Mutation of Arg-166 of Alkaline Phosphatase Alters the Thio Effect but Not the Transition State for Phosphoryl Transfer. Implications for the Interpretation of Thio Effects in Reactions of Phosphatases†

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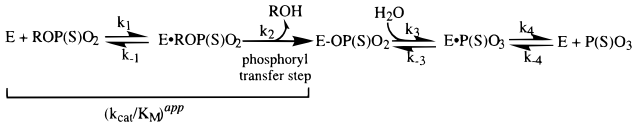
ABSTRACT: It has been suggested that the mechanism of alkaline phosphatase (AP) is associative, or triester-like, because phosphorothioate monoesters are hydrolyzed by AP approximately 10^2-fold slower than phosphate monoesters. This "thio effect" is similar to that observed for the nonenzymatic hydrolysis of phosphate triesters, and is the inverse of that observed for the nonenzymatic hydrolysis of phosphate monoesters. The latter reactions proceed by loose, dissociative transition states, in contrast to reactions of triesters, which have tight, associative transition states. Wild-type alkaline phosphatase catalyzes the hydrolysis of *p*-nitrophenyl phosphate approximately 70 times faster than *p*-nitrophenyl phosphorothioate. In contrast, the R166A mutant alkaline phosphatase enzyme, in which the active site arginine at position 166 is replaced with an alanine, hydrolyzes *p*-nitrophenyl phosphate only about 3 times faster than *p*-nitrophenyl 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. The identical values for the 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 phosphoryl transfer reaction. This result has important implications for the interpretation of thio effects in enzymatic reactions.

Alkaline phosphatase from *Escherichia coli* (EC 3.13.1) is a homodimeric metalloenzyme containing two zinc (Zn_1 and Zn_2) and one magnesium (Mg) ion in each active site (*1*, *2*). Alkaline phosphatase hydrolyzes phosphate monoesters through a covalent phosphoenzyme intermediate to produce inorganic phosphate and an alcohol (Scheme 1) (*3*). In the detailed mechanism proposed on the basis of the crystal structure (*4*), Zn_1 activates the hydroxyl group of Ser-102 for nucleophilic attack on the substrate to form a covalent phosphoseryl intermediate. This intermediate is subsequently hydrolyzed in the second step by an activated water molecule coordinated to Zn_2 to form the noncovalent enzyme−phosphate complex. In the presence of other phosphate acceptors such as Tris or ethanolamine, the enzyme also catalyzes transphosphorylation (*5*, *6*). 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 (*3*, *7*).

In the X-ray crystal structure of wild-type alkaline phosphatase crystallized in the presence of inorganic phosphate,

Scheme 1



the phosphate ion is held in the active site by interactions with both zinc ions and Arg-166 (*4*). The importance of Arg-166 has been investigated through site-specific mutagenesis experiments (*8*, *9*). Based upon studies of the site-directed mutants R166A and R166S, Arg-166 is important for catalysis, but is not absolutely required (*8*). For example, in the absence of a phosphate acceptor, with the substrate *p*-nitrophenyl phosphate, the R166A enzyme exhibits a 40-fold reduction in activity and a 2-fold increase in the K_M value compared to the wild-type enzyme under the same conditions. In the presence of a phosphate acceptor (1 M Tris), the k_{cat} decreases by about 2.5-fold, and the K_M increases by about 128-fold relative to the wild-type enzyme under the same conditions. With alkyl phosphate substrates, the R166S mutant exhibits values of k_{cat}/K_M that are reduced about 10^4-fold compared to the wild-type enzyme (*10*).

In addition, the K_i for phosphate, a competitive inhibitor, is significantly increased in the R166A and R166S enzymes (*8*). Unlike the reaction catalyzed by the wild-type enzyme, the rate-limiting step of the reaction of phosphate ester substrates with the R166A enzyme at both acidic and alkaline

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pH is the hydrolysis of the covalent phosphoenzyme intermediate (*11*).

A large body of evidence indicates that uncatalyzed phosphoryl transfer reactions of monoesters take place by a loose, or dissociative, transition state that is characterized by extensive bond cleavage to the leaving group and little bond formation to the nucleophile [for reviews of the extensive evidence for this mechanism, see (*12, 13*)]. It has often been suggested that cationic arginine groups may make the mechanism for enzymatic phosphoryl transfer more associative, by promoting electron withdrawal from phosphorus and promoting nucleophilic attack.

Phosphorothioates are phosphate esters in which one of the nonbridging oxygen atoms is replaced by sulfur. This substitution significantly increases the hydrolysis rates of monoesters, but significantly decreases reaction rates of triesters. The thio effect for the AP-catalyzed reaction resembles that of triesters. This has been cited as evidence that the reaction catalyzed by alkaline phosphatase proceeds by a triester-like, or associative, transition state.

In this paper, the effect of the mutation of Arg-166 to Ala on the thio effect and on the nature of the transition state is investigated using the substrates *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate, and by measuring linear free energy relationships (the Brønsted β_{lg}) with a series of aryl phosphorothioates. The enzymatic hydrolysis of *p*-nitrophenyl phosphorothioate by the wild-type enzyme has been previously characterized (*14*). The pre-steady-state and steady-state kinetic behavior of the chromophoric substrate analogue *p*-phenylazophenyl phosphorothioate has also been investigated with wild-type alkaline phosphatase (*15*−*17*). Both studies report the slower steady-state hydrolysis of the phosphorothioate substrate compared to the corresponding phosphate substrate by 2 orders of magnitude. In this study, the R166A mutant alkaline phosphatase is used to probe the basis for the observed slower hydrolysis of phosphorothioate substrates compared to phosphate substrates.

EXPERIMENTAL SECTION

Chemicals. Agar, agarose, ampicillin, *p*-nitrophenyl phosphate, magnesium chloride, zinc chloride, tribasic sodium thiophosphate, dibasic sodium phosphate, dibasic potassium phosphate, and CHES[1] were purchased from Sigma Chemical Co. Cyclohexylammonium salts of aryl phosphorothioates were prepared from thiophosphoryl chloride and the appropriate phenol as previously described (*18*). Tris, sucrose, and enzyme-grade ammonium sulfate were supplied by ICN Biomedicals. Tryptone and yeast extract were obtained from Difco Laboratories.

Enzyme Preparations. Expression and purification of the wild-type enzyme and the mutant enzymes have been previously described (*8*). The wild-type, R166A, and K328A enzymes were isolated from the plasmid/strain combinations pEK154/SM547, pEK145/SM547, and pEK86/SM547, respectively. The concentration of purified wild-type enzyme was determined using an extinction coefficient of 0.71 mg/mL for the ultraviolet absorption at 280 nm (*19*). The concentration of purified mutant enzymes, R166A and K328A, was determined by the Bio-Rad version of Bradford's dye binding assay (*20*) with wild-type alkaline phosphatase as the standard.

Determination of Enzymatic Activity. Alkaline phosphatase activity was measured spectrophotometrically using *p*-nitrophenyl phosphate or *p*-nitrophenyl phosphorothioate as the substrate. The release of the *p*-nitrophenolate chromophore was monitored at 410 nm. Assays were performed on a Beckmann DU-64 spectrophotometer. Temperature was regulated at 25 ± 0.5 °C using a circulating constant-temperature bath. Assays were performed in 0.01 M Tris, 0.5 M NaCl, pH 8.0.

Inhibition Studies. The inhibition constants for thiophosphate and phosphate with the wild-type and the R166A mutant enzymes were determined by the method of Segel (*21*). Data were fit to the theoretical equation for competitive inhibition. For the inhibition experiments, *p*-nitrophenyl phosphate was the substrate. In these experiments, the substrate concentration was kept below the respective K_M values of the two enzymes. For the wild-type enzyme ($K_M = 3$ μM), inhibition experiments were carried out in the presence of 2 μM *p*-nitrophenyl phosphate, and for the R166A mutant enzyme ($K_M = 17$ μM), a concentration of 10 μM was used.

Pre-Steady-State Kinetics. Pre-steady-state kinetics were performed using a KinTek Inc. stopped-flow spectrophotometer at 25 °C with a dead time of approximately 1 ms using the procedure of Xu and Kantrowitz (*22*). Data were collected at 410 nm directly by a computer via an analog/digital interface. Enzymes were dialyzed against buffer containing 10 mM Tris, 0.5 M NaCl, pH 8.0. Substrates were prepared in the same buffer. Enzyme and substrate were loaded in separate syringes and introduced into the mixing chamber in equal volumes.

Linear Free Energy Relationships. The leaving groups for the aryl phosphorothioate substrates used for construction of the linear free energy relationship, and the pK_a values, were as follows: *p*-nitrophenol, 7.14; *p*-chloro, *m*-nitrophenol, 7.78; *p*-cyanophenol, 7.95; *m*-nitrophenol, 8.35; *m*-cyanophenol, 8.61; *m*-chlorophenol, 9.02; *p*-chlorophenol, 9.38; phenol, 9.95. The appearance of the phenolate chromophores was monitored at the λ_{max} on a Beckman DU-64 spectrophotometer following the addition of R166A enzyme into the reaction mixture maintained at 25.0 ± 0.5 °C. The experimental conditions were as previously described for the linear free energy relationships of the wild-type enzyme (*23*). Enzymatic reactions were carried out in 0.45 M potassium CHES, pH 10, buffer and in the presence of 45 mM potassium phosphate (~70 × the K_i value of 640 μM for inorganic phosphate) to ensure k_{cat}/K_M conditions and a constant level of inorganic phosphate. Under these conditions, the concentration range of phosphorothioate substrate used in the experiments (60−400 μM) is well below the apparent K_M values. Kinetic data were collected for each substrate at least in duplicate and were fit by nonlinear regression to an equation for a first-order reaction. The pseudo-first-order rate constants obtained from this analysis were plotted against enzyme concentration to determine k_2, the apparent values for the second-order rate constants between the enzyme and the phosphorothioate substrate. The

[1] Abbreviations: AP, alkaline phosphatase; Tris, tris(hydroxymethyl)-aminomethane; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; *p*NPP, *p*-nitrophenyl phosphate; *p*NPT, *p*-nitrophenyl phosphorothioate.

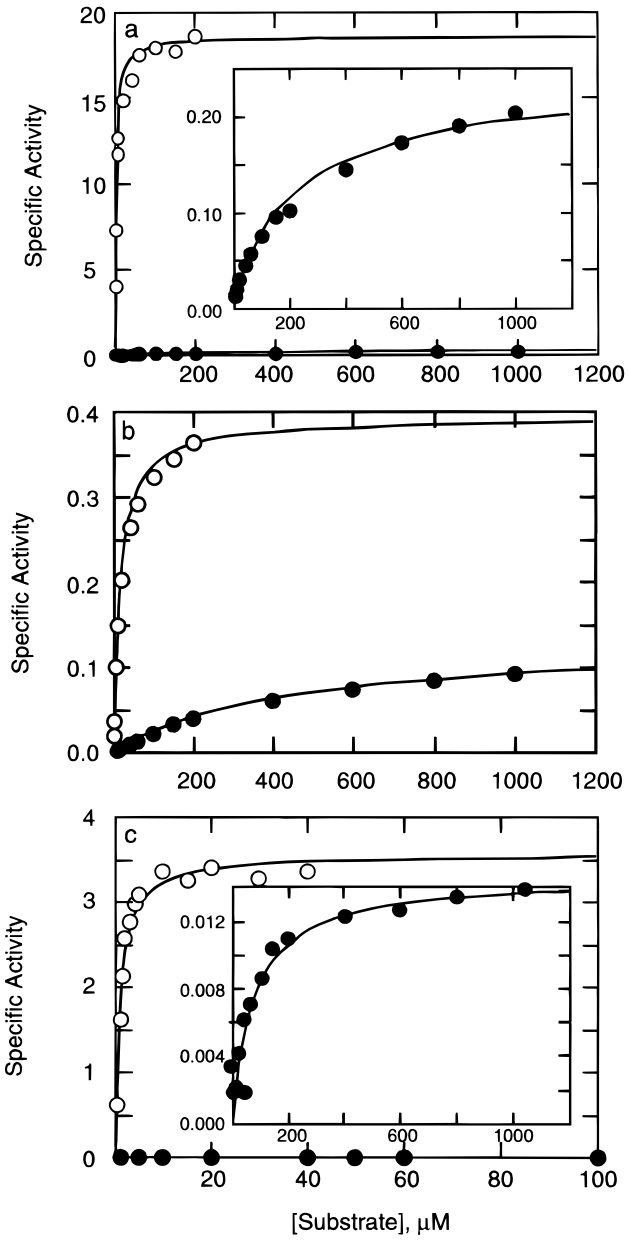


FIGURE 1: Kinetic behavior of the wild-type, the R166A mutant, and the K328A mutant enzymes toward *p*-nitrophenyl phosphate (○) and *p*-nitrophenyl-phosphorothioate (●) in 0.01 M Tris, 0.5 M NaCl, pH 8.0 at 25 °C. (a) The wild-type enzyme hydrolyzes the phosphate substrate approximately 70 times faster than the corresponding phosphorothioate substrate. (b) The R166A mutant enzyme hydrolyzes the phosphate substrate approximately 3 times faster than the corresponding phosphorothioate substrate. (c) The K328A mutant enzyme hydrolyzes the phosphate substrate approximately 300 times faster than the corresponding phosphorothioate substrate.

value of k_2 for the hydrolysis of *p*-nitrophenyl phosphate by the R166A enzyme was also obtained in the same manner. Values of k_2 for the phosphorothioate substrates were then normalized as described previously (*23*, *24*) using the k_2 value for *p*-nitrophenyl phosphate and the k_{cat}/K_M value of 2.4×10^4 M^{-1} s^{-1} for *p*-nitrophenyl phosphate hydrolysis by the R166A enzyme in the absence of added inorganic phosphate (*8*). The relative values of k_{cat}/K_M for the hydrolysis of the different phosphorothioate substrates by the R166A enzyme were compared to the relative values of the wild-type enzyme obtained in the same manner (*23*).

Table 1: Kinetic Parameters for the Wild-Type and Mutant Enzymes[a]

	*p*NPP hydrolysis		*p*NPT hydrolysis		k_{cat}(*p*NPP)/
enzyme	k_{cat}[b] (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)	k_{cat}(*p*NPT)
wild-type	13.7 (0.9)	2.9 (0.8)	0.20 (0.03)	41 (13) 205 (15)[c]	70
R166A	0.29 (0.02)	17 (2)	0.11 (0.03)	506 (133)	3
K328A	3.0 (0.5)	1.3 (0.3)	0.01 (0.02)	54 (4)	300

[a] Assays were performed at 25 °C in 0.01 M Tris, 0.5 M NaCl, pH 8.0. [b]The k_{cat} values are calculated per active site from the V_{max} using a dimer molecular weight of 94 000. [c]Two different slopes are observed with this enzyme and *p*-nitrophenyl phosphorothioate. Three independent determinations made for each slope were averaged to give the values reported in the table.

RESULTS

Steady-State Kinetics of the Wild-Type and R166A Mutant Enzymes with p-Nitrophenyl Phosphate and p-Nitrophenyl Phosphorothioate. The steady-state kinetics of the wild-type enzyme with the substrates *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate are shown in Figure 1a. *p*-Nitrophenyl phosphate is hydrolyzed ~70-fold faster than the corresponding phosphorothioate substrate (see Table 1). Previous results reported a 100-fold increase for the hydrolysis of *p*-nitrophenyl phosphate compared to *p*-nitrophenyl phosphorothioate (*14*). For a similar set of substrates, *p*-phenylazophenyl phosphorothioate and *p*-phenylazophenyl phosphate, the hydrolysis of the phosphate substrate is reported to be ~200 times faster than that of the corresponding phosphorothioate substrate (*16*, *17*).

The steady-state kinetics of the R166A mutant enzyme with *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate are shown in Figure 1b. In contrast to the wild-type enzyme, the R166A enzyme hydrolyzes *p*-nitrophenyl phosphate only 3-fold faster than *p*-nitrophenyl phosphorothioate, and the K_M of *p*-nitrophenyl phosphorothioate increases about 30-fold relative to *p*-nitrophenyl phosphate (see Table 1).

The hydrolysis of *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate by the K328A mutant enzyme was also measured, and is shown in Figure 1c. The K328A mutant alkaline phosphatase serves as a control in comparing the kinetic behavior of the R166A and the wild-type enzymes, which have different rate-limiting steps in the alkaline hydrolysis of *p*-nitrophenyl phosphate. Lys-328 interacts with phosphate in the active site of the wild-type enzyme through a water molecule. Substitution of Lys-328 with an alanine changes the rate-limiting step to the hydrolysis of the covalent enzyme−phosphate intermediate, the same rate-limiting step as the R166A mutant (*11*, *22*). The kinetic results for the relative hydrolysis rates of *p*-nitrophenyl phosphorothioate and *p*-nitrophenyl phosphate by K328A follow the same trend as the wild-type enzyme. *p*-Nitrophenyl phosphorothioate is hydrolyzed approximately 300-fold slower than *p*-nitrophenyl phosphate, and the K_M value for *p*-nitrophenyl phosphorothioate increases about 42-fold relative to *p*-nitrophenyl phosphate (see Table 1).

Linear Free Energy Relationships with R166A and Wild-Type Alkaline Phosphatase. The chemical step of phosphoryl transfer from the substrate to the serine nucleophile has been shown to be rate-limiting for k_{cat}/K_M in the reaction of wild-

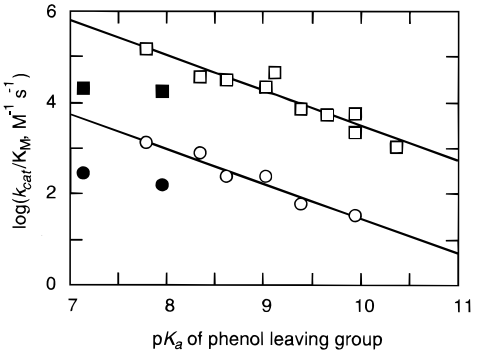


FIGURE 2: Dependence of the k_{cat}/K_M for a series of aryl phosphorothioates by wild-type (□, ■) and R166A (○, ●) alkaline phosphatase. The values for k_{cat}/K_M were measured under the special conditions described under Experimental Section. The solid lines are least-squares fits to the (□) wild-type and (○) R166A data. The R166A data yield a slope of -0.78 ± 0.06 ($r = 0.96$) for the β_{lg}, compared with that of -0.77 ± 0.09 ($r = 0.94$) for the wild type. The data for the reactions of the phosphorothioates with the wild-type enzyme are taken from Hollfelder and Herschlag (*23*). The filled symbols are data points for the *p*-nitro- and the *p*-cyano-substituted substrates, and were omitted from the correlations.

type AP with phosphorothioate substrates (*23*). Figure 2 shows the dependence of the k_{cat}/K_M on the pK_a of the leaving group for a series of aryl phosphorothioates. The values for the apparent second-order rate constants between R166A and the phosphorothioate substrates (k_{cat}/K_M) were obtained in the same manner as those previously reported for the native enzyme (*23*).

The parameter k_{cat}/K_M includes the portion of the enzymatic mechanism up to and including the irreversible step of phosphoryl transfer to the enzymatic nucleophile (Scheme 1). Since the chemical step of phosphoryl transfer is rate-limiting for k_{cat}/K_M, the Brønsted slope represents the effect of the pK_a of the leaving group on this step. Substrates with strongly electron-withdrawing para substituents (nitro and cyano) show deviations, and are hydrolyzed more slowly than expected from their pK_a compared with the other substrates. The same behavior was observed with the wild-type enzyme (*23*). The slope of the line for the R166A gives a value of β_{lg} of -0.78 ± 0.06, compared with the value of -0.77 ± 0.09 for the native enzyme.

Phosphate and Thiophosphate Inhibition Studies with the Wild-Type and the R166A Alkaline Phosphatase Enzymes. Both wild-type alkaline phosphatase and the R166A mutant enzyme are inhibited by inorganic phosphate and inorganic thiophosphate. Figures 3a and 3b show data from the inhibition studies of the wild-type alkaline phosphatase enzyme and the mutant enzyme, respectively. Thiophosphate ($K_i = 4.6$ μM) and phosphate ($K_i = 3.6$ μM, see Table 2) bind with similar affinities to wild-type alkaline phosphatase. These results are similar to the reported K_i values of Chlebowski et al. (*16*) of 5.8 and 5.0 μM for thiophosphate and phosphate, respectively. In contrast, the R166A enzyme binds thiophosphate ($K_i = 119$ μM) 5 times tighter than phosphate ($K_i = 643$ μM, see Table 2).

Pre-Steady-State Kinetic Behavior of the Wild-Type and the R166A Alkaline Phosphatase Enzymes with p-Nitrophenyl Phosphate and p-Nitrophenyl Phosphorothioate. The pre-steady-state behavior of both the wild-type enzyme and the R166A enzyme was evaluated with the two different substrates. The stopped-flow traces for the wild-type enzyme

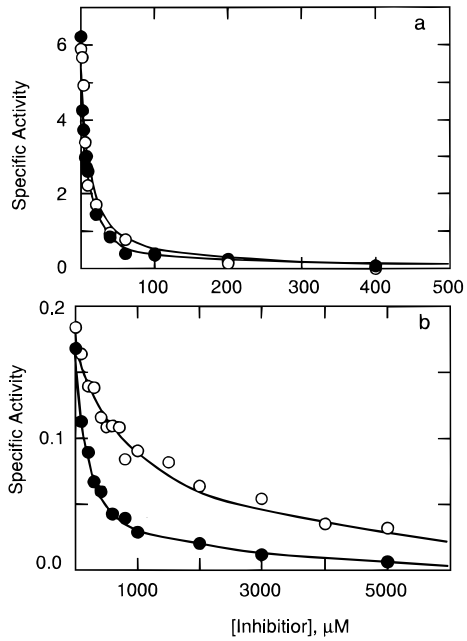


FIGURE 3: Inhibition of the wild-type and the R166A mutant enzymes by inorganic phosphate (○) and inorganic thiophosphate (●). Activities were measured in 0.01 M Tris, 0.5 M NaCl, pH 8.0 at 25 °C. Inhibition data were fit to an equation for competitive inhibition. (a) Inhibition data for the wild-type enzyme, and (b) inhibition data for the R166A mutant enzyme.

Table 2: Inhibition of the Wild-Type and Mutant Enzymes by Phosphate and Thiophosphate[a]

enzyme	phosphate K_i (μM)	thiophosphate K_i (μM)	K_i(phosphate)/ K_i(thiophosphate)
wild-type	3.6	4.6	0.78
R166A	643	119	5.4

[a] Assays were performed at 25 °C in 0.01 M Tris/0.5 M NaCl buffer, pH 8.0.

with *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate are shown in Figures 4a and 4b, respectively. A linear increase in absorbance with time is observed with *p*-nitrophenyl phosphate, as expected for the wild-type enzyme containing residual phosphate (*25*). In contrast, a very small burst of alcohol production is observed when *p*-nitrophenyl phosphorothioate is the substrate. The magnitude of the burst amplitude is small since the enzyme was not purged of residual phosphate. The pre-steady-state behavior of the enzyme with *p*-phenylazophenyl phosphorothioate substrate is similar (*16*).

The stopped-flow traces for the R166A mutant enzyme with *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate are shown in Figures 4c and 4d, respectively. When *p*-nitrophenyl phosphate is the substrate, an initial burst of *p*-nitrophenol is observed in the stopped-flow trace followed by a linear steady increase. In contrast, no significant burst is observed with the *p*-nitrophenyl phosphorothioate substrate.

DISCUSSION

The mechanisms of uncatalyzed phosphoryl transfer reactions in solution have been well studied using a variety of physical organic techniques [for reviews, see (*12*, *13*)]. This work has provided considerable evidence for a loose, or

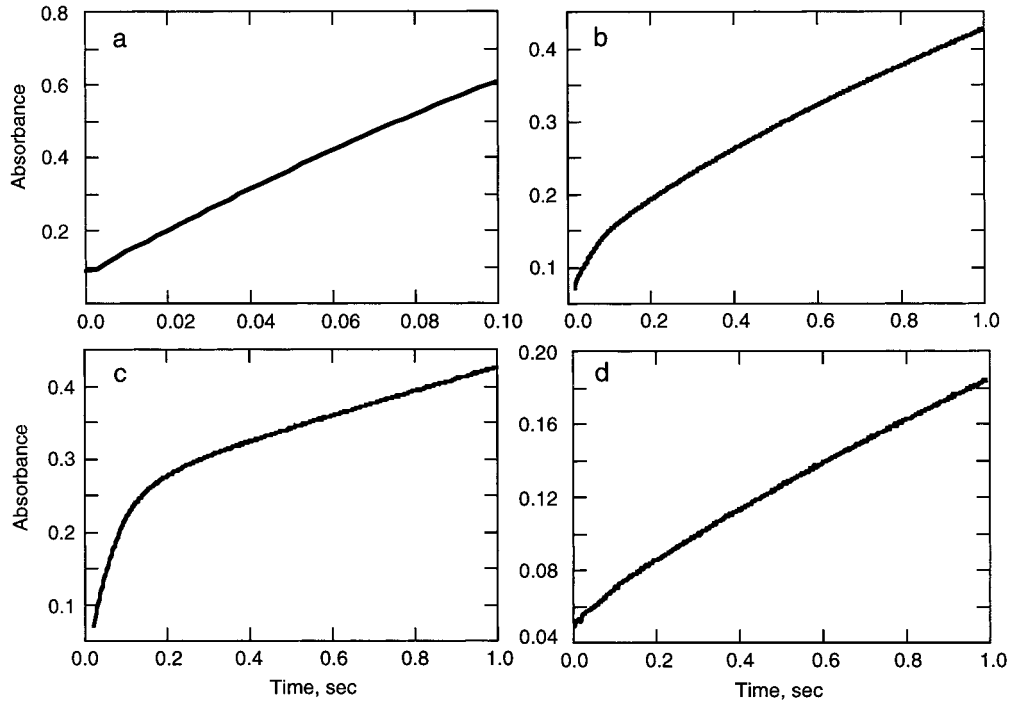
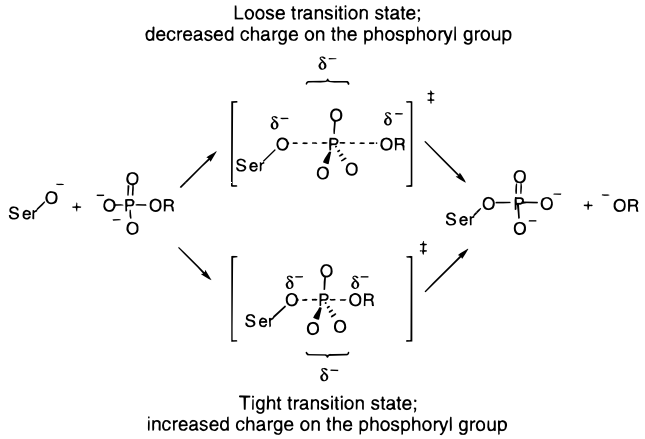


FIGURE 4: Pre-steady-state kinetic behavior of the wild-type and the R166A mutant enzymes toward *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate. Enzyme and substrate solutions were prepared in 0.01 M Tris, 0.5 M NaCl, pH 8.0. Data were collected at 25 °C at 410 nm. (a) Hydrolysis of *p*-nitrophenyl phosphate (0.1 mM) by the wild-type enzyme (0.8 mg/mL) is shown. (b) Hydrolysis of *p*-nitrophenyl phosphorothioate (1.0 mM) by the wild-type enzyme (3 mg/mL) is shown. (c) Hydrolysis of *p*-nitrophenyl phosphate (1.0 mM) by the R166A mutant enzyme (1 mg/mL) is shown. (d) Hydrolysis of *p*-nitrophenyl phosphorothioate (1 mM) by the R166A mutant enzyme (3 mg/mL) is shown.

Scheme 2



dissociative, transition state for nonenzymatic reactions of phosphate monoesters. In the transition states for these reactions, the bond to the leaving group is largely broken, and there is little bond formation to the nucleophile. By contrast, phosphotriesters undergo reaction by a tight, or associative, transition state characterized by a much smaller degree of bond cleavage to the leaving group and considerable bond formation to the nucleophile (Scheme 2). The reactions of phosphodiesters have transition states intermediate between those of monoesters and triesters. In general, the phosphoryl unit bears about a unit negative charge in the transition state (*26*).

The transition states for all three classes of phosphate esters share a trigonal bipyramidal geometry, but differ in the bond orders to the axial groups. The mechanisms carry different implications for the change in charge density on the phosphoryl group during the reaction (Scheme 2). In a loose transition state, the negative charge on the phosphoryl group decreases in the transition state; in an associative, triester-like transition state, this negative charge density increases. This has led some to conclude that the presence of positively charged groups at the active sites of phosphatases connotes a triester-like transition state.

Phosphorothioates are analogues of phosphate esters in which a sulfur atom has been substituted for a nonbridging oxygen atom. Data from linear free energy relationships (*23*), activation parameters (*18*, *27*), and stereochemical studies (*27*−*30*) indicate that phosphorothioate monoesters react via two-step mechanism with a thiometaphosphate intermediate. The transition state of the rate-limiting first step is late, with extensive bond cleavage to the leaving group.

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 3). 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 3. The thio effect is the ratio of the reaction rate with the phosphate substrate divided by that of the corresponding phosphorothioate, or k_O/k_S. For monoesters, this ratio is $\ll 1$, while for triester reactions, this ratio is $\gg 1$.

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 (*14*, *17*, *31*). However, a large negative value

Table 3: Correlation of Thio Effects, Brønsted β_{lg} Values, and Transition State Structures in Alkaline Hydrolysis Reactions of Phosphate and Phosphorothioate Esters[a]

phosphate ester	range of thio effects (k_O/k_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)

[a] The thio effect is reported as k_O/k_S, the ratio of the rate of phosphoryl transfer to that for thiophosphoryl transfer. The thio effect data come from the compilation reported in ref *34*, from references cited therein. The Brønsted β_{lg} values are those for the alkaline hydrolysis reactions of corresponding phosphate and phosphorothioate esters (*23*, *40*−*44*).

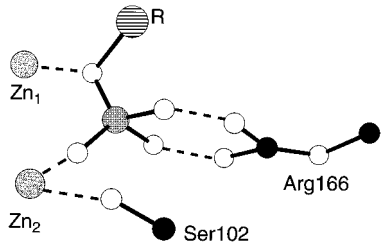


FIGURE 5: Model for substrate binding in the active site of wild-type alkaline phosphatase based on the structure of Kim and Wyckoff (*4*). Arg-166 forms a bidentate interaction with two oxygen atoms of the phosphate monoester. The third oxygen of the substrate coordinates Zn_2, while the alkoxide portion of the substrates coordinates Zn_1 through the fourth oxygen atom.

of −0.77 for the β_{lg} for the reaction of AP with *O*-aryl phosphorothioates is more consistent with a loose, monoester-like transition state (*23*).

The question of whether thio effects are reliable reporters for transition state structures in enzymatic reactions has not been tested by experiment. The slower catalytic rates observed with phosphorothioate substrates could be due to poorer complementarity between the active site and the substrate and/or the transition state. The bond orders and bond lengths, charge distribution, hydrogen bonding properties, and steric requirements of phosphorothioates differ substantially from those of phosphates (*32*). These factors may adversely affect substrate binding, as well as the complementarity of the transition state within the active site. A triester-like thio effect observed for the protein-tyrosine phosphatase from *Yersinia* has been attributed to impaired complementarity between the transition state and the active site with phosphorothioate substrates (*33*). Additional factors that can contribute to thio effects in enzymatic reactions have been pointed out, including different rate-determining steps and differences in hydrogen bonding or protonation (*34*, *35*).

Interactions of Active Site Residues with the Phosphoryl Group in the Transition State. The X-ray crystal structures of the wild-type enzyme with bound inorganic phosphate (*4*) and vanadate (*36*) indicate that there exists a bidentate interaction between Arg-166 and the nonbridging oxygen atoms of the phosphoryl group in the substrate, and in the transition state. In the proposed model for substrate binding based on the structure of the noncovalent enzyme phosphate intermediate (*4*), the guanidinium group of Arg-166 forms two hydrogen bonds to two phosphate oxygen atoms of the substrate (see Figure 5). The alcohol group of the phosphomonoester substrate is directed out into solution and is thought to coordinate to Zn_1 as an alkoxide ion during the reaction based on evidence implicating the zinc ion in activating a water molecule for nucleophilic attack in the second step of the reaction (*4*, *37*, *38*). The fourth interaction between phosphate and the Zn_2 ion of the enzyme is deeper into the rather shallow active site pocket and therefore excludes the alcohol group of the substrate.

Mutation of Arginine-166 to Alanine Changes the Thio Effect but Not the Transition State for Phosphoryl Transfer. To further explore the role of Arg-166 in stabilizing the transition state of the reaction, and on the effect of this residue on the thio effect, the kinetics of the site-directed mutant R166A and the wild-type enzyme with *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate were compared. In addition, values for k_{cat}/K_M were measured with a series of aryl phosphorothioates, and the results were used to construct a Brønsted plot to determine the value for β_{lg}. It has been previously demonstrated that the chemical step of phosphoryl transfer is rate-limiting for k_{cat}/K_M in the reaction of wild-type AP with phosphorothioate substrates (*23*). Thus, these β_{lg} values reflect the transition state for phosphoryl transfer to the serine nucleophile.

The kinetic data show that the R166A mutant enzyme catalyzes the hydrolysis of *p*-nitrophenyl phosphorothioate and of *p*-nitrophenyl phosphate at comparable rates. The ratio of *p*-nitrophenyl phosphate hydrolysis to *p*-nitrophenyl phosphorothioate is about 3, compared to the ratio of 70 for the wild-type enzyme. Despite the significant change (∼23-fold) in the magnitude of the thio effect, the β_{lg} for the reaction catalyzed by the R166A mutant is indistinguishable from that of the wild-type enzyme. The identical β_{lg} values imply that the transition state for the alkaline phosphatase-catalyzed hydrolysis of aryl phosphorothioates is unchanged by the mutation of arginine-166 to alanine. These results indicate that the magnitude of the thio effect is not a reliable reporter for the nature of the transition state of the enzymatic reaction, and that other factors influence the relative activities of the enzyme with phosphate versus phosphorothioate substrates.

Another recent study using a series of alkyl phosphate substrates has reported that the values for the β_{lg} of wild-type alkaline phosphatase and the R166S mutant are unaltered (*10*). The conclusion that interactions of arginine with the phosphoryl group do not alter the transition state thus holds for both phosphorothioate and for phosphate substrates.

Replacing the arginine side chain at position 166 with alanine removes the two hydrogen bonds observed between the enzyme and phosphate in the X-ray crystal structure of the wild-type enzyme. Whether phosphorothioate substrates bind with the sulfur atom coordinated to one or both of the zinc ions, or oriented toward the guanidinium group of the arginine, is unknown. The known preference of zinc ions to coordinate sulfur over oxygen in complexes with phosphates (*39*) would be expected to thermodynamically favor the former one. For either orientation, the additional steric requirements of phosphorothioates (*32*) will require displacements of active site residues, and/or alterations in the positioning of the substrate, relative to phosphate substrates. The loss of the guanidinium group and its hydrogen bonding interactions will be deleterious to the complementarity between the active site and the phosphoryl group in the transition state. However, the removal of this side chain will allow for better accommodation of the larger phosphorothio-

ate group in the active site. This is the most likely molecular basis for the observation that the R166A mutation reduces the rate of phosphate ester hydrolysis by more than it reduces the rate with phosphorothioates, and thus exhibits a smaller thio effect.

Negative deviations are present in the Brønsted plots of the rates for substrates bearing leaving groups with strongly electron-withdrawing substituents. These polar substituents may have unfavorable interactions with the hydrophobic surface of the enzyme. It is also possible that these strongly electron-withdrawing groups provide significant charge delocalization in the transition state, decreasing the charge density on the phenolic oxygen atom and thereby weakening the interaction with the catalytic zinc ion. In a prior study with the wild-type enzyme, it was shown that the deviations do not arise from a change in rate-limiting step (*23*).

Thiophosphate Inhibition of R166A Suggests a Specific Binding Orientation. Inhibition studies show that phosphate and thiophosphate have the same K_i within experimental error for the wild-type enzyme. In the case of the R166A mutant, thiophosphate is a tighter binding inhibitor than phosphate. The replacement of Arg-166 with alanine results in the loss of two hydrogen bonds to phosphate or thiophosphate. The loss of these interactions, together with greater available space in the active site, may allow a more specific interaction between the sulfur of the thiophosphate ion and the zinc ions. The preferential binding of a thiol group over a phosphonate group has been observed in the X-ray crystal structure of a bifunctional inhibitor, mercaptomethyl phosphonate, bound in the active site of wild-type alkaline phosphatase (unpublished observation). In addition, zinc is known to prefer to coordinate sulfur over oxygen in complexes with phosphate esters (*39*). The stronger sulfur−zinc interaction may be responsible for the greater affinity of thiophosphate for the R166A enzyme.

The Rate-Determining Step in the Catalytic Mechanism with Phosphate Substrates of the R166A Enzyme Differs from That of the Wild-Type Enzyme. The rate-limiting step in the hydrolysis of *p*-nitrophenyl phosphate by the wild-type enzyme under alkaline conditions is the dissociation of phosphate from the noncovalent enzyme−phosphate complex. In contrast, the rate-limiting step across the pH range for hydrolysis of *p*-nitrophenyl phosphate by the R166A mutant enzyme is the hydrolysis of the covalent phosphoenzyme intermediate. Since the rate-determining step in the catalytic reaction at alkaline pH differs for these two enzymes, the k_{cat} ratios with phosphorothioate and phosphate substrates do not reflect the respective rates of the same chemical step with the two substrates. The kinetic behavior of the active site mutant K328A was evaluated with both *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate. Like the R166A mutant, for K328A the hydrolysis of the covalent phosphoenzyme intermediate is rate-limiting in the hydrolysis of *p*-nitrophenyl phosphate. Unlike the R166A mutant enzyme, the K328A mutant enzyme exhibits a thio effect for k_{cat} that follows the same trend as the wild-type enzyme. Namely, *p*-nitrophenyl phosphorothioate hydrolysis is reduced, compared to the hydrolysis of *p*-nitrophenyl phosphate, by a factor that is even greater than that of the wild-type enzyme (see Table 1). This thio effect represents the ratio of the relative rates of hydrolysis of the phosphoserine and the thiophosphoserine intermediates.

Pre-Steady-State Behavior in the Alkaline Phosphatase Reaction. The pre-steady-state behavior of the wild-type enzyme was evaluated with *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate. Based upon the stopped-flow traces for the wild-type enzyme, the slow step in the hydrolysis of *p*-nitrophenyl phosphate is the dissociation of inorganic phosphate from the noncovalent complex, while the slow step in the hydrolysis of *p*-nitrophenyl phosphorothioate is the hydrolysis of the covalent enzyme−thiophosphate intermediate. Since thiophosphate and phosphate bind to the wild-type enzyme with similar affinities, it is assumed that the shift in the slow step of the reaction is not due to a decrease in the dissociation rate of thiophosphate from the noncovalent complex. Therefore, the slower hydrolysis of the enzyme−thiophosphate intermediate compared to the enzyme−phosphate intermediate is probably a direct consequence of the slower hydrolysis of the thiophosphoenzyme intermediate.

The pre-steady-state kinetic behavior of the R166A enzyme was also evaluated with both *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate. The large burst observed in the hydrolysis of *p*-nitrophenyl phosphate indicates that the rate-limiting step follows formation of the phosphoenzyme intermediate. Given the large K_i value for phosphate in the R166A mutant, phosphate release should be fast; thus, the rate-limiting step is most likely hydrolysis of the covalent enzyme−phosphate intermediate. In the hydrolysis of *p*-nitrophenyl phosphorothioate, the absence of a significant burst indicates that an earlier step, most likely formation of the thiophosphoenzyme intermediate, becomes rate-limiting or at least mostly so. The significantly lower K_i value for thiophosphate compared to that for phosphate implies that dissociation of thiophosphate from the noncovalent product complex should be slower than dissociation of phosphate in the corresponding hydrolysis of *p*-nitrophenyl phosphate. Thus, in order for thiophosphoenzyme intermediate formation to be rate-limiting, this step must also be slowed substantially by the R166A mutation.

CONCLUSIONS

The kinetic behavior of wild-type alkaline phosphatase and the R166A mutant, in which the active site arginine is replaced with an alanine, has been evaluated with both *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate, and with a series of aryl phosphorothioates. This mutation results in a significant change in the thio effect. However, the Brønsted β_{lg}, and by implication the transition state, is unaltered. This demonstrates that the magnitude of the thio effect in the alkaline phosphatase reaction is affected by factors other than the transition state structure. This implies that the thio effect cannot reliably be used to infer the degree of associative or dissociative character of the transition state for enzymatic phosphoryl transfer.

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