effects are only predicted by computational studies in very late transition states in which the TDF contribution from bonding is dominant.[43]

In contrast, equilibrium isotope effects are only the result of contributions from the TDF and can often be inverse (the heavier isotope is favored), when there is an increase in bonding between the two relevant species. For example, the equilibrium effect upon going from an O—H to an O—C bond is ca. 4% inverse due to the stronger bond to carbon[44] and equilibrium protonation of the nonbridging phosphate oxygens is ca. 2% inverse.[45] Similarly, metal ion coordination of water favors $H_2^{18}O$ and overall effects can be as large as ca. 2.5% inverse in the direction of formation of the metal hydrate.[46,47] Thus, understanding whether upstream equilibria are part of the reaction mechanism is crucial since they can have an important influence on the observed isotope effect.

Analysis of primary isotope effects on the nucleophile ($^{18}k_{nuc}$) and leaving group ($^{18}k_{lg}$), and secondary effects on the nonbridging oxygen atoms ($^{18}k_{nonbridge}$) for phosphodiester hydrolysis to date have focused on reactions with nitrophenyl leaving groups (Figure 3). This focus is due in part to the requirement for measurable reaction rates, as well as technical considerations in measuring leaving group and nonbridging oxygen effects. Because the perturbations in rate and equilibria are relatively small, these effects are generally measured by comparing the isotopic ratios in the reactant and product populations using an ^{15}N remote label in the nitro leaving group. A detailed description of this methodology, and the remote label approach and the technical considerations involved, can be found in several recent reviews.[14,30,35,48] To explain briefly: Due to the high precision required for isotope effect analysis, the optimal measurements are made using an isotope ratio mass spectrometer (IRMS). In the remote label approach the reactants are labeled at two positions: one label is incorporated into the atom under examination, and the second "reporter" label is at a position that is easily isolated and analyzed by IRMS. Although this method has been extraordinarily useful in examining nonenzymatic reactions and enzymatic reactions where small defined leaving groups can be used, there exist significant technical barriers to applying this approach to phosphodiesterase enzymes that act on large macromolecular substrates.

KIE Analysis of Acid- and Base-Catalyzed Phosphodiester Hydrolysis Reactions

Overall, the isotope effect data for phosphodiester hydrolysis reactions support the conclusion from Brønsted analysis that these reactions proceed by a simultaneous addition-displacement mechanism with partial bonding to both the nucleophile and leaving group in the transition state. Specifically, the attacking nucleophile, the leaving group, and nonbridging oxygens all show significant effects upon substitution with ^{18}O.[35,37] Importantly, the magnitude and direction of these effects (Table I) provides more detailed information about the degree of charge development on the leaving group and the associative nature of the transition state.

In general, the magnitude of ^{18}O substitution of the leaving group oxygen ($^{18}k_{lg}$) provides information on the extent of P—O bonding in the transition state. However, buildup of negative charge on the departing oxygen due to bond cleavage can result in charge delocalization onto the phenol ring, which in turn results in secondary ^{15}N effects on the nitro group (^{15}k) (Figure 3). Thus, in concert with $^{18}k_{lg}$, measurement of this effect provides information on whether the leaving group departs as the anion, or if protonation occurs to alleviate buildup of negative charge. For diester reactions $^{18}k_{lg}$ is relatively small (0.4–0.6%) compared to analogous reactions of monoesters dianions (2%)[14,35,48] (Table I). Similarly, the secondary ^{15}k effect is generally small (0.07–0.16%), which, together with $^{18}k_{lg}$ reflects a more associative transition state than monoesters (although less associative than triesters) with less bond cleavage to the leaving

Table I ^{18}O and ^{15}N Kinetic Isotope Effects for Phosphodiester, Monoester, and Triester Hydrolysis Reactions[a]

Reaction	$^{18}k_{lg}$	^{15}k	$^{18}k_{nonbridge}$	$^{18}k_{nuc}$
Diesters				
alkyl-pNP (alkaline)[35,37]	1.0063	1.0017	0.9945	1.027 (1.068)
alkyl-pNP (acid)[35]	1.0039	1.0009	1.0139	
alkyl-pNP (phosphodiesterase)[35]	1.0073	1.0017	0.9842	
3′pNP-UMP (acid)[50]	1.0063	1.001		
3′pNP-UMP (imidazole)[50]	1.0067	1.0009		
3′pNP-UMP (carbonate)[50]	1.0059	1.0001		
3′MNB-UMP (acid) cleavage[52]	1.0019	0.9885		
3′MNB-UMP (neutral) cleavage[52]	1.009	0.9983		
3′MNB-UMP (neutral) isomerization[52]	1.0004	0.9988		
3′MNB-UMP (alkaline) cleavage[52]	1.0272			
ethyl-pNP (Eu3+)[71]	1.016	1.0030		
ethyl-pNP (Zn2+)[71]	1.0095	1.0005		
Dinuclear Co complex[72]	1.029	1.0026		0.937
3′MNB-UMP(RNase A)[83]	1.0016	1.0004	1.005	
Monoesters				
pNPP(dianion)[85]	1.0189	1.0028	0.9994	
pNPP(monoanion)[85]	1.0087	1.0004	1.0184	
Triesters[86,87]	1.0060–1.0052	1.0007	1.0063–1.033	

[a] Abbreviations: alkyl-pNP, alkyl-*p*-nitrophenyl phosphate; 3′pNP-UMP, uridine-3′-*p*-nitrophenyl phosphate; 3′MNB-UMP, uridine-3′-*m*-nitrobenzyl phosphate; ethyl-pNP, ethyl-*p*-nitrophenyl phosphate; pNPP, *p*-nitrophenyl phosphate.

group and generally little neutralization of negative charge on the departing oxygen.

The acid-catalyzed reactions of both aryl [*p*-(*t*-butyl)phenyl] and alkyl (3,3-dimethylbutyl) phosphodiesters of *p*-nitrophenyl both show relatively small, inverse nonbridging oxygen isotope effects ($^{18}k_{nonbridge} = -0.8\%$). This inverse effect likely arises from equilibrium protonation under such strongly acidic conditions. Even though the pK_a of these nonbridging oxygens is quite low (-0.3), this value is likely to be higher in the transition state, particularly if it has an associative character. A similarly inverse $^{18}k_{nonbridge}$ is observed for snake venom phosphodiesterase, which has been interpreted as reflecting protonation by an active site functional group. Thus, such inverse effects can serve as an important diagnostic tool for evaluating proton transfer to one of the nonbridging oxygens of the reactive phosphate. Taking the equilibrium contribution into account yields normal intrinsic $^{18}k_{nonbridge}$ effects for acid hydrolysis of 0.88–1.4%, consistent with a more associative transition state. The low magnitude of $^{18}k_{lg}$ (0.4%) may also reflect this more associative transition state. Additionally, it could also indicate partial protonation of the leaving group in the transition state.

The alkaline hydrolysis reaction also displays a low $^{18}k_{lg}$ (0.6%), but the effect is slightly greater in magnitude to that observed for the acid-catalyzed reaction. Also, the secondary ^{15}N effect is significantly greater than that observed for the acid-catalyzed reaction (0.1–0.16% vs 0.07–0.09%). Here, the larger ^{15}k value for alkaline hydrolysis indicates that cleavage of the P—O bond to the leaving group results in increased electron density on this oxygen, which is partially delocalized onto the nitro group. For acid hydrolysis, the protonation of the leaving group oxygen diminishes this effect. The observation of significant effects for $^{18}k_{lg}$ and $^{18}k_{nonbridge}$ demonstrates that both the acid and base reactions are concerted. The small magnitudes of $^{18}k_{lg}$ indicate relatively little bond cleavage to the leaving group in the transition state. Such an "early" or reactant-like transition state is also supported by nucleophile isotope effects that are consistent with relatively little contribution to the observed effects coming from bond formation[37] (Figure 4).

However, the most intriguing results are the significant differences seen in the nonbridging oxygen effects for the alkyl vs the aryl diesters. As indicated above, both diesters show a greater secondary $^{18}k_{nonbridge}$ for the acid-catalyzed reaction, consistent with an associative, triester-like transition state (Table I). However, $^{18}k_{nonbridge}$ is inverse for the alkaline hydrolysis of the alkyl diester (-0.5%), while the aryl diester still shows a normal effect (0.4%). Perhaps the ability to delocalize electrons onto the aryl group results in a more associative transition state in that increased overall bond-

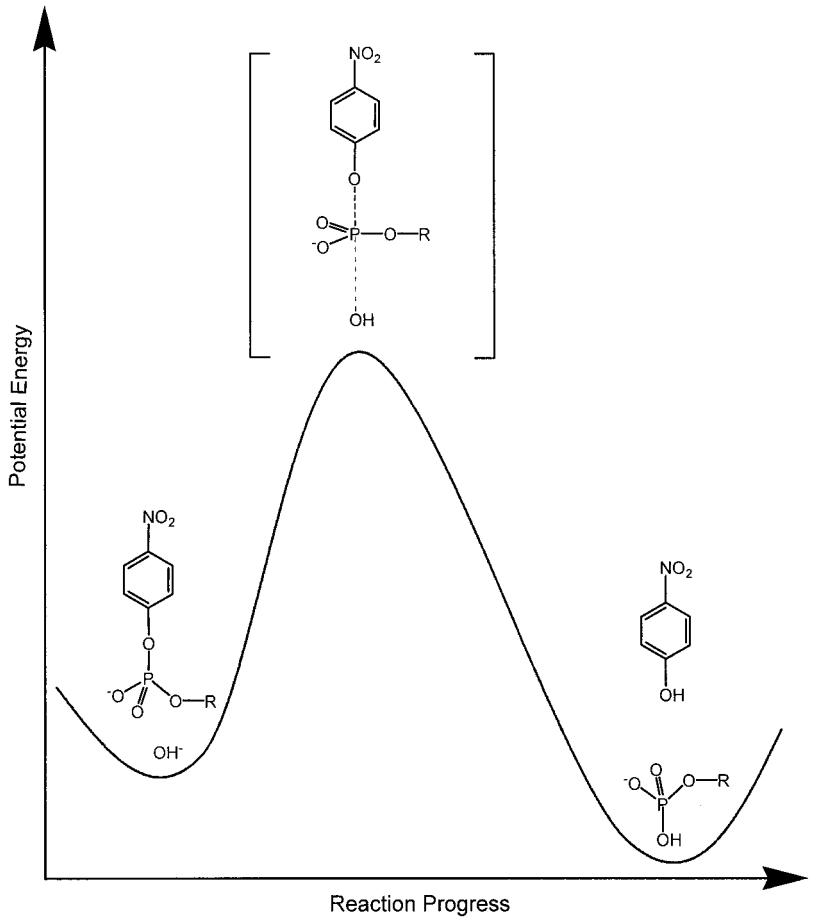

FIGURE 4 Potential energy diagram for hydroxide-catalyzed phosphodiester hydrolysis with a *p*-nitrophenyl leaving group. The early or reactant-like transition state for this reaction is depicted in both the model for the transition state structure and the steeper incline of the leading edge of the potential energy barrier compared to the trailing edge.

ing to phosphorus can be compensated for by this effect. Nonetheless, for biological reactions involving nucleic acids the "secondary" R group (the 3′ or 5′ position) is necessarily an alkyl substituent. Thus, the observation of an inverse effect for the alkaline hydrolysis reaction may mean that such reactions will have a more dissociative character than previously appreciated. However, such reactions still show much smaller leaving group effects (and smaller β_{lg}) than those observed for monoester dianions consistent with less bond cleavage. Although both nucleophile and leaving group effects also indicate an early transition state, it may be that for these reactions bond formation to the nucleophile lags behind leaving group departure, such that a compensating increase in bonding to the nonbridging oxygens occurs. Regardless, the associative–dissociative distinction between monoester and diester reactions should be considered general in that different conditions can influence the associative or dissociative character of the transition state. Herschlag and Jencks have proposed such a continuum of transition states for phosphate monoesters, and a similar paradigm appears to be useful for considering diester reactions.[36,49]

TRANSITION STATES INVOLVING INTRAMOLECULAR ATTACK BY AN ADJACENT 2′ HYDROXYL

Insight into how inter- vs intramolecular nucleophilic attack results in differences in the transition state structures and mechanisms for phosphodiester cleavage comes, in part, from analysis of KIE on reactions involving 2′ hydroxyl attack of 3′ phosphorylated uridine compounds.[50–52] Such reactions are of significant biological interest because certain protein ribo-

nucleases as well as the small self-cleaving ribozymes employ this general mechanism.[16–18,53] It has long been recognized that diesters of uridine 3′ phosphate can under go both cleavage and isomerization reactions: in the former case yielding the 2′ 3′ cyclic phosphate, while in the latter the 2′ phosphodiester is formed. Evidence from isotopic labeling and stereochemical and chemical kinetic studies shows that under certain conditions these compounds undergo a stepwise mechanism involving not merely a trigonal bipyrimidal transition state, but a true phosphorane intermediate characterized by a single bond to both the nucleophile and leaving group, with sufficient lifetime to undergo pseudorotation.[54] Do the intrinsic constraints imposed by intramolecularity result in this mechanism rather than the concerted transition states of intermolecular reactions? Or is the phosphorane intermediate important for expelling leaving groups with higher pK_as? Understanding the factors involved in promoting one or the other of these two reaction channels is important for understanding why an enzyme employs a specific mechanism. Additionally, this class of ribonucleases can often act without active site metal ions, underscoring the importance of understanding how acid and base influences the reaction mechanism.

Both acid and base can catalyze displacement reactions of uridine 3′-nitrophenyl phosphate, and both reactions proceed via cyclization with elimination of nitrophenol. Acid catalysis proceeds via nucleophilic attack by the adjacent 2′OH on the protonated neutral diester.[50] In contrast, catalysis by general bases results in partial deprotonation of the attacking 2′OH, while during specific base catalysis the nucleophile is the fully deprotonated alkoxide.[38] However, the degree of protonation, its effect on mechanism, as well as the charge distribution in the transition state are not well understood.

A more detailed understanding of the different transitions states for these model reactions comes from analysis of $^{18}k_{lg}$ and $^{18}k_{nonbridge}$ for reactions of uridine 3′-nitrophenyl phosphate (summarized in Table I).[50] Overall, the values of $^{18}k_{lg}$ and ^{15}k for both the acid-catalyzed and general base-catalyzed reactions of this compound show no large-scale deviation from that measured for intermolecular acid- and base-catalyzed reactions of other model phosphodiesters.[35,48] Thus, the intramolecular nature of the reaction does not by itself have a large effect on reaction mechanism or transition state structure. The relatively small leaving group and ^{15}k effects reveal that the cyclization reactions occur with early transition states and only moderate bond cleavage to the bridging oxygen, similar to intermolecular reactions of phosphodiesters with the same good leaving group. Interestingly, the specific base-catalyzed reaction differs in that it shows a smaller $^{18}k_{lg}$ compared to the acid- and general base-catalyzed reactions and the ^{15}k is essentially unity. A ^{15}k effect of unity suggests that there is essentially no charge development on the leaving group. This observation raises the possibility that under these conditions the reaction proceeds with a different rate-limiting step—for example, formation of a phosphorane intermediate. However, analysis of the stereochemistry of the reaction provides no evidence of pseudorotation, and thus such a mechanism seems unlikely. Similarly, linear free energy experiments indicate that the reaction mechanism is indeed concerted.[38] Thus, the picture that emerges for the specific base-catalyzed reaction is one in which the transition state is even earlier, or more reactant-like with P—O bond cleavage less advanced and less negative charge on the leaving group compared to the acid- and general base-catalyzed reactions (as in Figure 4). The authors suggest that this difference arises from the stronger nucleophilic character of the fully deprotonated 2′ alkoxide relative to the less potent, partially, or fully protonated species in the acid and general base reactions.

At this point, however, the effects of isotopic substitution on the nonbridging oxygens have not been examined, and so information concerning the associative or dissociative nature of the transition state is lacking. Like most phosphodiester reactions, information about the nucleophile is missing as well. Here, the effect would be influenced by nucleophile protonation as well as participation in the reaction coordinate. Unfortunately, labeled compounds in which these intramolecular nucleophile effects can be measured have not yet been described. Nonetheless, together with physical chemical data, these reactions are currently viewed as occurring via a concerted mechanism, with a typically early transition state similar to other phosphodiester reactions with good, aryl leaving groups.

What might these results mean for enzymes that carry out this cleavage mechanism? Clearly, for reactions with activated leaving groups, which are likely to occur in enzyme active sites, the degree of deprotonation of the nucleophile can still have an important influence on the reaction rate and, potentially, the transition state charge distribution. Additionally, given sufficient leaving group activation by enzyme active site residues, reactions involving attack by the 2′ OH can be concerted, as opposed to stepwise. However, the relevance of such model systems is sometimes questioned since the leaving group for this class of RNA and protein enzymes is the ribose 5′

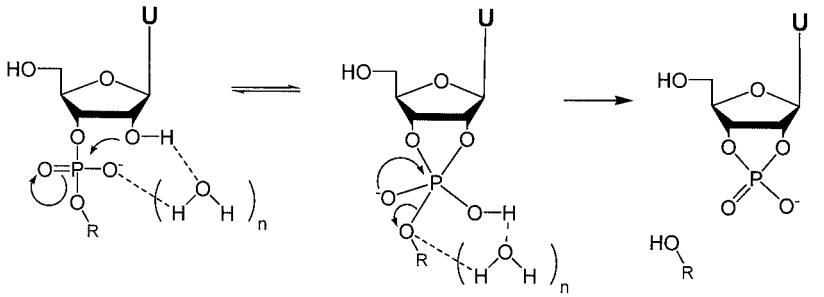

FIGURE 5 Intramolecular cleavage reaction for a phosphodiester with a poor leaving group at neutral pH. Water acts as a general acid/base catalysis in both the formation and breakdown of the phosphorane intermediate.

oxygen, which as a pK_a much higher than *p*-nitrophenol (15 vs 7).

Earlier experiments with similar 3′ phosphorylated uridine compounds with poor leaving groups demonstrate that reactions of this kind can proceed under neutral or acidic conditions with retention of stereochemistry, and are thus considered stepwise with formation of a phosphorane intermediate[12] (Figure 5). Model compounds of RNA, like ribonucleoside 3′ alkyl phosphates, in acidic or neutral pH undergo two distinct transesterification reactions that can yield either the 2′-isomer or the 2′,3′-cyclic phosphate with elimination of the alkyl group.[54] Although a phosphorane intermediate is widely accepted for this reaction, competition between the concerted and stepwise mechanisms still appears to be condition dependent. In enzymes, active site constraints likely restrict pseudorotation even if an intermediate forms, and where it has been examined enzyme-catalyzed reactions appear concerted as they occur with inversion of stereochemistry.[55–57] Formation of a phosphorane is clearly not a prerequisite even for the nonenzymatic reactions, as under alkali pH the reaction appears concerted in that no isomerization is observed.[52] These observations indicate that the intramolecular reaction can occur via a concerted mechanism, even with a poor leaving group.

Insight into the factors that govern these transition states, and thus what might be expected for enzyme active sites, comes from comparison of the leaving group and nonbridging oxygen isotope effects for the cleavage and isomerization reactions of uridine 3′ *m*-nitrobenzyl phosphate (Table I). [52] Under acidic conditions the primary $^{18}k_{lg}$ effect of ca. 2% and the slightly inverse secondary $^{18}k_{nonbridge}$ effect appear consistent with a proposed phosphorane intermediate. The authors propose that the normal $^{18}k_{lg}$ arises from opposing effects on O—P bond cleavage, which is normal, and an inverse contribution from protonation of the leaving group in the transition state. The inverse $^{18}k_{nonbridge}$ indicates an increase in bonding to the nonbridging oxygens attributable to formation of a neutral phosphorane via protonation (Figure 5). In fact, the magnitude of this effect is close to that expected for equilibrium protonation of a nonbridging oxygen of phosphate. Taken together, these results indicate that the resultant phosphorane is "late" or product-like, likely due to the poor leaving group.

In contrast to the acid-catalyzed reaction, at neutral pH the nonbridging oxygen effect is not inverse but unity for both the isomerization and cleavage reactions. This result indicates that protonation of the nonbridging oxygen has not occurred, and that the phosphate in the proposed intermediate still carries a single negative charge as in the ground state. This conclusion is consistent with the observation that the reaction is largely pH independent from pH 4 to 8, indicating no net protonation (or deprotonation) in the transition state. Also, the bridging isotope effect is smaller for the isomerization reaction than for cleavage (0.04 vs 0.9%). This difference makes sense in light of the fact that for the isomerization reaction the bond to the bridging oxygen remains unchanged, while the cleavage reaction results in breakage of the P—O bond. However, these results do not reveal whether the cleavage reaction is concerted or stepwise, since a primary effect would be observed in either case.

The hydroxide-catalyzed reaction does not yield any isomerization product. This observation strongly suggests that no phosphorane intermediate exists to undergo pseudorotation, and therefore the hydroxide-catalyzed reaction is concerted. The $^{18}k_{lg}$ effect is normal with a magnitude of 2.7%. This very large magnitude is much greater than those observed for both the inter- and intramolecular cleavage mechanisms with *p*-nitrophenyl leaving groups (Table I). This difference may indicate a significantly later transition state for expulsion of the less reactive *m*-nitrobenzyl leaving group.

NUCLEOPHILE ISOTOPE EFFECTS

The studies described in the preceding sections provide an enhanced understanding of phosphodiester cleavage reactions and the influence of acid and base on reaction mechanism. However, until recently, a crucial piece of this puzzle has been missing—that is, the effects of isotopic substitution on the nucleophile ($^{18}k_{nuc}$). General aspects of nucleophilic participation have previously been inferred from structure–reactivity kinetic studies and the combined leaving group and nonbridging oxygen isotope effects. Nonetheless, the magnitude of $^{18}k_{nuc}$ could provide novel insight into the formation of potential intermediates, as well as the mechanism of nucleophilic activation and test predictions about the overall charge distribution in the transition state.

For example, hydroxide ions can potentially catalyze hydrolysis of phosphodiesters by acting directly as the nucleophile, or indirectly by acting as a general base to facilitate the deprotonation of a water molecule. As indicated above, the more potent 2′ alkoxide nucleophile is associated with a concerted mechanism, and an early transition state for the intramolecular cyclization reaction. On the other hand, this reaction is also catalyzed by imidazole via a general base mechanism in which the nucleophile retains partial protonation in the transition state on the pathway to a phosphorane intermediate, as evidenced by observation of the competing isomerization reaction pathway. Thus, it follows that transition states of *inter*molecular phosphodiester hydrolysis reactions might also be influenced by the mechanism of nucleophilic activation. Furthermore, both specific and general base mechanisms are proposed for nucleophilic activation in enzymes that catalyzed the hydrolysis of phosphodiester bonds in DNA and RNA. Thus, understanding the precise mechanism of nucleophilic attack for nonenzymatic reactions provides the backdrop for testing specific hypotheses concerning nucleophilic activation by enzyme active site residues.

In light of the strong nucleophilic character of hydroxide, a specific base mechanism for phosphodiester hydrolysis seems most likely. However, this conclusion does necessarily hold for hydrolysis reactions involving other organic esters. Previous studies of primary kinetic ^{18}O isotope effects on the nucleophile for both ester and amide hydrolysis have been analyzed in detail by Marlier and colleagues.[58–60] Here the measured ^{18}O KIE is, in general, around 2%. If hydroxide were to act directly as the nucleophile, then the measured effect should include an equilibrium isotope effect on deprotonation in addition to the intrinsic kinetic effects arising from on bond formation and reaction coordinate motion. The equilibrium effect on deprotonation is large (ca. 4%) and normal due to the loss of an O—H bond.[61] Since the observed $^{18}k_{nuc}$ of ca. 2% for ester and amide hydrolysis is the product of $^{18}K_{OH}$ and the intrinsic kinetic effect, if hydroxide were to act directly as the nucleophile, then the intrinsic kinetic effect would necessarily be inverse. As discussed above, kinetic effects for nucleophiles are derived from two competing effects. The effect due to bond formation is inverse because the transition state includes bond formation to the nucleophile, and more strongly bonded environments favor the heavier isotope. However, this effect is generally considered to be offset by the effect due to reaction coordinate motion. Because the attacking nucleophile is less massive than the chemical group undergoing reaction, it is argued that it will experience the bulk of this effect. Computational studies of ester hydrolysis indicate that inverse effects can arise, but only in very late transition states with differences in ground state and transition state bonding to the nucleophile sufficiently advanced to overcome the effect from reaction coordinate motion.[43] In light of these arguments and additional primary and secondary KIEs, hydroxide ion appears to act as general base in catalysis of ester and amide hydrolysis.

These results clearly raise the question of whether base-catalyzed phosphodiester hydrolysis involves a general or specific base mechanism. To explore this issue we examined the mechanism of hydrolysis of a model phosphodiester (thymidine-5′-*p*-nitrophenyl phosphate; T5PNP) by measuring $^{18}k_{nuc}$.[37] Until recently, technical considerations limited the ability to measure $^{18}k_{nuc}$ for reactions involving relatively complex chemical species such as phosphate esters. For any hydrolysis reaction the measurement of $^{18}k_{nuc}$ requires assessing the isotopic ratio in the reactant, in this case water, and the product phosphate. Because in phosphodiester hydrolysis the attacking oxygen becomes one of four in the product phosphomonoester, measuring the product isotope ratio for this reaction represents a significant challenge. Previous work examining $^{18}k_{nonbridge}$ avoided this issue by linking the ^{18}O-labeled nonbridging oxygen atoms to an ^{15}N label on the leaving group that serves as a reporter.[35] This remote label method allowed the ^{18}O isotope effect to be measured using the reporter ^{15}N label, eliminating the additional oxygen atoms from the analysis. Unfortunately, the remote label method is not possible since the ^{15}N content in the substrate/product cannot be directly connected to the isotopic ratio in the solvent like it can be for the nonbridging and leaving group. Thus, direct measurement of the ^{16}O/^{18}O ratio in the product molecule is required. Recently, this

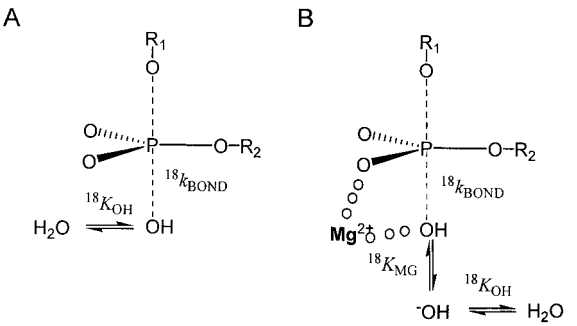

FIGURE 6 Contributions from bond formation and upstream equilibria to observed $^{18}k_{nuc}$ values. A: The $^{18}k_{nuc}$ for nucleophilic attack by hydroxide includes contributions from both the kinetic effect on bond formation ($^{18}k_{BOND}$) and the equilibrium effect on deprotonation ($^{18}K_{OH}$). B: The $^{18}k_{nuc}$ for nucleophilic attack by magnesium coordinated hydroxide also includes the additional equilibrium isotope effect on coordination ($^{18}K_{MG}$).

challenge has been met by the application of whole molecule mass spectrometry, which permits ratios to be measured with precision of ±0.5% for relatively large molecules.[62,63] In fact, the limit (ca. 1000–5000 MW) is set by the requirement that the mass spectrometer be capable of resolving the isotopic peaks. Precision, and thus usefulness for KIE analysis, diminishes as the mass increases due to increased corrections for overlap between adjacent peaks and for subtraction of natural abundance contributions to the isotopomers of interest.

By using this approach we find that the base-catalyzed hydrolysis of T5PNP gives a normal nucleophile isotope effect of 6.8%.[37] Such an effect is significantly larger than other precedents for intrinsic KIEs on bond formation. Maximal ^{18}O nucleophile effects are predicted by computational analysis to be around 3% for carbonyl addition,[43] and observed effects on ester and amide hydrolysis are in this range as described above. Thus, the observed effect must necessarily include a contribution from equilibrium deprotonation to yield hydroxide (Figure 6). This interpretation is also supported by analysis of solvent deuterium isotope and ionic strength effects.

Taking $^{18}K_{OH}$ into account, the ^{18}O-KIE is 2.7%, a large, but reasonable value based on computational and experimental studies of ester and amide solvolysis. The large normal $^{18}k_{nuc}$ is likely to arise from a dominant contribution from reaction coordinate motion, and little contribution from bond formation. Extracting quantitative information on bond order is not yet possible because we currently lack both quantum mechanical studies of these reactions and sufficient precision of the measurement to make such studies useful (addressing these limitations will greatly improve the current picture for nucleophilic participation in phosphodiester hydrolysis). Nonetheless, the current interpretation is consistent with the leaving group and nonbridging isotope effects for other alkyl esters of *p*-nitrophenyl phosphate that show an early transition state without significant associative or dissociative character (Figure 4). Importantly, hydroxide-catalyzed phosphodiester cleavage is the only phosphoryl transfer reaction where $^{18}k_{lg}$, $^{18}k_{nonbridge}$, and $^{18}k_{nuc}$ have all been measured. The consistency between these values confirms the utility of KIE in probing transition state structure and chemical mechanism. Thus differences observed in the KIE for phosphodiester cleavage under different conditions or in an enzyme active site will represent observable differences in the transition state structure and provide valuable insight into the catalytic mechanism.

A similar analysis of nucleophile isotope effects would clearly provide important information on the isomerization and cyclization reactions involving nucelophilic attack by the adjacent 2′ hydroxyl. The proposed concerted and stepwise mechanisms make specific predictions about what nucleophile isotope effects will be observed. For the concerted base- catalyzed reaction a large normal effect, much like that observed for the intermolecular reaction, is anticipated, and most of the arguments will be similar. In contrast, for the stepwise mechanism, it is anticipated that departure of the leaving group is rate limiting. Thus, the nucleophile isotope effect will in essence be entirely an equilibrium effect for going from an O—H to an O—P bond. Thus, a large inverse effect is predicted, and so one of the most simple and direct tests of intermediate formation would be the magnitude and direction (inverse vs normal) of $^{18}k_{nuc}$. These effects could be determined by whole molecule mass spectrometry using a population of substrates enriched for ^{18}O at the 2′ hydroxyl. Alternatively, the intramolecular nature of the reaction makes it possible, in principle, to relate the isotopic composition of the nucleophile represented by the remote label in the ring nitrogen.

INFLUENCE OF METAL IONS ON PHOSPHODIESTER REACTION TRANSITION STATES

A widespread and important feature of enzymes that catalyze the hydrolysis of phosphodiester bonds in nucleic acids is the presence of essential active site metal ions.[3,16,64] Thus, the interactions between phosphodiesters and active site metal ions and the mech-

anisms by which these interactions promote catalysis are of intense interest. Our current understanding of the mechanisms by which metal ions enhance the rates of phosphodiester reactions holds that they manifest their influence by (a) Lewis acid activation, (b) by enhancing formation of an anionic nucleophile, (c) by positioning the nucleophile for in-line attack via what has been called "induced intramolecularity," or (d) by stabilization of the leaving group.[26,39,49] A large body of structural data position one, two, or more active site metal ions in coordination variously with the nucleophile, leaving group, and nonbridging oxygens. Further, numerous structure–function studies carried out on both protein and RNA phosphodiesterases demonstrate that metal ion interactions with the reactive phosphate contribute significantly to transition state stabilization.[3,64–67] However, even for some of the most well characterized enzymes it is difficult to unambiguously identify the precise number and role of active site metal ions and to define their precise contribution to catalysis. Additionally, we are only just beginning to understand how, or whether interactions between phosphodiester transition states and metal ions result in differences in chemical mechanism or charge distribution.

As it turns out, the nonenzymatic hydrolysis of model phosphodiester compounds can also be catalyzed by adding metal ions to the reaction, providing an arena for evaluating how metal ions influence transition state structure. In fact, a rather large range of metal ions and metal ion complexes are effective at promoting phosphodiester hydrolysis, and several systems have been developed with the goal of generating chemical reagents that can cleave specific nucleic acid sequences. The structure, chemistry, and application of such complexes have recently been reviewed.[68–70] However, the transition state structures of these reactions and the precise influence of metal ion interactions are only just now coming into focus. Interestingly, Hengge, Cleland, and colleagues find that some of these systems show surprising, in some cases dramatic, changes in mechanism and transition state structure.

To date, KIE analyses have focused primarily on catalysis by lanthanides, Co(II) and Cu(II) (Table I) as complexes of these metal ions can show relatively large catalytic effects. Lanthanides alone can catalyze the cyclization reaction of uridine 3′ *p*-nitrophenyl phosphate with a concentration dependence described by dissociation constants ranging from 0.1 to 1 m*M*.[51] Reactions catalyzed by Er^{3+} and Eu^{3+} result in much higher ^{15}k effects as compared to both the general base- (imidazole) and specific base-catalyzed reactions (e.g., 0.3 vs 0.15 and 0.01%). Such impressively large secondary effects have only been observed for monoester hydrolysis, in which the transition state is dissociative, or metaphosphate-like, where bond cleavage to the leaving group is very advanced. Thus, it follows that in the lanthanide-catalyzed reaction a very large degree of negative charge development occurs relative to other reactions of phosphodiesters. A similar, surprisingly large ^{15}k effect is observed for lanthanide-catalyzed hydrolysis of ethyl *p*-nitrophenyl phosphate as well as catalysis by Co(III)-cyclen.[71] These results contrast with the more typical concerted, early transition state observed in base-catalyzed hydrolysis and in catalysis by Zn^{2+} and the Cu-containing compound copper(II)-1,4,7-triazacyclononane. Thus, there appear to be significant underlying differences in how metal ions accelerate phosphodiester reaction by interacting with the transition state.

The concentration dependence of the Eu^{3+}-catalyzed reaction indicates that two metal ions bind the transition state potentially bridging the phosphate and nucleophile[71]; however, the precise geometry of this complex is still unclear. The strong, polarizing interaction between the metal ion and the nonbridging oxygens are proposed to result in development of positive charge on the phosphorus. Catalysis would then come from the induced intramolecularity of nucleophilic attack by simultaneous interaction of the metal ion with the diester and nucleophile and from the Lewis acid activation resulting from metal coordination of the nonbridging oxygen atoms (Figure 7A). In this transition state dominated by nucleophile activation, the partial positive charge on the phosphorous induced by this metal ion coordination to the nonbridging oxygens would make it more difficult to break the P—O bond to the leaving group, requiring greater development of negative charge on the leaving group in the transition state. The large degree of negative charge development on the leaving group oxygen is reflected in the large secondary ^{15}k effect on the nitro group. For enzymes, it seems unlikely that active site metal ion interactions would result in the transition state observed in lanthanide catalysis since Zn^{2+} and other complexes do not show these effects. Further, interactions between phosphodiester transition states with Mg^{2+}, the most common metal ion observed in phosphodiesterase active sites, are not expected to result in such strong interactions with the non-bridging oxygens. In fact, Ca^{2+} and Mg^{2+} alone can catalyze the hydrolysis of thymidine 5′ *p*-nitrophenyl phosphate (Cassano, Anderson, and Harris, unpublished), and it is hoped that further investigation of these reactions will help extend our understanding

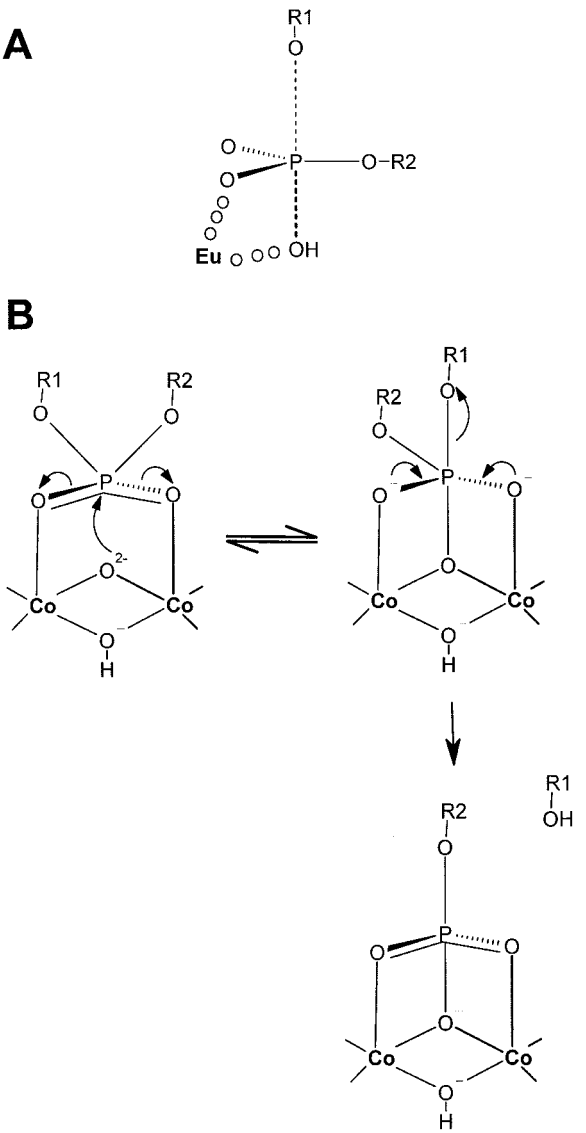

FIGURE 7 Mechanisms of metal ion catalysis in phosphodiester hydrolysis. A: Europium (III) catalyzes phosphodiester hydrolysis by coordinating both the nonbridging and nucleophile oxygen atoms. The interaction with the nonbridging oxygens engenders Lewis-acid catalysis while the coordination of the nucleophile facilitates deprotonation and provides induced intramolecularity. The late or product-like transition state is characterized by the strong bond to the nucleophile and the weak bond to the leaving group. B: Catalysis by the cobalt (III) dinuclear cluster alters the intermolecular mechanism from concerted to stepwise with a phosphorane intermediate. Formation of this intermediate may be aided by the strong interactions between the metal ions and the nonbridging oxygen atoms. Additionally, double coordination to the nucleophile stabilizes the O^{2-} species.

of the intrinsic ability of divalent metal ions to catalyze phosphodiester reactions.

Some enzyme active sites contain dinuclear metal centers, and several dinuclear compounds are potent catalysts of phosphodiester hydrolysis. The influence of such a metal ion center on phosphodiester reaction mechanisms is provided by the recent report by Hengge and co-workers of KIEs for hydrolysis of an alkyl phosphodiester by a dinuclear Co complex (Table I).[42] Here, these investigators take advantage of the fact that the stability of the Co–water coordination permits the isotopic composition of the nucleophile to be followed using the nitrogen of the nitrophenol of as a remote label.

Surprisingly, the $^{18}k_{nuc}$ measured for this reaction is large and inverse (-7%). As described in the preceding section, even though bond formation to the nucleophile will favor the heavier isotope, nucleophile isotope effects are generally normal due to dominant reaction coordinate motion contributions, which are always normal. The observation of normal $^{18}k_{nuc}$ values for hydroxide-catalyzed ester, amide, and phosphodiester hydrolysis is consistent with this point of view. The authors propose that the unexpected inverse effect results from a change in the reaction mechanism from concerted to stepwise, with formation of a phosphorane intermediate with full bond formation to the nucleophile (Figure 7B). In this case, the observed $^{1}k_{nuc}$ effect would essentially be an equilibrium effect on going from an O—H to and O—P bond in the context of the coordination complex. This effect should be inverse by several percent due to the stronger O—P bond, and observed fractionation factors for formation of O—C bonds.[44] It is important to note that the strong coordination of the nonbridging phosphate oxygen atoms by Co(III) has been compared with alkylation.[49] In this scenario, the transition state would be expected to resemble the more associative triester transition state. The forces driving formation of the phosphorane intermediate in this specific case may therefore differ significantly to the forces driving formation of the phosphorane intermediate in the intramolecular reaction.

The ^{15}k and $^{18}k_{lg}$ values have also been measured for this reaction.[72] The ^{15}k and $^{18}k_{lg}$ values are both much greater than the corresponding effects in the hydrolysis of uncomplexed phosphodiesters. In fact, these large values are more in line with values obtained for hydrolysis of phosphomonoester dianions. The observation of a measurable $^{18}k_{lg}$ demonstrates that departure of the leaving group is occurring in the rate-limiting step of the reaction, which is consistent with the stepwise mechanism described above since breakdown of the phosphorane intermediate is con-

sidered to be rate-limiting (Figure 7B). Monoester transition states are characterized by an advanced breakage of the leaving group bond, and thus the large $^{18}k_{lg}$ and ^{15}k indicate that the leaving group bond is largely broken in the rate-limiting step, resulting in a corresponding buildup of negative charge on the departing oxygen. However, the KIE results do not formally exclude a concerted mechanism with a very late transition state, as proposed for lanthanide-catalyzed reactions.

In this regard a comparison of the $^{18}k_{nuc}$ for other metal ion-catalyzed phosphodiester hydrolysis reactions would be extremely useful. Interactions with metal ions can introduce their own contributions to the observed isotope effect. As illustrated in Figure 6B, the observed nucleophile effect will contain equilibrium contributions from metal ion coordination, and in cases where metal-coordinated hydroxide is the nucleophile, an equilibrium effect from deprotonation of the metal ion bound water. Effects from metal ion coordination are inverse in the direction of metal ion coordination due to the more strongly bonded environment.[46,47] Presently, these important fractionation factors have not yet been thoroughly examined for the metal ions of interest in biological systems (e.g., Mg^{2+}, Ca^{2+}, or Mn^{2+}). Thus, understanding these values and the impact of metal ion coordination on nonenzymatic transition states remain important goals in this area.

OUTLOOK: APPLICATIONS TO RNA AND PROTEIN PHOSPHODIESTERASES

We know that enzyme active sites comprise a stereospecific environment, imposing specific geometries on bound substrates before and during formation of the transition state. Studies of nonenzymatic reactions of phosphodiesters clearly demonstrate that their mechanisms and transition states are very sensitive to both geometric constraints and interactions with metal ions, acid, and base. It seems likely, then, that interactions with different active site functional groups will have a similarly significant, and unique impact on the transition states of enzymatic reactions. Clearly, useful information on phosphodiesterase mechanism can be obtained by examining the effects of isotopic substitution at the reactive phosphate for specific phosphodiesterase enzymes. Also, as studies of monoesterase mechanism using KIE analysis have shown, comparison of results obtained between enzyme mutants can be used to test specific hypotheses concerning enzyme catalytic mechanism.[48] To date, only a few experiments have been done targeting phosphodiesterases, and this area of research remains a very fertile ground for further study.

In principle, phosphodiesterase active sites can manipulate the charge distribution of the transition state by interacting with the oxygens of the reactive phosphate. Catalysis is proposed to arise from interactions with (a) the nucleophile to facilitate anion formation and position it for in-line attack; (b) the nonbridging oxygens to promote Lewis acid catalysis, or to stabilize the anionic transition state via electrostatic interactions; and (c) the leaving group oxygen to stabilize developing electron density via protonation, or electrostatic interactions.[3,26,39,49,64] The magnitude of the isotope effects for these oxygen atoms will certainly be influenced by these interactions. In basic terms, the magnitude of $^{18}k_{lg}$ is proportional to the degree of bond cleavage, but is also sensitive to charge neutralization, which will lessen the observed isotope effect. For the nonbridging oxygens, effects for enzymes could range from large normal effects, for associative phosphorane-like transition states, to inverse values for dissociative, metaphosphate-like ones. Additionally, large inverse nonbridging effects can also arise from protonation. As with the nonenzymatic reaction, the nucleophile isotope effect for enzymatic phosphodiester hydrolysis will potentially contain contributions from the intrinsic effect on bond formation, from deprotonation (an equilibrium effect if it occurs prior to the chemical step, or a lesser kinetic effect if it occurs in the transition state), and potentially from an equilibrium effect from metal ion coordination. Because there are interesting differences between the active site architectures, the apparent catalytic strategies, and the number and roles of active site metal ions for this class of enzymes, comparisons between selected enzymes would shed valuable light on the general as well as the idiosyncratic means by which these enzymes work to effect phosphodiester bond cleavage.

Before such comparisons can be made, however, it is essential to establish that the selection of the heavy or light isotope in product formation occurs at the chemical step rather than a step upstream of catalysis such as binding or a conformational change. If a nonchemical step is partially rate limiting, then the intrinsic isotope effect will be masked by a "commitment factor" or "commitment to catalysis" by the upstream step. Detailed descriptions of how to understand and account for these "commitments" in analyzing enzyme isotope effects are available.[30,73,74] The general magnitude and sign of isotope effects can be reasonably predicted from measurements on model reactions. Because commitments to catalysis can mask these intrinsic effects, detection of a measurable

isotope effect provides the most direct test of whether the chemical step is indeed rate limiting. Thus although commitment factors may preclude obtaining information about the *chemical* mechanism, they provide valuable information regarding the enzyme's *kinetic* mechanism.

To overcome the potential limitation imposed by complex enzyme reaction mechanisms, it is important to understand in as much detail as possible the reaction pathway. One method for testing for upstream commitments is to compare effects determined under optimal reaction conditions with effects determined under conditions where the rate of chemistry is slower. Changes in the observed isotope effect under conditions where the rate of chemical catalysis is reduced, or with slow reacting substrates, indicate changes in the commitment factors that contribute to the measured effect. Often using a slow substrate or enzyme mutant is the only way to measure intrinsic isotope effects on the chemical step of the reaction.

Applying KIE analysis to enzymes, in particular those that work on macromolecular substrates, is nontrivial for two additional reasons. First, a prerequisite for most KIE experiments is the synthesis of substrates with the appropriate site-specific isotopic labels. For phosphodiesterases, this will generally involve enrichment, or depletion of individual phosphate oxygens. Although such compounds are certainly not commercially available, the methodology for generating isotopically into the phosphates of ATP has been accomplished,[75–78] and the general syntheses of isotopically labeled pentoses have been optimized.[79] Second, the isotope ratio of the reactants and products must be compared with great precision. The large molecular weights of most phosphodiesterase substrates necessitate isolation of a specific residue, or functional group containing the atom of interest in sufficient yield and purity for mass spectrometry. Fortunately, some phosphodiesterases will accept model diesters of nitrophenol as substrates, and so the same remote label approach used in the nonenzymatic analyses is directly applicable to these enzymes. For the majority, significant advances in substrate labeling and product mass analysis will be required. Nonetheless, recent advances in instrumentation and the development of new labeling approaches for analysis of KIEs indicate that although these challenges are significant, they are not insurmountable.

To date, isotope effect measurements have only been made for reactions catalyzed by phosphodiesterase I and by RNase A, which employ intermolecular and intramolecular nucleophilic mechanisms respectively. Phosphodiesterase I employs a two-step mechanism in which an active site threonine is the nucleophile in the first step resulting in a covalent enzyme–substrate intermediate that is subsequently hydrolyzed.[80] Because nitrophenol is released in the first irreversible step, it is this aspect of the mechanism that is reflected in the observed isotope effects. The enzymatic reaction shows measurable normal effects for ^{15}k and $^{18}k_{lg}$ demonstrating that bond cleavage to the leaving group is occurring in the transition state, consistent with either a concerted mechanism or a stepwise one in which breakdown of the intermediate is rate limiting.[35] The magnitude of $^{18}k_{lg}$ is larger for the enzyme reaction compared to both alkaline and acid hydrolysis, indicating a later transition state with respect to bond cleavage. Interestingly, the phosphodiesterase I catalyzed reaction gives rise to a large (1.7%) inverse isotope effect on the nonbridging oxygens. This result contrasts with both the small inverse effects observed for the dissociative transition state of monoester dianions, and the normal effects observed for the more associative nonenzymatic diester reactions. This large inverse value for the enzymatic reaction could be due to a very dissociative transition state (even more so than for monoester reactions) with a metaphosphate-like structure; however, the magnitude of $^{18}k_{lg}$ is smaller than that observed for monoester reactions, arguing that a lesser (not greater) extent of leaving group bond cleavage has occurred. As an alternative, the authors argue that the large inverse effect arises from protonation of one of the nonbridging oxygens by enzyme active site residues. Formation of the new bond to hydrogen will favor the heavier isotope resulting in an inverse contribution. Additionally, the magnitude of the value obtained for the enzyme reaction is similar to that for equilibrium protonation of phosphate.

The interpretation is that the enzymatic reaction is concerted, as for the nonenzymatic reactions, however, the transition state is more associative with a more phosphorane-like structure compared to nonenzymatic reactions. Protonation of the nonbridging oxygens is likely to make the phosphorus more susceptible to nucleophilic attack, but might also result in more difficulty in leaving group expulsion, and thus greater bond cleavage in the transition state as suggested by the isotope effect data. The constraints provided by active site interactions might also render the transition state more phosphorane-like similar to that observed for the constrained compounds like uridine 3′ *m*-nitrobenzyl phosphate that undergo intramolecular displacement by a ribose hydroxyl group.

This is precisely the reaction catalyzed by a large number of ribozyme and protein nucleases. However, whether these enzymes catalyze the formation of

phosphorane intermediates such as those observed for nonenzymatic reactions remains the subject of significant interest. RNase A, an archetypical example of this class, cleaves the RNA phosphodiester backbone in a two-step mechanism in which the adjacent 2′ OH attacks the phosphate in the first step yielding characteristic 5′ hydroxyl and 2′,3′ cyclic phosphate termini. In the second step the cyclic phosphate is hydrolyzed. Both steps proceed with inversion of configuration, suggesting that the basic mechanism is concerted.[81,82] Although a large body of structural and biochemical data demonstrate that the enzyme accomplishes catalysis using general acid–base mechanisms, there continues to be debate over whether this enzyme, and other enzymes in this class, promote the formation of phosphorane-like transition states, or potentially intermediates as observed for analogous nonenzymatic reactions.

Fortuitously, the active site of RNase A will accept model compounds such as uridine 3′-*m*-nitrobenzyl phosphate, which are amenable to KIE analysis using the remote label technique (Table I). Sowa et al. show that there is a normal isotope effect of 1.6% for the leaving group oxygen at both pH 5.0 and 8.0, indicating that the chemical step is indeed rate limiting and that there is a large degree of bond cleavage in the transition state.[83] This result does not necessarily distinguish between a concerted mechanism and one in which a phosphorane intermediate forms, but does place an important constraint on any stepwise mechanism. Observation of a significant $^{18}k_{lg}$ shows that bond cleavage to the leaving group is occurring in the transition state, thus, if an intermediate forms, breakdown to yield the cleaved phosphodiester bond must be largely rate limiting.

Analysis of $^{18}k_{nonbridge}$ shows that there is a 0.5% normal effect observed, indicating a loss in bond order in the transition state. If the mechanism involved rate-limiting breakdown of a phosphorane then its structure could be either dianionic, or more likely monoanionic with protonation of the nonbridging oxygens as observed in the nonenzymatic reactions involving this compound. Ab initio calculations for the hydrolysis of ethylene phosphate indicate that formation of dianionic phosphoranes is unlikely, whereas singly charged pentacovalent species do form.[84] Even were a dianionic intermediate to form, then relative to the ground state there would be a loss of double bond character to the nonbridging oxygens resulting in a large, normal isotope effect. Similarly, protonation of the nonbridging oxygens to give a monoanionic phosphorane would result in an overall increase in bonding, and thus a large inverse isotope effect. Neither of these scenarios for formation of a phosphorane intermediate is consistent with the small normal effect observed for the RNase A reaction. Thus, together with the information from $^{18}k_{lg}$, the data suggest a concerted mechanism, with a small decrease in bonding to the nonbridging oxygens due to an associative transition state.

The above examples illustrate the potential of heavy atom isotope effects to provide detailed mechanistic information, even on enzyme systems as intensely studied as RNase A. However, only a small fraction of this potential has been realized in the investigation of phosphodiesterases. To date, phosphodiesterase I and RNase A are the only phosphodiesterases where heavy atom isotope effects have been applied. These enzymes were studied largely because their broad substrate specificities allow utilization of the remote label method. Phosphodiesterases with more complex substrate specificities, such as restriction enzymes, will require new techniques to ascertain isotope effects. Additionally, methods for determining nucleophile isotope effects must be improved to provide enhanced precision and adapted for applications to intramolecular reactions. These challenges will include devising new synthetic methods to insert isotopic labels into macromolecular substrates and establishing new instrumental techniques to allow measuring isotope ratios in nucleosides and nucleotides. Overcoming these obstacles represents the critical first steps in fulfilling the potential of heavy atom isotope effects.

Once these technical challenges are met, a comprehensive model for enzymatic catalysis of phosphodiester cleavage will require an examination of specific enzymes that utilize characteristic, or distinct catalytic mechanisms. Because the majority of naturally occurring ribozymes also catalyze this reaction, several catalytic RNAs must be investigated. The power of heavy atom isotope effects to provide a detailed characterization of transition state structure presents the opportunity to detect the influence of specific catalytic strategies (such as general acid/base catalysis or metal ion catalysis). Ultimately, KIE analysis could provide a diagnostic tool for identifying which specific strategies are utilized by a particular phosphodiesterase. Such concrete conclusions about enzyme mechanism often remain elusive, even when high-resolution crystal structures exist for the enzyme–substrate complex. As an additional dividend, comparison of the transition states for the RNA- and protein-catalyzed reactions is likely to provide insight into both the intrinsic differences between these two types of active site and the universal features that underlie phosphodiesterase structure and function.

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