

Isotope effects on enzymatic and nonenzymatic reactions of phosphorothioates

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Abstract Kinetic isotope effects have been measured for the aqueous hydrolysis reactions of *p*-nitrophenyl phosphorothioate (pNPPT) and the diester ethyl *p*-nitrophenyl phosphorothioate, and for the alkaline phosphatase-catalyzed reaction with pNPPT. The results show that the transition states of the uncatalyzed reactions of the phosphorothioate mono- and diesters are very similar to those of the corresponding phosphate ester reactions. The secondary ^{18}O nonbridge isotope effects in reactions of phosphate esters become more normal as the mechanism changes from dissociative, metaphosphate-like to associative, phosphorane-like. The opposite trend occurs in phosphorothioate esters, due to differences in the relative contributions of bond-order changes and bending modes to this isotope effect. The KIEs for the alkaline phosphatase-catalyzed reaction of pNPPT are most consistent with a tight, triester-like transition state, probably a result of perturbations resulting from the larger size of sulfur that lead to a nucleophile attack angle that is unfavorable for an in-line process with a loose transition state.

Key words alkaline phosphatase • isotope effect • phosphoryl transfer • phosphorothioate • thio effect

Introduction

O-phosphorothioate analogs of phosphate monoesters have long been used to probe kinetic and stereochemical aspects of phosphoryl transfer. Both uncatalyzed reactions in solution and enzymatic reactions have been studied, the latter most notably with alkaline phosphatase. Large thio effects, defined as the ratio of the reaction rate with a phosphate substrate over that of the corresponding phosphorothioate, or $k_{\text{O}}/k_{\text{S}}$, observed in alkaline phosphatase have been used to infer an associative, triester-like mechanism [4, 10, 19]. Alternative conclusions favoring a dissociative mechanism have been proposed from linear free energy relationships obtained from reactions with phosphorothioate esters [24]. The use of phosphorothioates as probes of the mechanisms of enzymatic phosphoryl transfer stems in part from the observation that the chemical step is rate-limiting in the alkaline phosphatase-catalyzed reactions of phosphorothioates, while non-chemical steps are rate limiting with phosphate ester substrates. This allows the chemical step of reactions with phosphorothioates to be probed by such means as linear free energy relationships (LFER) and kinetic isotope effects (KIEs).

Phosphate monoesters typically react in solution by mechanism A in Figure 1. This is a concerted reaction, termed $\text{A}_{\text{N}}\text{D}_{\text{N}}$ in the IUPAC nomenclature [18]. This pathway for the dianions of phosphate monoesters is supported by a very small entropy of activation [29], a large (-1.2) β_{g} [30] a small β_{nuc} [29], and by the occurrence of inversion of configuration when the phosphoryl group is made chiral [17]. An essential difference occurs in the phosphoryl transfer from the dianion of *p*-nitrophenyl phosphate (pNPP) to

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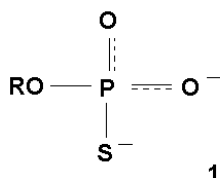
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Received: 11 July 2001, Accepted: 8 January 2002

anhydrous *tert*-butanol, where this acceptor also is the reaction solvent. Under these conditions racemization at phosphorus is observed when the reactant is made chiral by the use of oxygen isotopes [17]. This outcome implicates the formation of a free metaphosphate intermediate in the $D_N + A_N$ mechanism B in Fig. 1 [17]. This outcome was in contrast to that in more nucleophilic solvents such as methanol, where the product had inverted configuration consistent with nucleophilic participation in the transition state of the concerted mechanism A [16].

The reactions of phosphate monoanions are believed to proceed by a mechanism in which the proton is transferred to the leaving group either in a preequilibrium step as shown at the bottom of Fig. 1 or, for less basic leaving groups, simultaneously with P-O bond cleavage [30]. The reaction of pNPP monoanion in methanol proceeds with inversion of configuration at phosphorus [6].

Experimental [27] and theoretical [32] evidence regarding the bonding in phosphorothioates indicates that structure 1 is the most accurate representation of the charge distribu-



tion and bonding, with greater negative charge and less double bond character on the sulfur atom than each of the two nonbridge oxygens. A study of the hydrolysis reactions of O-aryl phosphorothioate dianions using linear free-energy relationships found a value for $\beta_{\text{leaving group}}$ of -1.1 [24] suggesting that the transition state resembles that for reactions of the aryl phosphate ester counterparts, which exhibit a value of -1.2 for this parameter [30].

In contrast to stereochemical results with chiral phosphate monoesters, stereochemical studies with the dianion and the monoanion of chiral *p*-nitrophenyl [^{18}O , ^{16}O]phosphorothioates show that ethanolysis [12, 13] and hydrolysis [8, 16] both proceed with a large degree of racemization, indicating the formation of free thiometaphosphate as an intermediate. The observation of a large volume of activation for the hydrolysis of the dianion of 2,4-dinitrophenyl thiophosphate, in contrast to the near zero value measured with the corresponding phosphate ester, also is indicative of a difference in mechanism and is consistent with formation of a thiometaphosphate intermediate [8]. These results indicate that phosphorothioate monoesters typically react via mechanism B in Fig. 1. The opposite mechanistic extreme is shown in mechanism C, an addition-elimination process. A concerted reaction may in principle proceed by either a loose transition state or a tight one resembling a phosphorane. Triester reactions proceed with more nucleophilic participation and tighter transition states than monoesters.

Measurement of the isotope effects

Figure 2 shows the position at which isotope effects have been measured in reactions with the substrate *p*-nitrophenyl phosphorothioate (pNPPT). The conventional nota-

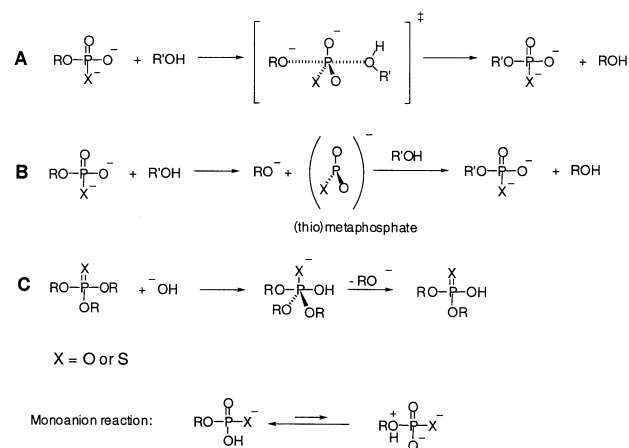


Fig. 1. Pathways observed for phosphoryl transfer from monoester dianions (top, A and B) and monoanions (bottom). A – is a concerted mechanism with a loose transition state, with the bond to the leaving group largely broken and a small degree of bond formation with the nucleophile. B – is a stepwise mechanism proceeding by way of a (thio)metaphosphate intermediate. C – is an addition-elimination pathway, that is followed by some phosphotriesters. At the bottom is the first step of the mechanism for reaction of the monoanionic species. Proton transfer may occur in a preequilibrium step as shown before nucleophilic attack, or proton transfer can occur simultaneous with leaving group departure, depending upon the basicity of the leaving group.

tion for isotope effects used, in which a leading superscript of the heavier isotope indicates 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 .

The kinetic isotope effects were measured by the competitive method, using an isotope ratio mass spectrometer, using the same methods as previously reported for *p*-nitrophenyl phosphate [22]. 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 natural abundance of ^{15}N is sufficient). The reaction is stopped at some measured fraction f of reaction, and the $^{15}\text{N}/^{14}\text{N}$ 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 Equations (1) or (2) from the isotope ratio of the residual starting material, or the product, respectively [3].

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

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

A high degree of precision is required in the measurement of the isotope ratios. Several methods have been utilized [34] the most precise of which is the use of an isotope ratio

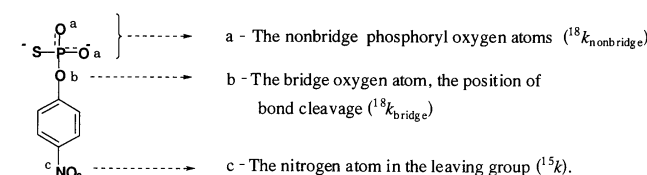


Fig. 2. Diagram of *p*-nitrophenyl phosphorothioate showing the positions where isotope effects were measured.

Phosphate ester KIEs	^{15}k	$^{18}k_{\text{bridge}}$	$^{18}k_{\text{nonbridge}}$
pNPP Dianion, H ₂ O, 95°C	1.0028 (2)	1.0189 (5)	0.9994 (5)
pNPP Dianion, <i>t</i> -butanol, 30°C	1.0039 (3)	1.0202 (8)	0.9997 (16)
Diesters	1.0007–1.0016	1.0042–1.0063	1.0028–1.0056
Triesters	1.0007	1.0063 ^a	1.0063–1.0250
pNPP Monoanion, 35°C	1.0005 (1)	1.0094 (3)	1.0199 (3)
Phosphorothioate ester KIEs	^{15}k	$^{18}k_{\text{bridge}}$	$^{18}k_{\text{nonbridge}}$
pNPPT Dianion, 50°C	1.0027 (1)	1.0237 (7)	1.0135 (13)
Ethyl pNPPT diester, 95°C	1.0010 (2)	1.0020 (6)	1.0019 (4)
pNPPT Monoanion, 30°C	1.0005 (1)	1.0091 (7)	1.0221 (4)

^a Data from Ref. [9].

mass spectrometer. The major drawback to the use of this instrument is that it measures only small molecular weight gases that do not undergo fragmentation in the mass spec (since such fragmentation will potentially have isotope effects)! Thus, one must convert the atom of interest into a form the instrument is built to handle, which includes H₂, N₂, CO, CO₂, or SO₂. Such conversion must be made cleanly and quantitatively to insure that there is no isotopic fractionation. This can present a formidable barrier; however the use of the remote label technique can alleviate this problem [33]. In this method a substrate is prepared that is isotopically labeled in two positions. One label is incorporated into the position of interest, which may not be amenable to isolation for analysis. A second label at a position that is not involved in the reaction, and which is easily accessible, accompanies the label at the atom of interest. In our work we use a nitrogen atom as our remote label as a reporter for O-18 isotope ratios. Most isotope ratio mass spectrometers incorporate a combustion system which converts nitrogen to N₂, 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 synthesis from available highly enriched starting materials, and be inert to the reaction conditions. Since the nonenzymatic reactions of phosphate and sulfate esters are very slow and require fairly harsh conditions, we have used the nitro group in this capacity. The isotope effects $^{18}k_{\text{bridge}}$ and $^{18}k_{\text{nonbridge}}$ are measured by the remote label method. The observed KIE is corrected for the ^{15}k KIE and for incomplete levels of isotopic incorporation.

Isotope effects in phosphoryl transfer reactions

The KIEs for reactions of phosphorothioates can be considered in light of a body of KIE data for reactions of pNPP in water and in *tert*-butanol, and for a number of uncatalyzed hydrolysis reactions of diesters and triesters in which the leaving group is *p*-nitrophenol. These data, summarized in the upper part of Table 1, indicate that isotope effects can reliably distinguish the loose, metaphosphate-like transition state operative in the monoester reaction from the tighter,

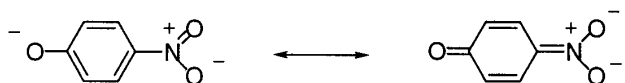


Fig. 3. Diagram of resonance contributors of *p*-nitrophenolate ion, showing involvement of the nitro group in charge delocalization that gives rise to the N-15 isotope effect.

Table 1. The range of isotope effects measured for reactions of phosphate esters of *p*-nitrophenol, including data for the triester diethyl (4-carbamoylphenyl) phosphate. Much of the difference between the KIE data for the dianion reactions in water and in *t*-butanol arises from the difference in temperature. The standard errors in the last digit(s) are in parentheses.

phosphorane-like transition state which occurs in diester and triester reactions.

The primary isotope effect $^{18}k_{\text{bridge}}$ gives a measure of the degree of cleavage of the P-O bond in the transition state. As a primary isotope effect it will have contributions from other than zero point energy and thus cannot be interpreted as a linear ruler for P-O bond cleavage, but it gives a reasonable estimate for the loss of P-O bond order in the transition state. Our interest has been in distinguishing between tight and loose transition states, which result in considerably different magnitudes in this isotope effect.

The secondary isotope effect ^{15}k is sensitive to the amount of negative charge borne by the leaving group. The *p*-nitrophenolate anion has contributions from a quinonoid resonance form, shown below (Fig. 3). Because the N-O bonds are stiffer in terms of force constants than N-C bonds, the nitrogen atom is more tightly bonded in the neutral species than in the phenolate anion. The ^{15}k EIE for deprotonation of *p*-nitrophenol is thus normal, 1.0023 ± 0.0001 [20]. Although small, this isotope effect is readily measured using an isotope ratio mass spectrometer. The magnitude of ^{15}k 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 bond rupture.

Isotope effects are not solely determined by bond order considerations, but also by bending and torsional vibrational modes and the latter effects can be dominant for secondary isotope effects at atoms bonded to a site that undergoes a hybridization change. The bond order changes of the nonbridge oxygen atoms in the mechanisms in Fig. 1 would lead one to expect an inverse $^{18}k_{\text{nonbridge}}$ for dissociative transition states, and normal ones for the 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 [36]. BEBOVIB calculations predict normal values for $^{18}k_{\text{nonbridge}}$ in associative transition states, and inverse values for dissociative ones [38]. More recent *ab initio* calculations we have carried out reaffirm the BEBOVIB predictions. Thus the secondary $^{18}k_{\text{nonbridge}}$ reveals whether the phosphoryl group resembles metaphosphate in a loose transition state, or if it has a phosphorane-like structure in an associative mechanism.

The experimental results (Table 1) follow the trend predicted by the calculations. This suggests that for phosphate

esters bond order changes seem to be the dominant contributors to $^{18}k_{\text{nonbridge}}$, since these are normal for diester and triester reactions (with a single exception, which may be anomalous) and inverse (though very small) for dissociative reactions [9, 21–23, 37]. The value for $^{18}k_{\text{nonbridge}}$ is very small and inverse in the loose transition states of monoester reactions, in contrast to the phosphorane-like transition states of phosphate diesters and triesters, where this isotope effect becomes successively more normal and reaches values of 2.5% in triester reactions. Intermediate values are seen for the diester reactions which are $A_N D_N$ -like and more synchronous.

One can conclude from the collected data that nucleophilic participation in the more associative transition states reveals itself in normal values for $^{18}k_{\text{nonbridge}}$ in contrast to the small inverse effects seen in dissociative reactions. 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 in aryl diesters and triesters than in the monoester reactions [2, 14]. The LFER data indicate substantially less bond cleavage to the leaving group is present in transition states of diester and triester reactions, and this is borne out by the reduced magnitudes of the isotope effects in the leaving group, ^{15}k and $^{18}k_{\text{bridge}}$.

The KIEs for the hydrolysis of the pNPP monoanion are also shown in Table 1. In the reaction of the monoanion, the leaving group is protonated in the transition state. Thus the magnitude of $^{18}k_{\text{bridge}}$ is significantly lower than in the dianion reaction, because loss of the P-O bond is partially compensated for by formation of the O-H bond. The very small value for ^{15}k indicates the leaving group remains essentially neutral. The value for $^{18}k_{\text{nonbridge}}$ in this reaction primarily reflects the known normal isotope effect for deprotonation of a phosphoryl group [31].

Isotope effects in reactions of phosphorothioates

The KIEs for the uncatalyzed hydrolysis of the monoester *p*-nitrophenyl phosphorothioate, and of the diester ethyl *p*-nitrophenyl phosphorothioate, are shown in the lower part of Table 1. Considering the reaction of the dianion of the monoester first, comparisons of the isotope effects in the leaving group (^{15}k and $^{18}k_{\text{bridge}}$) with those for pNPP hydrolysis reveals the two sets of numbers to be essentially the same. This indicates that the transition states for the two reactions have a similar degree of P-O bond cleavage and amount of charge borne on the leaving group. Prior data described above suggest that the phosphate ester reaction is a concerted nucleophilic substitution with only minimal nucleophilic participation in the transition state, but that the phosphorothioate reaction proceeds by initial formation of thiometaphosphate. Evidently the transition state of the latter reaction is essentially identical to that of the phosphate ester but lacking the minimal amount of nucleophilic participation.

Interestingly, the $^{18}k_{\text{nonbridge}}$ KIE in the pNPPT reaction is normal, not inverse as in the phosphate ester reaction. Since a preponderance of data indicates that the thiophosphoryl group resembles thiometaphosphate in the transition state, why is this KIE normal in this reaction, not inverse as it is in the phosphate case? The most likely explanation is that the larger size of the sulfur atom makes the bending modes the dominant contributors to this secondary isotope effect. Our computational results confirm this. We have modeled the transition state of the dissociative reaction of a phosphorothioate esters. The computations predict a normal $^{18}k_{\text{nonbridge}}$ KIE for the dissociative process, in contrast to the inverse value for the corresponding reaction of a phosphate ester. In the two transition states there is no significant difference in the bond orders between phosphorus and the nonbridge oxygens in the phosphorothioate compared to the phosphate case. The only significant differences are in the frequencies of several bending modes, supporting the hypothesis that the opposite trend in $^{18}k_{\text{nonbridge}}$ in the phosphorothioate reaction arises from a greater contribution of bending modes to the isotope effect.

If the hypothesis that the bending modes are the major contributors to the secondary $^{18}k_{\text{nonbridge}}$ KIEs in reactions of phosphorothioates is correct, then $^{18}k_{\text{nonbridge}}$ for reaction of a diester should move in the inverse direction relative to that for the more dissociative monoester reaction. This, in fact, is just what is observed; the value of $^{18}k_{\text{nonbridge}}$ for the diester ethyl pNPPT is 1.0019 ± 0.0004 (Table 1). Phosphorothioate diesters with aryl leaving groups are thought to react by a concerted mechanism intermediate between the dissociative one of the monoester, and the tighter transition state of triester reactions. A phosphorothioate triester has no nonbridge oxygen atoms, so $^{18}k_{\text{nonbridge}}$ for this even more associative reaction cannot be measured, but is expected to be inverse.

The isotope effects in the leaving group were also measured for the alkaline hydrolysis of ethyl pNPPT (Table 1). The value for ^{15}k is in the middle of the range measured for reaction of phosphate diesters, while $^{18}k_{\text{bridge}}$ is a bit below the low end of the range. This suggests a transition state that is not very different from the corresponding reactions of phosphate diesters, though P-O bond cleavage is somewhat less advanced. LFER results on phosphate diester reactions indicate that though the reaction is concerted, there is charge buildup on the phosphoryl group in the transition state, which implies development of partial phosphorane character [14, 15].

Enzymatic thiophosphoryl transfer

Alkaline phosphatase from *Escherichia coli* (EC 3.13.1) is a metalloenzyme containing two zinc ions and one magnesium ion in each active site [11]. Alkaline phosphatase hydrolyzes phosphate monoesters through a covalent phosphoenzyme intermediate to produce inorganic phosphate and an alcohol (Scheme 1). In the detailed mechanism proposed on the basis of the crystal structure [28], Zn_2 activates the hydroxyl group of Ser-102 for nucleophilic attack on the substrate to form a covalent phosphoserine intermediate (Fig. 4). This intermediate is subsequently hydrolyzed in the second step by an activated water molecule coordinated to

Table 2. Correlation of thio effects, Brønsted β_{lg} values, and transition state structures in alkaline hydrolysis reactions of phosphate and phosphorothioate esters. The thio effect is reported as $k_{\text{O}}/k_{\text{S}}$, the ratio of the rate of phosphoryl transfer to that for thiophosphoryl transfer. The thio effect data comes from the compilation reported in reference [34], from references cited therein. The Brønsted β_{lg} values are those for the alkaline hydrolysis reactions of corresponding phosphate and phosphorothioate esters [1, 5, 14, 24, 26, 30].

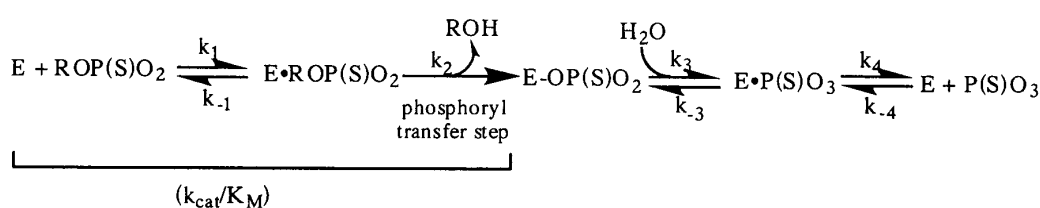
Phosphate ester	Range of thio effects ($k_{\text{O}}/k_{\text{S}}$)	Range of β_{lg} values	Transition state
Triester	10–160	-0.35 to -0.43	associative (tight)
Diester	4–11	-0.55 to -0.63	intermediate
Monoester	0.1–0.3	-1.1 to -1.2	dissociative (loose)

Zn₁ to form the non-covalent enzyme phosphate complex. The identity of the rate-limiting step is pH-dependent. The dissociation of inorganic phosphate is rate-limiting at alkaline pH, while the hydrolysis of the covalent phospho-intermediate is rate-limiting at acidic pH [35].

The substitution of sulfur for oxygen in a nonbridging position significantly increases the rates of hydrolysis of phosphate monoester dianions, but significantly decreases reactions of triesters, with diesters exhibiting intermediate thio effects (Table 2). For monoesters this $k_{\text{O}}/k_{\text{S}}$ ratio is $\ll 1$, while for triester reactions, it is $\gg 1$. The Brønsted β_{lg} values for reactions of phosphates and of phosphorothioates are very similar within the three classes of phosphate esters. Collected values of β_{lg} and thio effects for alkaline hydrolysis reactions are shown in Table 2. The thio effect for the AP-catalyzed reaction resembles that of triesters, but is the inverse of the typical thio effect of monoesters. This has been cited by some as evidence that the AP-catalyzed reaction proceeds via a tight, triester-like transition state [19]. However, a large negative value of -0.77 for the β_{lg} for the reaction of AP with O-aryl phosphorothioates has been cited as evidence for a loose, monoester-like transition state [24].

In order to settle this issue and to assess the validity of thio effects as a reporter for the enzymatic mechanism, we measured the KIEs for the reaction of pNPPT with AP. We also compared the β_{lg} for the native AP and the arginine mutant R166A using a series of aryl phosphorothioate substrates [25]. The chemical step of phosphoryl transfer has been shown to be rate-limiting for the AP-catalyzed hydrolysis of phosphorothioate esters [24].

Wild-type alkaline phosphatase catalyzes the hydrolysis of *p*-nitrophenyl phosphate approximately 70 times faster than *p*-nitrophenyl phosphorothioate. In contrast, the R166A mutant AP, in which the active site arginine at position 166 is replaced with alanine, hydrolyzes *p*-nitrophenyl phosphate only about 3 times faster than *p*-nitrophenyl phosphate.



Scheme 1

Table 3. KIEs for AP-catalyzed reaction of *p*-nitrophenyl phosphorothioate, at pH 9.0. The standard errors in the last digit(s) are in parentheses.

	$^{15}(\text{V}/\text{K})$	$^{18}(\text{V}/\text{K})_{\text{bridge}}$	$^{18}(\text{V}/\text{K})_{\text{nonbridge}}$
pNPPT, pH9	1.0005 (2)	1.0094 (4)	0.9760 (22)

phosphorothioate. Despite this ~ 23 -fold change in the magnitude of the thio effects, the magnitudes of Brønsted β_{lg} for the native AP (-0.77 ± 0.09) and the R166A mutant (-0.78 ± 0.06) are the same [25]. The identical values for β_{lg} indicate that the transition states are similar for the reactions catalyzed by the wild-type and the R166A mutant enzymes. The fact that a significant change in the thio effect is not accompanied by a change in the β_{lg} indicates that the thio effect is not a reliable reporter for the transition state of the enzymatic reaction.

Is the transition state of the AP-catalyzed reaction of pNPPT dissociative or associative in nature? The KIEs (Table 3) are not those expected of a dissociative process. Most notably, the $^{18}k_{\text{nonbridge}}$ isotope effect is ‘inverse’. If our interpretation of this isotope effect presented earlier is correct, this argues for a highly phosphorane-like transition state. In addition, the isotope effects in the leaving group indicate only a small degree of loosening of the P-O bond, and essentially no negative charge developed on the leaving group. The dianion of the substrate is the reactive species, AP does not utilize general acid catalysis, and reaction occurs at a pH above the $\text{p}K_{\text{a}}$ of the leaving group, thus protonation of the leaving group cannot be responsible for these low KIEs. It is possible that coordination of the leaving group to the zinc ion could reduce the isotope effects to the same degree as protonation; this would require the formation of an essentially full covalent bond between zinc and *p*-nitrophenolate. Isolable zinc-phenolate complexes are known. Although formation of a tight complex in the enzymatic reaction might make product release difficult, if the complex were kinetically labile, such an interaction would be viable. We are seeking to experimentally measure the isotope effect for coordination of *p*-nitrophenolate to a zinc complex.

One must not forget the consequences of the sulfur for oxygen substitution. Sulfur has both a larger van der Waals radius (1.09 Å vs. 0.65), as well as longer bond lengths. Accommodating a thiophosphoryl group places new demands on the active site of a phosphatase. In an effort to understand these consequences for binding to AP, we carried out computational modeling of phenyl and of methyl phosphorothioate in the active site at the PM3 level. The active site from the X-ray structure by Kim and Wyckoff [28] was used as the starting point, with the bound inorganic phosphate replaced by the substrates before optimization. There are three possible orientations for the sulfur atom in

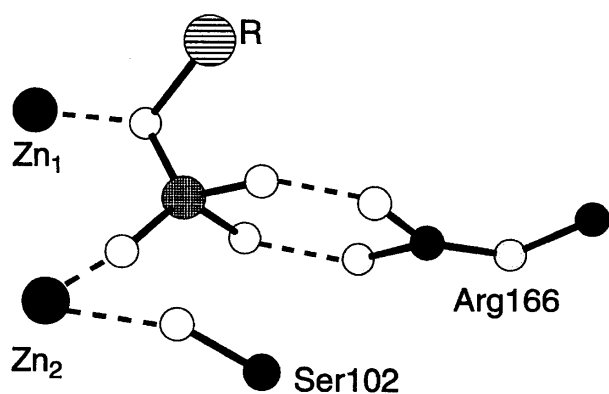


Fig. 4. Diagram of the active site of alkaline phosphatase showing the likely mode of substrate binding.

the nonbridging position, and all three geometries were optimized. As expected, the most energetically favorable binding mode has the sulfur atom coordinated to zinc rather than in either of the two positions hydrogen bonded to the guanidinium group of arginine. Though energetically favored by a considerable amount, this enzyme-substrate complex has an angle between the nucleophile oxygen of serine, phosphorus, and the leaving group oxygen atom of 146 degrees. The corresponding angle in the complex with the phosphate esters is 161 degrees. The additional deformation of this angle from the ideal one of 180 degrees may disfavor a concerted in-line displacement, requiring a transition state with considerably more nucleophilic participation, and the assumption of a phosphorane-like geometry in order to expel the leaving group. Even though phosphorothioate monoesters react by a thiometaphosphate intermediate in solution, the stabilizing effects of metal coordination and hydrogen bonding may disfavor this pathway, making some nucleophilic participation necessary.

There is precedent that geometrically restricting the angle of nucleophilic attack can change the mechanism for phosphoryl transfer from a phosphate monoester from the normally dissociative, concerted mechanism to a two-step mechanism with a phosphorane intermediate [7]. The KIE data do not prove that a (thio)phosphorane intermediate forms, but the reaction seems to have a triester-like transition state with considerable nucleophilic participation implied by the inverse value for $^{18}k_{\text{nonbridge}}$ and only a small degree of bond cleavage to the leaving group.

Could the inverse $^{18}k_{\text{nonbridge}}$ in the AP-catalyzed reaction be a result of binding interactions? For this to be the case, hydrogen bonding interactions with the phosphoryl group in the active site would have to be considerably stronger than in aqueous solution; the magnitude of $^{18}k_{\text{nonbridge}}$ is larger than that expected even for protonation of the phosphoryl group. In preliminary results we have measured the kinetic isotope effects for the reaction of pNPPT with two arginine mutants of AP and have obtained isotope effects little different from the data obtained with the native enzyme. This rules out hydrogen bonding interactions with the nonbridge oxygen atoms as the source of the inverse $^{18}k_{\text{nonbridge}}$ isotope effect. Methylation of the sulfur atom of phosphorothioate monoesters does not change the bond order between phosphorus and the nonbridging oxygen

atoms [27]. This result indicates that the zinc-sulfur interaction should not be able to cause such a change. Thus, binding interactions are unlikely to explain the observed inverse $^{18}k_{\text{nonbridge}}$ isotope effect.

Thus, the interpretation originally inferred from thio effects, that phosphorothioates react via a tight transition state, may well be correct, even though thio effects are not reliable indicators of mechanism. What does this imply about the AP-catalyzed reaction with its natural substrates, phosphate esters? Our results indicate that one must be extremely cautious in using results with phosphorothioates to draw conclusions about enzymatic phosphoryl transfer, since the significant perturbations on binding that result from the sulfur substitution are likely responsible for the particular mechanism (or transition state) that must be followed with phosphorothioate substrates. Whether the natural reaction is similar or different awaits isotope effects studies with a more natural phosphate substrate with a poor leaving group, for which chemistry is rate-limiting. Such experiments are underway.

Acknowledgment. The assistance of Dr. Richard H. Hoff in carrying out some of the isotope effect experiments is gratefully acknowledged. We also thank the reviewers for insightful comments. This work was supported by a grant from the National Institutes of Health (GM 47297).

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