Alkaline Phosphatase Revisited: Hydrolysis of Alkyl Phosphates[†]

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ABSTRACT: *Escherichia coli* alkaline phosphatase (AP) is the prototypical two metal ion catalyst with two divalent zinc ions bound ∼4 Å apart in the active site. Studies spanning half a century have elucidated many structural and mechanistic features of this enzyme, rendering it an attractive model for investigating the potent catalytic power of bimetallic centers. Unfortunately, fundamental mechanistic features have been obscured by limitations with the standard assays. These assays generate concentrations of inorganic phosphate (P_i) in excess of its inhibition constant ($K_i \approx 1\ \mu M$). This tight binding by P_i has affected the majority of published kinetic constants. Furthermore, binding limits k_{cat}/K_m for reaction of *p*-nitrophenyl phosphate, the most commonly employed substrate. We describe a sensitive ^{32}P-based assay for hydrolysis of alkyl phosphates that avoids the complication of product inhibition. We have revisited basic mechanistic features of AP with these alkyl phosphate substrates. The results suggest that the chemical step for phosphorylation of the enzyme limits k_{cat}/K_m. The pH−rate profile and additional results suggest that the serine nucleophile is active in its anionic form and has a pK_a of ≤5.5 in the free enzyme. An inactivating pK_a of 8.0 is observed for binding of both substrates and inhibitors, and we suggest that this corresponds to ionization of a zinc-coordinated water molecule. Counter to previous suggestions, inorganic phosphate dianion appears to bind to the highly charged AP active site at least as strongly as the trianion. The dependence of k_{cat}/K_m on the pK_a of the leaving group follows a Brønsted correlation with a slope of $\beta_{lg} = -0.85 \pm 0.1$, differing substantially from the previously reported value of −0.2 obtained from data with a less sensitive assay. This steep leaving group dependence is consistent with a largely dissociative transition state for AP-catalyzed hydrolysis of phosphate monoesters. The new ^{32}P-based assay employed herein will facilitate continued dissection of the AP reaction by providing a means to readily follow the chemical step for phosphorylation of the enzyme.

A large number of structurally diverse enzymes employ two metal ions in their catalytic centers [for review, see refs *1* and *2*]. Although many different types of reactions are catalyzed, they share in common two metal ions bound ∼4 Å apart. This constellation of metal ions appears to be particularly well-suited for catalyzing phosphoryl transfer, as many phosphomonoesterases and phosphodiesterases, as well as the bacterial phosphotriesterase, have two metal ions in their active sites. The rate enhancements of two metal ion catalysts are among the largest known (*3*, *4*).

E. coli alkaline phosphatase (AP)[1] is the most extensively studied two metal ion catalyst and thus represents an attractive system for investigation of the catalytic power and mode of action of bimetallic centers (*5*−*8*). The wealth of structural information available for this enzyme includes structures with inorganic phosphate (P_i) product bound both covalently and noncovalently and with a pentavalent vanadyl transition state analogue [Figure 1; *6*, *9*, *10*]. These structures suggest central roles for the two Zn^{2+} ions in the catalytic mechanism (*5*, *6*, *10*). AP also contains a third metal ion, typically Mg^{2+}, that appears to be less intimately involved in the reaction, although removal of this metal ion has deleterious effects on catalysis and on the stability of AP (*11*−*14*).

A large body of kinetic data and biochemical characterization has established a two-step reaction mechanism with formation of a phosphoserine intermediate [Scheme 1; for review, see refs *5*, *7*, *8*)]. The two zinc ions are favorably placed to simultaneously activate the nucleophile and stabilize the leaving group (Figure 1). In the first chemical step (k_2; Scheme 1), Zn_{II} is thought to facilitate formation of the more reactive serine alkoxide, and Zn_I is thought to stabilize the developing negative charge on the leaving group. In the second chemical step (k_3; Scheme 1), which is analogous to the reverse of the first step, Zn_I would facilitate generation of a hydroxide molecule for attack on the phosphoryl group and Zn_{II} would stabilize the serine leaving group.

Initial kinetic investigations of AP reported complex kinetics, that were interpreted by some investigators as indicative of rate-limiting conformational change or of anticooperative behavior between the two active sites of the

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[1] Abbreviations: AP, *E. coli* alkaline phosphatase; DNPP, 2,4-dinitrophenyl phosphate; E, enzyme; LFER, linear free-energy relationship; NaMES, sodium 4-morpholineethanesulfonate; NaMOPS, sodium 4-morpholinepropanesulfonate; NaCHES, sodium 2-(cyclohexylamino)-ethanesulfonate; NaCAPS, sodium 3-cyclohexylamino-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; P_i, inorganic phosphate; PNPP, *p*-nitrophenyl phosphate; S, substrate.

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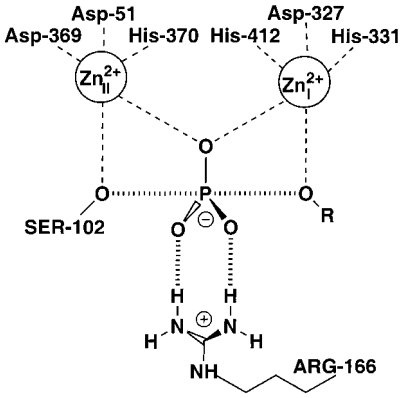


FIGURE 1: Transition state model for phosphoryl transfer by AP based upon the structure of AP covalently bound to a pentavalent vanadate ester (*10*). The two zinc ions are ~4 Å apart (*6*, *10*).

AP dimer. Much of this confusion was resolved when it was discovered that AP binds P_i tightly [$K_i = 1$ μM at pH 8.0; *15*, *16*] and contains stoichiometric quantities of P_i when isolated (*17*). The implications of tight binding by product have been well documented for pre-steady-state kinetics (*17*−*19*), but the effect on steady-state turnover continues to be overlooked. As a consequence, published rate constants are often in poor agreement between independent studies.

The majority of studies of AP have employed *p*-nitrophenyl phosphate (PNPP) as the substrate, because it offers a convenient colorimetric assay. The extinction coefficient for *p*-nitrophenolate is 1.62×10^4 M^{-1} cm^{-1} at its absorbance maximum at pH 8.0 (*20*), corresponding to an absorbance change of 0.016 for complete hydrolysis of 1 μM PNPP. Thus, saturating concentrations of P_i are required to observe a substantial absorbance change upon hydrolysis. Concentrations of substrate >1 μM are routinely used and are presumably responsible for the 40-fold range of reported values of k_{cat}/K_m for PNPP under the standard assay conditions [e.g., refs *16*, *21*−*24*)]. Similarly, the formation of the P_i product during the course of the assay causes the inhibition by added P_i to be less pronounced and may be responsible for the 50-fold range of K_i values for P_i inhibition [e.g., refs *7*, *12*, *15*, *16*, *25*].

An additional limitation of aryl phosphates as substrates for AP is that the chemical step for phosphorylation of the enzyme is not rate-limiting for steady-state hydrolysis. Binding of substrate or an associated conformational change limits k_{cat}/K_m (*26*−*28*), and k_{cat} is limited by product release at pH > 7.5 and by breakdown of the covalent intermediate at pH < 7.5 [for review, see refs *5*, *7*, and *8*)]. Even pre-steady-state methods are not able to measure the rate of the chemical step, as it is too fast for conventional rapid mixing methods, $k_2 > 10^3$ s^{-1} (*19*). Experiments with alternative substrates could provide much more insight into the mechanism of AP catalysis if the chemical step were rate-limiting. Alkyl phosphates are good candidates, because they are inherently less reactive than aryl phosphates.

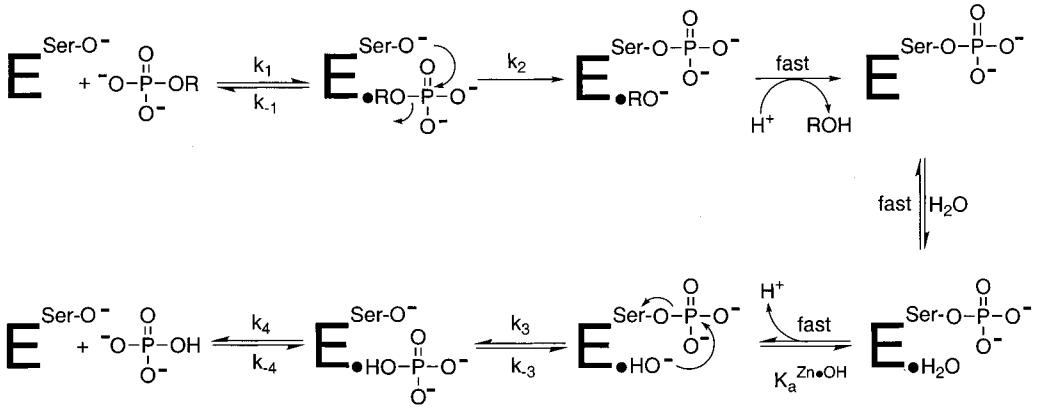
Previous studies that employed alkyl phosphates disagree as to whether they are poorer substrates than PNPP, with reported k_{cat}/K_m values that vary from the same as that for PNPP (*15*, *29*) to 10^4-fold lower than that of PNPP (*30*, *31*). Values of k_{cat} for alkyl phosphates also range from the same as PNPP (*15*, *31*, *32*) to 50-fold lower (*30*, *32*). The assay used to detect reaction of alkyl phosphates [$\epsilon_{660} = 4 \times 10^3$ M^{-1} cm^{-1}; *30*] is 4-fold less sensitive than detection of *p*-nitrophenolate, and thus 25 μM P_i must be formed to give an absorbance increase of 0.1. The requisite use of high concentrations of substrate and the formation of high concentrations of P_i, which inhibits the reaction, is presumably responsible for the wide range of reported k_{cat}/K_m values.

A necessary step toward a full understanding of enzymatic catalysis is the characterization of the transition state for the enzyme-catalyzed reaction. It has been widely suggested that coordination of active site metal ions or other positively charged groups to the nonbridging phosphoryl oxygens renders enzymatic transition states for phosphoryl transfer more associative, whereas phosphoryl transfer reactions in solution proceed through dissociative transition states [e.g., (*30*, *33*−*46*)].[2] The idea that interactions of positively charged enzymatic groups change the nature of the transition state was tested for AP by mutating the active site arginine and evaluating the effect on the leaving group dependence. The results strongly suggested that R166 does not render the transition state more associative [*47*, *48*; see also ref *49*]. Divalent metal ions have been shown not to affect the transition state for phosphoryl transfer in solution (*50*, *51*), but the possibility that the Zn^{2+} ions at the AP active site might stabilize a more associative transition state than in solution has not been directly addressed.

As AP is a nonspecific phosphatase with a shallow binding pocket, it is a good candidate for linear free energy relationship (LFER) approaches, and several studies have previously addressed this question (*26*, *30*, *32*, *52*, *53*). AP-catalyzed hydrolysis of aryl *O*-phosphorothioates follows a steep Brønsted leaving group dependence [$\beta_{lg} = -0.77$; *26*]. This value is similar to that for the nonenzymatic reaction ($\beta_{lg} = -1.1$), suggesting that the AP-catalyzed reaction is also largely dissociative.[2] In contrast, studies employing aryl and alkyl phosphates found little dependence upon the pK_a of the leaving group [$\beta_{lg} = -0.19$; *30*, *54*]. Subsequent work showed that reactions of aryl phosphates are not limited by the chemical step [k_2; Scheme 1; *26*−*28*], whereas the values of k_{cat}/K_m for alkyl phosphates were considerably lower, consistent with a rate-limiting chemical step for reaction of alkyl phosphates (*30*, *54*). The simplest interpretation of the shallow leaving group dependence for reaction of alkyl phosphates would be that AP renders the transition state more associative than that observed for the solution reaction (*30*). However, as the assay is expected to be greatly affected by P_i inhibition these results and conclusions are called into question.

[2] Two transition state extremes can be considered for phosphoryl transfer: a dissociative extreme in which there is no bond formation to the nucleophile and complete bond cleavage to the leaving group, and an associative extreme in which there is full bond formation to the nucleophile and no bond cleavage to the leaving group (*66*, *67*, *89*). A continuum of possible transition states lies between these extremes, but it is useful to adopt an operational definition for dissociative and associative transition states. A dissociative transition state is defined as one in which the sum of the bonding between the incoming nucleophile and outgoing leaving group is less than one (i.e., bond order decreases in going from the ground state to the transition state). An associative transition state is defined as one in which the sum of the bonding between the incoming nucleophile and outgoing leaving group is greater than one (i.e., bond order increases in going from the ground state to the transition state). Considerable evidence suggests that nonenzymatic reactions of phosphate monoesters are highly dissociative, i.e., have transition states that are close to the dissociative extreme (*66*, *67*, *89*, *90*).

Scheme 1



We describe a ^{32}P-based assay to follow hydrolysis of alkyl phosphates. The high sensitivity of this method has allowed the AP-catalyzed reaction to be followed at extremely low concentrations of substrate, far below the range where product inhibition by P_i occurs. Reinvestigation of the basic features of AP-catalyzed hydrolysis of alkyl phosphates has provided information about the ionizable groups important for binding and catalysis. The results also suggest that k_{cat}/K_m is limited by the chemical step for phosphorylation of the enzyme, thereby allowing the reaction rate to be followed as a function of the leaving group pK_a to provide information about the nature of the transition state for AP-catalyzed phosphate ester hydrolysis.

MATERIALS AND METHODS

Buffers were prepared at 25 °C. Substituted alcohols were of the highest purity available (≥95%) and used in the synthetic protocol without further purification. Disodium salts of ethyl phosphate and methyl phosphate were obtained by hydrolysis of the alkyl phosphorodichloridates with excess sodium hydroxide. Dicyclohexylammonium salts of ethyl phosphate, 2-fluoroethyl phosphate, and 2,2,2-trifluoroethyl phosphate were synthesized as previously described and recrystallized from ethanol/water mixtures (*55*). Structures and purity (≥98%) were confirmed by ^{1}H and ^{31}P NMR (data not shown). The di-2,6-lutidinium salt of 2,4-dinitrophenyl phosphate (DNPP) was a gift from F. Hollfelder. The plasmids for expression of AP (pEK48) and R166S AP (pEK1152) and the phoA$^-$ strain of *E. coli* (SM547) were provided by E. R. Kantrowitz (*56*).

Purification of Alkaline Phosphatase. AP was purified via a modification of a previously described protocol (*56*). *E. coli* strain SM547 harboring pEK48 was grown to saturation (~24 h) in 1× YT media, 150 μg/mL carbenicillin. Following osmotic shock and centrifugation, the supernatant was adjusted to 100 μM $MgCl_2$, 10 μM $ZnSO_4$, 10 mM Tris, pH 7.0, heated rapidly to 80 °C, incubated for 10 min to denature proteins other than AP, and cooled to 0 °C. Two centifugations (30 min at 20 000 rpm in a JA-20 rotor) cleared the solution of precipitated protein. The sample was filtered with a 0.45 μm cellulose filter and passed over a HiTrap Blue column (5 mL, Pharmacia) in the same buffer, and the flow-through was collected. This sample was loaded onto a 40-mL Source Q FPLC column (Pharmacia), washed with 4 column volumes of buffer A (10 mM Tris, pH 7.0, 10 μM $ZnSO_4$, 100 μM $MgCl_2$), and eluted with a linear gradient of 10−120 mM NaCl in buffer A. Following concentration by centrifugation through a filter (50 kD cutoff, Amicon), the sample was loaded onto an FPLC gel filtration column (Superose12, Pharmacia) and chromatographed with 10 mM NaMOPS, pH 8.0, 50 mM NaCl, 100 μM $MgCl_2$, and 10 μM $ZnSO_4$.

A single band was observed by Coomassie Blue staining of SDS/polyacrylamide gels, with an estimated purity of greater than 99%. Protein concentration was determined from the absorbance at 278 nm using $\epsilon_{278,AP} = 6.7 \times 10^4$ M^{-1} cm^{-1} (*57*). Activity with saturating PNPP (k_{cat}) confirmed that the specific activity is the same as that reported previously (*56*, *57*).

Spectroscopic Assays for PNPP and Phenyl Phosphate Hydrolysis. The standard assay conditions for AP (0.1 M NaMOPS, pH 8.0, 0.5 M NaCl at 25 °C) were employed unless otherwise indicated. At low concentrations of AP, it was necessary to add Mg^{2+} and Zn^{2+} ions for full activity, and thus 1 mM $MgCl_2$ and 100 μM $ZnSO_4$ were present in all reaction buffers. For determination of k_{cat}/K_m, the reaction velocity was first order in enzyme concentration and in substrate concentration, each of which were varied by at least 5-fold. Reactions were carried out in quartz cuvettes, and the production of *p*-nitrophenolate was followed at 410 nm. By following the full course of the reaction (≥6 half-lives), excellent data were obtained with as little as 0.1 μM PNPP ($R^2 \geq 0.97$), which yields an absorbance change of 0.0016 at pH 8.0 [$\epsilon_{410} = 1.62 \times 10^4$ M^{-1} cm^{-1} at pH 8.0; *20*]. Reaction of phenyl phosphate was monitored at pH 10 using a value of $\epsilon_{288} = 1190$ M^{-1} cm^{-1} for phenolate. The value of k_{cat}/K_m obtained at pH 10 was corrected to pH 8 using the values of k_{cat}/K_m for PNPP at pH 8.0 and 10.0.

Preparation of ^{32}P-Labeled Alkyl Phosphates. Typically, ^{32}P-γ-labeled ATP (100 μCi; 6000 Ci/mmol) was mixed with 10 μL of 50 mM NaMES, pH 6.0, dried down, and resuspended in 100 μL of neat alcohol. The major ^{32}P-labeled product of solvolysis at 70 °C for 6−12 h was the alkyl phosphate (eq 1). Reaction progress was monitored by thin-layer chromatography on PEI cellulose (Aldrich), developed with 1 M LiCl. The sample was dried under vacuum to remove the alcohol, resuspended in 10 mM triethylammonium acetate, pH 10, loaded onto an anion exchange column (5-mL Source Q; Pharmacia), and eluted with a linear gradient of triethylammonium acetate, pH 11, from 0.01 to 0.5 M. Fractions were analyzed via thin-layer chromatography, and peak fractions of the alkyl phosphate were pooled.

After removal of triethylammonium acetate with 2−3 cycles of evaporating to dryness with a vacuum concentrator and resuspending in water, the sample was brought up to a final volume of 0.1−1 mL in 10 mM NaMOPS, pH 8.0. As ATP and ADP are expected to have similar reactivity with alcohol nucleophiles, as much as half of the alkyl phosphate produced does not contain the ^{32}P label (eqs 1 and 2). It is not necessary to know the exact specific activity as the rate constant k_{cat}/K_m can be determined independent of the total concentration of alkyl phosphate with enzyme in excess, provided that subsaturating concentrations are maintained, and there is no product inhibition under these conditions.

$$\text{AMP}-\text{P}-\text{P}^* + \text{ROH} \rightarrow \text{AMP}-\text{P} + \text{ROP}^* \quad (1)$$

$$\text{AMP}-\text{P} + \text{ROH} \rightarrow \text{AMP} + \text{ROP} \quad (2)$$

k_{cat}/K_m for AP-Catalyzed Hydrolysis of Alkyl Phosphates. The typical reaction volume was 20 μL. Unless otherwise specified, the reaction buffer was 0.1 M NaMOPS, pH 8.0, 0.5 M NaCl, 1 mM $MgCl_2$, 100 μM $ZnSO_4$, and the temperature was 25 ± 1 °C. Enzyme, buffer, and trace ^{32}P-labeled alkyl phosphate (≤100 pM) were mixed and incubated in a circulating water bath. Reactions were usually initiated by addition of substrate, but control reactions in which enzyme was added last gave identical rate constants. Aliquots of 3 μL each (*5*−*6*) were removed from the reaction mixture and quenched in 3 μL of 0.4 M NaOH, 20% glycerol, 0.5% xylene cyanol FF, and kept at 4 °C. Samples were loaded onto 20% polyacrylamide gels [37.5:1 acrylamide to bis-acrylamide; 0.5× TBE buffer; *58*] and electrophoresed 45−55 min at 25 W with 0.5× TBE running buffer. After drying, gels were exposed to Phosphorimager cassettes, and the ratio of counts in the P_i product band and the alkyl phosphate band was determined. A concern with drying polyacrylamide gels is that small molecules could diffuse out of the gel during the drying process. If there were differential loss of the substrate versus the product, then artifactual rate constants would be obtained. The observation that the total amount of radioactivity per lane was constant over the course of the reactions shows that this was not a problem (data not shown). In addition, we directly assayed for the loss of radioactivity via diffusion out of the gel by placing multiple sheets of filter paper beneath the gel during drying; there was no significant transfer of radioactivity to the filter paper (data not shown). Finally, reactions of several alkyl phosphates were followed by both gel electrophoresis and thin-layer chromatography (using polyethyleneimine cellulose, developed with 1 M LiCl), and the same rate constants were obtained in each case (± 10%). Because of the poor resolution of methyl phosphate and P_i under the above gel conditions, reactions of methyl phosphate were monitored by thin-layer chromatography.

Reactions were followed to completion (≥5 half-lives), and rate constants were obtained by nonlinear least-squares fit to a single exponential (Kaleidagraph, Abelbeck software). Reactions were first-order in substrate and enzyme concentration in all cases. Under subsaturating conditions, with [S] ≪ K_m, the Michaelis−Menten equation (eq 3) simplifies to eq 4. The reaction progress curve was plotted as a function of time and the fractional extent of reaction and fit by a single-exponential function (eq 5) to yield k_{obs}, which is the product of the enzyme concentration and the apparent second-order rate constant k_{cat}/K_m (eq 6).

$$V_{obs} = [\text{E}][\text{S}]k_{cat}/(K_m + [\text{S}]) \quad (3)$$

$$V_{obs} = (k_{cat}/K_m)[\text{E}][\text{S}] \quad (4)$$

$$\text{fraction } P_i = 1 - e^{-k_{obs}t} \quad (5)$$

$$k_{obs} = (k_{cat}/K_m)[\text{E}] \quad (6)$$

The observed rate constant was independent of labeled substrate across a concentration range of at least 100-fold for each alkyl phosphate. This demonstrated that substrate was not saturating and that no contaminants were present in the alkyl phosphate stocks that affected AP activity. At very high concentrations of labeled substrate, greater than the concentrations used to obtain k_{cat}/K_m values, inhibition could be detected with some substrates. Addition of unlabeled ethyl phosphate and methyl phosphate up to 1 μM had no effect on the observed rate constant, demonstrating that the K_m for these substrates is greater than 1 μM under these conditions.

pH Dependence for k_{cat}/K_m. The following buffers were used for the indicated pH ranges: NaAcetate (4.4−6.0), NaMES (4.8−6.9), NaMOPS (5.9−7.9), NaCHES (8.0−9.8), and NaCAPS (9.4−11.4). Reactions were typically carried out with 100 mM buffer and 500 mM NaCl, and the pH of the diluted buffer in 500 mM NaCl was measured with a pH meter. Control reactions with 25−200 mM buffer showed that the different buffers that were employed did not exert any significant effects on the AP reaction and that differences in reaction rate reflected the different pH values. Trace concentrations of ^{32}P-labeled ethyl phosphate and 2-fluoroethyl phosphate (≤1 nM) were employed. The rate constants obtained at each pH value for multiple reactions (*6*−*12*) were averaged, and the standard deviations were ≤15% of the average. A model for two rate-controlling ionizations, described by eq 7, was fit to the data.

$$(k_{cat}/K_m)^{obs} = (k_{cat}/K_m)^{max}/(1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+]) \quad (7)$$

To test whether the acidic limb was caused by deprotonation of the substrate or the enzyme, we determined the pH dependence for k_{cat}/K_m with 2,4-dinitrophenyl phosphate (DNPP), which has a lower pK_a value than the alkyl phosphates, under the same conditions as described above for reaction of alkyl phosphates. As the K_m for reaction of DNPP is very low at pH values less than 7, assays were carried out in the presence of an inhibitor to ensure k_{cat}/K_m conditions [*26*, *52*, and data not shown]. The presence of tungstate (1 mM) at a concentration ~1000-fold above the K_i value raised the apparent K_m for DNPP sufficiently to prevent saturation of the enzyme with substrate. As tungstate binding is essentially independent of pH from 4.5 to 7 (see below), the pH dependence of the apparent k_{cat}/K_m reflects that of free enzyme and of DNPP. If binding of tungstate and reactivity of DNPP are both reduced by an enzymatic pK_a of 8, then the apparent k_{cat}/K_m for DNPP in the presence of a given concentration of tungstate will be pH independent above pH 8 as well.

Reactions of DNPP were carried out in quartz cuvettes and the change in absorbance at 360 nm was monitored for

more than five half-lives. Although the absorbance change was small (0.006−0.024), exponential fits to the entire reaction progress curve gave good fits with a correlation coefficient of $R^2 \geq 0.95$, and the standard deviation for replicates was less than 10%. The observed rate constants were linearly dependent on the enzyme concentration between 5 and 25 nM, and independent of the concentration of DNPP between 0.4 and 1.6 μM, as expected for k_{cat}/K_m conditions (eq 6). The observed rate constants were independent of the buffer concentration, which was varied by 4-fold.

pH Dependence for Inhibition by P_i and Tungstate. The standard assays and conditions described above were used to monitor k_{cat}/K_m for hydrolysis of ^{32}P-alkyl phosphates and PNPP in the presence and absence of inhibitor. A range of inhibitor concentrations was employed from at least 5-fold below to 10-fold above the inhibition constant. Nonlinear least-squares fits of the equation for competitive inhibition (eq 8) gave excellent fits in all cases, and the standard error was typically less than 10%. As expected, values of K_i at a given pH that were determined with PNPP as a substrate were the same within error as those determined with an alkyl phosphate substrate. For plotting these data, each observed rate constant, corresponding to the apparent k_{cat}/K_m at a given inhibitor concentration, was normalized by dividing by the rate constant in the absence of inhibitor.

$$(k_{cat}/K_m)^{obs} = (k_{cat}/K_m)/(1 + [I]/K_i) \quad (8)$$

Determination of Alkyl Phosphate pK_a Values. The second ionization constants for inorganic phosphate, ethyl phosphate, 2-fluoroethyl phosphate, and 2,2,2-trifluoroethyl phosphate were determined by direct titration. Solutions containing 10 mM cyclohexylammonium alkyl phosphate and 0.5 M NaCl were stirred at room temperature. Addition of a small quantity of NaOH ensured that all of the alkyl phosphate was in the dianionic form. Titration was accomplished with small aliquots of HCl standard solution and the pH was recorded with a standard pH meter. Titration of the same solution in the reverse direction with NaOH gave the same pK_a values within 0.02 pH units. The pK_a value for inorganic phosphate of 6.43 ± 0.05 is in good agreement with previous measurements under these conditions [6.47 ± 0.02; *59*]. The pK_a values for the alkyl phosphates were as follows: ethyl phosphate, 6.36 ± 0.02; 2-fluoroethyl phosphate, 6.03 ± 0.02; and 2,2,2-trifluoroethyl phosphate, 5.62 ± 0.02 (data not shown).

Viscosity Effects for AP-Catalyzed Reactions. The standard assays described above were used for reactions of PNPP and ^{32}P-labeled alkyl phosphates. Several sugars and organic solvents were previously used to probe the viscosity dependence of AP-catalyzed PNPP hydrolysis (*27*). Although some of the additives gave additional solvent effects that could not be accounted for solely by viscosity effects, sucrose appeared to be well-behaved. We therefore evaluated the effect of added sucrose [0%−35% w/v, corresponding to relative solvent viscosities of $\eta/\eta^o = 1.0-2.85$; *60*]. Data were analyzed as previously described by plotting $(k_{cat}/K_m)^o/(k_{cat}/K_m)$ versus η/η^o (*27*, *61*). The theoretical slope of such a plot is 1.0 for reactions limited by diffusion (*62*). It was necessary to use reagent grade sucrose (Aldrich), as sucrose from other sources contained an inhibitor, presumably P_i, that obscured the effect from viscosity.

In agreement with previous results, we observed a viscosity effect of 1.0 ± 0.1 for k_{cat}/K_m with PNPP as a substrate and with sucrose as a viscosigen, consistent with a rate-limiting binding step or conformational change [*26*, *27*; and data not shown]. Viscosity effects of 0.5 ± 0.1 for ethyl phosphate and trifluoroethyl phosphate were observed (data not shown). A slope of zero is predicted for a nondiffusion controlled step and a slope of unity is predicted for a diffusion-controlled step; it is not clear why an intermediate viscosity effect is observed in these reactions. Nevertheless, the shallower dependence upon the concentration of sucrose for reactions of alkyl phosphates than for aryl phosphates suggests that a different step is rate-limiting. Observation of the same sucrose dependence for alkyl phosphates that span the range of observed reactivity suggests that the same step is rate-limiting for all of the alkyl phosphates (data not shown).

COMPARISON OF AP-CATALYZED AND NONENZYMATIC PHOSPHORYL TRANSFER REACTIONS

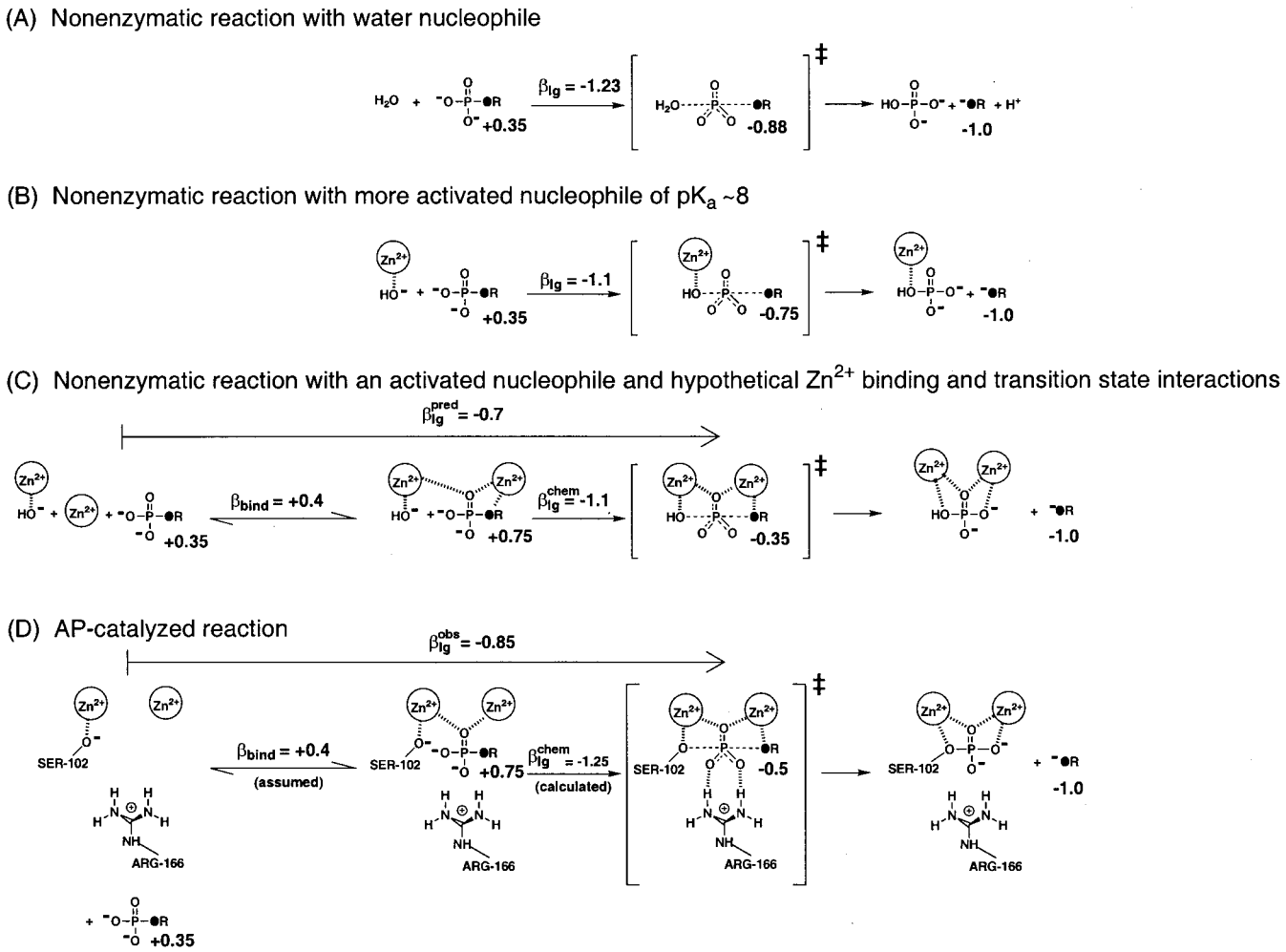
We have estimated the effective charge on the leaving group for a series of nonenzymatic reactions and for the AP-catalyzed reaction to compare the transition state for the AP-catalyzed reaction to that for nonenzymatic phosphoryl transfer (Scheme 2). In the transition state, the bond to the leaving group will be partially broken, and there will be an accumulation of negative charge on the oxygen of the leaving group, with the extent of charge accumulation dependent on the nature of the transition state. The charge accumulation at this position can be partially offset by local interactions. An effective charge derived from LFERs has been used to evaluate the extent of charge accumulation and the possible effects of local interactions [for review, see refs *63*−*65*].[3]

We first define the solution reaction that is analogous to the AP-catalyzed reaction and account for the effect of bound Zn^{2+} ions using literature results. We next describe an analysis of effective charge for the AP-catalyzed reaction assuming a transition state that is analogous to the dissociative solution reaction. Finally, we calculate the effective charge in diester- and triester-like reactions of a phosphate monoester to consider the possibility that the AP-catalyzed transition state is associative. These estimates are used to evaluate the results from the enzymatic LFERs in the Results and Discussion.

β_{lg} for the Solution Reaction Analogous to the AP Reaction. Reactions of the alkyl phosphate dianions that were employed herein have not been systematically studied, but nonenzymatic reactions of aryl phosphates have been extensively characterized using LFERs and kinetic isotope effects [for review, see refs *66* and *67*], and aryl and alkyl phosphates appear to have similar reaction mechanisms (*68*).

[3] The effective charge depicted on the leaving group oxygen is not simply the excess electron density on the atom; for example, the actual charge on the leaving group oxygen in the transition state for Scheme 2C is not −0.35. This value reflects the combination of the electrostatic contribution from this oxygen atom and the electrostatic contribution of the nearby Zn^{2+} ions that is expressed at the position of the oxygen atom, as described in the text.

Scheme 2: Effective Charge and Observed and Predicted Brønsted Value[3]



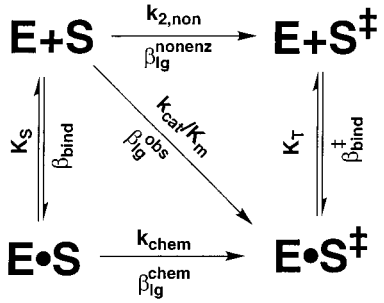
We have therefore used the nonenzymatic hydrolysis of aryl phosphate dianions as a starting point for comparison with the AP-catalyzed reactions of alkyl phosphates (Scheme 2A).

Water, the nucleophile in the nonenzymatic hydrolysis reaction, has a lower pK_a value than the activated serine used in the AP reaction. Comparison of the β_{lg} values for reactions of monosubstituted phosphoryl compounds with different nucleophiles suggests that β_{lg} becomes less negative by ~0.013 per pK_a unit increase in the pK_a of the nucleophile [eq 9; *69*−*71*].

$$p_{xy} = \Delta\beta_{lg}/\Delta pK_{Nuc} \approx 0.013 \qquad (9)$$

The value of $\beta_{lg} = -1.23$ for the hydrolysis of aryl phosphate dianions (*68*), eq 9, and the pK_a values of water and the serine nucleophile in the AP reaction can be used to calculate the value of β_{lg} for a nonenzymatic reaction with a comparably activated nucleophile. As the pK_a value of S102 in the reactive complex (E•S) is not known, we have used the pK_a of a typical Zn^{2+}-bound water molecule [pK_a ~ 8; *72*].[4] This leads to a correction of the nonenzymatic β_{lg} by +0.13 {$\Delta\beta_{lg}$ = $p_{xy} \times \Delta pK_{nuc}$ = 0.013 × [8 − (−1.7)] = 0.013 × 9.7 = 0.13} to yield a β_{lg} value of −1.1 (= −1.23 + 0.13). The smaller value of β_{lg} with a stronger nucleophile indicates a small reduction in the extent of bond cleavage to the leaving group in the reaction with a stronger nucleophile [Scheme 2B; *69*, *73*].

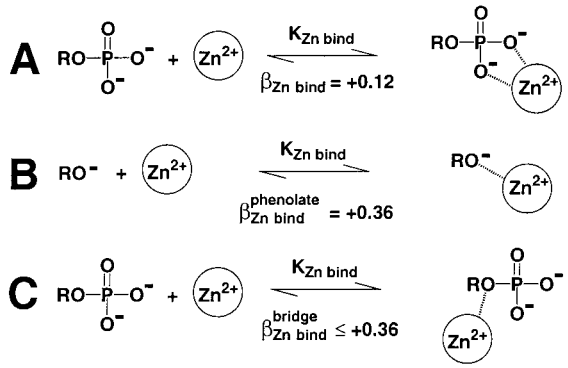
Scheme 3



Definition of $\beta^{\ddagger}_{bind}$ and General Strategy for Analysis of the AP LFER. It is necessary to consider effects on binding and on reaction of the bound substrate to evaluate the observed Brønsted leaving group dependence for k_{cat}/K_m (Scheme 3). Equation 10 describes the relationship between the observed β_{lg} value, β^{obs}_{lg}, and the value of β_{lg} for the chemical step, β^{chem}_{lg}. The magnitude of the binding effects, $\beta^{\ddagger}_{bind}$, must be estimated to interpret the component of the observed Brønsted correlation, β^{obs}_{lg}, that corresponds to the

[4] Although the pH dependence suggests a pK_a of ≤5.5 in the free enzyme, binding of the negatively charged substrate is expected to raise the pK_a by neutralization of the electropositive Zn^{2+} ion. The estimated pK_a value does not greatly affect the subsequent analysis, because the p_{xy} coefficient is small for reactions of phosphate monoesters [*69*−*71*; eq 9].

Scheme 4



extent of bond breaking in the transition state, β_{lg}^{chem} (eq 10). The electrostatic interactions between the positively charged Zn^{2+} ions at the AP active site and the negatively charged phosphate ester substrate are expected to result in a LFER, or Brønsted β value for binding; more strongly electron withdrawing substituents on the substrate's leaving group will decrease the negative charge potential at the phosphoryl group and therefore bind more weakly than phosphate esters with less strongly electron withdrawing substituents. Below we use available literature results to estimate the value of $\beta_{bind}^{\ddagger}$. The estimated values of $\beta_{bind}^{\ddagger}$ and the known values of β_{lg}^{chem} for model phosphoryl transfer reactions are then used to predict the value of β_{lg}^{obs} for dissociative and associative enzymatic mechanisms. As described in the Results and Discussion, these calculations provide the basis for evaluating the leaving group dependence for the AP-catalyzed reaction.

$$\beta_{lg}^{obs} = \beta_{bind}^{\ddagger} + \beta_{lg}^{chem} \quad (10)$$

Literature results were used to estimate the effect of substitutions on Zn^{2+} binding to phosphoryl oxygens of phosphate esters (Scheme 4). We considered Zn^{2+} interactions with the nonbridging phosphoryl oxygens as well as with the bridging oxygen that undergoes bond scission. Sigel and co-workers determined the stability constants for Zn^{2+} complexes of phosphate and phosphonate esters, which reflect the binding of Zn^{2+} to the nonbridging phosphoryl oxygens [*74*, *75*; Scheme 4A]. A plot of the logarithm of the association constant (K_{Zn}) versus the pK_a of the phosphate ester (pK_{ROP}) gives a slope of 0.35, as described by eq 11 (*74*, *75*). This relationship between Zn^{2+} affinity and the pK_a of the phosphate ester can be expressed in terms of the pK_a of the phosphoryl substituent (i.e., the leaving group) by relating the dependence of the pK_a of the phosphate ester (pK_{ROP}) to the pK_a of the leaving group (pK_{ROH}). Bourne and Williams determined this relationship for aryl phosphates [eq 12; *76*]. Combining eq 11 and 12 gives eq 13, the slope of which describes the dependence of the logarithm of the association constant for Zn^{2+} binding upon the pK_a of the substituted phosphate ester, and is defined herein as $\beta_{Zn\ bind}^{nonbridge}$ (= 0.12; Scheme 4A). Larger β values would be required for conversion of a monoester to diester- or triester-like species (see below, "*β_{lg}^{obs} for Reaction via a Diester- or Triester-Like Mechanism at the AP Active Site*").

$$\log (K_{Zn}) = 0.35 pK_{ROP} + p \quad (11)$$

$$pK_{ROP} = 0.36 pK_{ROH} + q \quad (12)$$

$$\log(K_{Zn}) = 0.12 pK_{ROH} + r \quad (13)$$

To estimate the β value for the interaction of a Zn^{2+} ion with the bridging oxygen (Scheme 4C), we used literature results for binding of substituted phenolates to Zn^{2+} in solution [$\beta_{Zn\ bind}^{phenolate} = +0.36$; Scheme 4B; *77*]. The $\beta_{Zn\ bind}^{phenolate}$ value of +0.36 was determined for binding of Zn^{2+} to phenolic oxygens containing a full negative charge.[5] To calculate the value of $\beta_{Zn\ bind}^{bridge}$ in the transition state in which there is only partial bond cleavage, the value of $\beta_{Zn\ bind}^{phenolate}$ must be multiplied by the fractional bond cleavage (eq 14). Fractional bond cleavage is given by the ratio β_{lg}/β_{eq} and will vary from 1 for a fully dissociative transition state to 0 for a fully associative one.[2] The fractional bond cleavage for mono-, di-, and triester-like reactions with a nucleophile of pK_a 8 are 0.81 (= |(−1.1)/1.36|); 0.57 (= |(−1.0)/1.74|); and 0.28 (= |(−0.51)/1.83|), respectively [The values of β_{eq} are from (*76*), the value of β_{lg} for a monoester-like reaction is described in "*β_{lg} for the Solution Reaction Analogous to the AP Reaction*", and values of β_{lg} for diester and triester-like reactions are described in "*β_{lg}^{obs} for Reaction via a Diester- or Triester-Like Mechanism at the AP Active Site*"].

$$\beta_{Zn\ bind}^{bridge} = \beta_{Zn\ bind}^{phenolate} \times (\text{fractional bond cleavage}) \quad (14)$$

We have defined $\beta_{bind}^{\ddagger}$ for binding of phosphate esters to the AP active site as the sum of the β for interaction with the nonbridging oxygen atoms and the β for interaction with the bridging oxygen atom (eq 15). As the exact nature of the binding interactions in the AP transition state are not known and the surrounding active site environment could perturb this value, this analysis provides only an estimate of $\beta_{bind}^{\ddagger}$.

$$\beta_{bind}^{\ddagger} = \beta_{Zn\ bind}^{nonbridge} + \beta_{Zn\ bind}^{bridge} \quad (15)$$

Analysis to Evaluate a Dissociative Mechanism for the AP-Catalyzed Reaction. The nonenzymatic hydrolysis reaction of aryl phosphate dianions is depicted in Scheme 2A. The effective charge on an alkoxide or phenoxide is defined as −1.0, and the effective charge on the alcohol or phenol oxygen is defined as zero. The effect of different leaving groups on the overall equilibrium for hydrolysis provides a value of $\beta_{eq} = +0.35$ relative to zero for the neutral alcohol (*76*). Thus, the effective charge on the leaving group of the substrate in the ground state is +0.35, presumably because of the electron-withdrawing nature of the phosphoryl group. The Brønsted parameter, β_{lg}, describes the change in effective charge in going from the ground state to the transition state. For hydrolysis of aryl phosphates β_{lg} has a value of −1.23 (*68*), yielding an effective charge in the transition state for the leaving group oxygen of −0.88 (= 0.35−1.23; Scheme 2A). The effective charge of −0.88 suggests that bond

[5] This value of $\beta_{Zn\ bind}$ is calculated from the association constants for three substituted salicylaldehydes, and the pK_a values for the phenolic oxygen have not been corrected for the effect of substitutions on the carbonyl oxygen of the aldehyde. Other divalent metal ions show similar β_{bind} values (*77*), and the effect of the neutral aldehydic oxygen on binding of Zn^{2+} is expected to be small as compared to that of the anionic oxygen. Thus, the value of $\beta_{Zn\ bind}$ provides a reasonable estimate for this analysis.

cleavage to the leaving group is almost complete in the transition state, as complete bond cleavage corresponds to an effective charge of −1.0. This large extent of bond cleavage is one of the pieces of evidence for a largely dissociative transition state for nonenzymatic reactions of phosphomonoester dianions (*34*, *66*, *67*).

The increased nucleophilicity of the enzymatic nucleophile will affect the value of β_{lg} for reactions of phosphate monoesters. A Zn^{2+}-coordinated hydroxide ion, which is analogous to the serine alkoxide in the AP active site, is the nucleophile in the reaction in Scheme 2B. The value of $\beta_{lg} = -1.1$ that is predicted for a nucleophile with a pK_a value of 8 (see above, "*β_{lg} for the Solution Reaction Analogous to the AP Reaction*") gives a predicted effective charge of −0.75 in the transition state (0.35−1.1 = −0.75; Scheme 2B). The slight reduction in effective charge in the transition state with a stronger nucleophile relative to a weaker nucleophile (Scheme 2B versus 2A) indicates a small decrease in the extent of bond cleavage to the leaving group in the transition state. Nevertheless, this transition state remains largely dissociative in nature.[2]

We next consider a model in which the two Zn^{2+} ions interact with the oxygens of the phosphoryl group in the transition state (Scheme 2C). We have used a value of $\beta_{bind}^{\ddagger} = 0.4$ (eq 15), crudely estimated from the value of $\beta_{Zn\ bind}^{nonbridge} = 0.12$ that is observed for Zn^{2+} binding to phosphate esters and the value of $\beta_{Zn\ bind}^{bridge} = 0.29$ [$= 0.36 \times (|-1.1/1.36|)$; eq 14] that is estimated from binding of Zn^{2+} to phenolate oxygens in solution (Scheme 4; see above, "*Definition of $\beta_{bind}^{\ddagger}$ and General Strategy for Analysis of the AP LFER*"), to calculate an effective charge of +0.75 (= 0.35 + 0.4) for the Zn^{2+}-bound substrate. For simplicity, we have depicted the same β_{bind} value for binding in the E·S complex as for $\beta_{bind}^{\ddagger}$ in the transition state. This is not likely to be the case, but does not effect the conclusions herein. This is because k_{cat}/K_m monitors the change in going from the ground state free in solution to the transition state on the enzyme; only the transition state binding interactions are relevant to the interpretation of β_{lg} (Scheme 3, diagonal arrow).

The value of $\beta_{bind}^{\ddagger}$ increases the effective charge in the transition state by +0.4 relative to the transition state in Scheme 2B to give an effective charge of −0.35 (= −0.75 + 0.4) in Scheme 2C. The change in effective charge of +0.75 to −0.35 in going from the E·S complex to the transition state gives a value of $\beta_{lg}^{chem} = -1.1$ (= −0.35 − 0.75). This is the same β_{lg} value as for the nonenzymatic reaction depicted in Scheme 2B, verifying that the corrections for binding followed our starting assumption that there is no change in the extent of bond cleavage in the transition state for Scheme 2C.

The effective charge in the transition state can also be used to calculate the predicted value of β_{lg}^{obs}, relative to the ground-state free of any binding interactions, $\beta_{lg}^{pred} = -0.7$ (= −0.35−0.35; Scheme 2C). This value can also be obtained from eq 10, in which the predicted value of β_{lg}^{obs} is designated β_{lg}^{pred} [$= -0.7 = +0.4 + (-1.1)$]. The effective charge in the transition state for this model reaction, which incorporates changes in nucleophilicity and the addition of binding interactions, can now be directly compared to the enzymatic reaction to evaluate whether AP changes the nature of the transition state from that of the nonenzymatic reaction.

The transition state for the AP-catalyzed hydrolysis of alkyl phosphates is depicted in Scheme 2D. The experimental value of $\beta_{lg}^{obs} = -0.85 \pm 0.1$ is similar to the predicted value (see Results and Discussion) and yields an effective charge of −0.5 in the transition state (−0.85 + 0.35 = −0.5). This value suggests a substantial net accumulation of negative charge on the oxygen of the leaving group despite the Zn^{2+} ion interactions in the AP active site, as described in the Results and Discussion.

β_{lg}^{obs} for Reaction via a Diester- or Triester-Like Mechanism at the AP Active Site. We also considered diester- and triester-like mechanisms for AP-catalysis and estimate the value of β_{lg} that would be observed for comparison with the β_{lg} value expected for a dissociative mechanism and the observed value for the AP-catalyzed reaction. As noted above, the kinetic parameter k_{cat}/K_m involves two steps, binding of substrate and reaction of the bound substrate (Scheme 3; eq 10). This analysis assumes that binding of phosphate monoester dianions to AP converts them to species that behave identically to phosphate diester monoanions or neutral phosphate triesters, albeit without formal protonation. Such effects have often been proposed in the literature because of the high density of positive charge in the active sites of AP and other phosphoryl transfer enzymes [*6*, *30*, *32*, *42*; see also ref *47* and refs therein]. We first describe the calculation for a diester-like mechanism and then the analogous calculation for a triester-like mechanism.

To consider the possibility that the phosphate monoester dianion substrate is converted to a diester-like species at the AP active site, we estimate the value of β_{bind} for such a conversion. This β value reflects binding interactions with the nonbridging oxygens of the phosphate esters that convert the monoester dianions into diester-like species. A Brønsted coefficient of +0.38 has been determined for protonation of a phosphate monoester dianion to give the corresponding monoanion for a series of aryl phosphates in solution, and we use this as a minimal estimate for $\beta_{Zn\ bind}^{nonbridge}$. We must also account for the binding interaction with the bridging oxygen, as was done for the dissociative mechanism above. As there is less bond cleavage in the transition state for a diester-like mechanism, as compared to a monoester-like mechanism, the value of $\beta_{Zn\ bind}^{bridge}$ is calculated to be +0.21 ($= +0.36 \times 0.57 = 0.21$; eq 14, see "*Definition of $\beta_{bind}^{\ddagger}$ and General Strategy for Analysis of the AP LFER*" above). Thus, the value of $\beta_{bind}^{\ddagger}$ predicted for Zn-mediated conversion to a diester-like species and a Zn^{2+} interaction with the bridging oxygen at the AP active site is $\beta_{bind}^{\ddagger} = +0.59$ (= +0.38 + 0.21; eq 15).

Next, the contribution from a diester-like transition state must be considered to estimate the value of β_{lg}^{obs} for a diester-like reaction at the AP active site. Hydrolysis of phosphate diester monoanions in solution is characterized by a β_{lg} value of −1.0 (*78*).[6] This value can be corrected for the stronger Zn^{2+}·alkoxide nucleophile in the AP-catalyzed reaction in a manner analogous to that described in "*β_{lg} for the Solution Reaction Analogous to the AP Reaction*" for the monoester reaction above, using a value of $p_{xy} = +0.016$ for reactions of phosphate diesters (*73*, *79*). This leads to a correction of +0.16 for a stronger nucleophile in an

enzymatic diester-like reaction with a nucleophile of pK_a 8 (eq 9; $0.016 \times 9.7 = 0.16$), yielding a corrected β_{lg}^{chem} value of -0.84 ($= -1.0 + 0.16$). Combining the values for binding (conversion of a monoester dianion to a diester monoanion at the AP active site and interaction with the bridging oxygen) and for the chemical step gives a predicted value of $\beta_{lg}^{pred} \approx -0.3$ [$\approx 0.59 + (-0.84) = -0.25$; eq 10] for AP-catalyzed hydrolysis of phosphate monoester dianions via a diester-like mechanism.

The value of β_{lg}^{pred} for reaction via a triester-like mechanism at the AP active site can be calculated in an analogous manner. Conversion of a monoester dianion to a triester involves a β value of $+0.47$ (*76*). This value is used as an estimate for $\beta_{Zn\ bind}^{nonbridge}$. The interaction at the bridge position is estimated from eq 14, $\beta_{Zn\ bind}^{bridge} = +0.1$ ($= 0.36 \times 0.28$; see "*Definition of* $\beta_{bind}^{\ddagger}$ *and General Strategy for Analysis of the AP LFER*" above). These give a value of $\beta_{bind}^{\ddagger} = +0.57$ ($= 0.47 + 0.1$; eq 15) for binding of substrates to AP in a triester-like mechanism. The solution reaction of dimethyl aryl phosphates with phenolate anion has a β_{lg}^{chem} value of -0.51 (*80*). This value was not corrected for a nucleophile of pK_a 8, because phenolate, with a pK_a of 10, has similar reactivity. Combining the values for binding and for the chemical step gives a predicted value of $\beta_{lg}^{pred} \approx +0.1$ [$\approx 0.57 + (-0.51) = 0.06$; eq 10] for AP-catalyzed hydrolysis of phosphate monoester dianions via a triester-like mechanism.

RESULTS AND DISCUSSION

Steady-State Kinetics for PNPP Hydrolysis and P_i Inhibition. The standard assay for AP activity is hydrolysis of PNPP. The good agreement in k_{cat} values from study to study verifies that fully active enzyme is readily obtained. Nevertheless, reported values of k_{cat}/K_m vary much more than expected for experimental error. As described in the introduction, the likely explanation for this variation is product inhibition from generation of P_i in excess of its inhibition constant.

We have reinvestigated the steady-state rate constants for hydrolysis of PNPP. By following the full course of reaction, reasonable data can be obtained with 0.1 to 0.5 μM PNPP, concentrations that are below the K_i for P_i of $\sim$1 μM ($\Delta Abs_{410} = 0.002-0.008$). The observed rate constant for the full reaction is independent of the concentration of PNPP in the range from 0.1 to 0.5 μM, indicating that the K_m for PNPP is >0.5 μM, and that k_{obs} reflects k_{cat}/K_m for PNPP. The results are shown in Table 1 ($O_2NC_6H_4O$-) and are in good agreement with prior studies that also used very low concentrations of substrate (*15*, *16*). Inhibition of the reaction of 0.2 to 0.5 μM PNPP with 0.1 to 100 μM P_i yields an inhibition constant for P_i of 1.1 ± 0.2 μM under these conditions [*81* and data not shown]. Although values of K_i ranging up to 44 μM have been reported for similar solution conditions in several investigations, these studies presumably employed higher concentrations of substrate (*24*, *25*, *56*, *82*, *83*). Other studies that employed submicromolar concentrations of substrate reported similar K_i values of $\sim$1 μM (*15*, *16*), in good agreement with the results reported herein. These findings lend support to the supposition that many prior kinetic studies with AP have been severely affected by P_i inhibition.

Table 1: Values of k_{cat}/K_m for Alkaline Phosphatase-Catalyzed Hydrolysis of Phosphate Esters

leaving group	pK_a[a]	k_{cat}/K_m (M^{-1} s^{-1})[b]
$O_2NC_6H_4O$-	7.1	$(3.3 \pm 0.5) \times 10^7$
C_6H_5O-	10.0	$(2.4 \pm 0.5) \times 10^7$
F_3CCH_2O-	12.4	$(2.0 \pm 0.2) \times 10^7$
$HC{\equiv}CCH_2O$-	13.6	$(2.8 \pm 0.2) \times 10^7$
$N{\equiv}CCH_2CH_2O$-	14.0	$(7.5 \pm 0.8) \times 10^6$
FCH_2CH_2O-	14.3	$(2.3 \pm 0.1) \times 10^6$
$CH_3OCH_2CH_2O$-	14.8	$(1.2 \pm 0.1) \times 10^6$
$CH_2{=}CH_2O$-	15.5	$(1.2 \pm 0.1) \times 10^6$
CH_3O-	15.5	$(1.2 \pm 0.4) \times 10^6$
CH_3CH_2O-	16.0	$(1.5 \pm 0.1) \times 10^5$
$CH_3CH_2CH_2O$-	16.1	$(1.3 \pm 0.2) \times 10^5$
$CH_3CH_2CH_2CH_2O$-	16.1	$(8.6 \pm 0.5) \times 10^4$
$(CH_3)_2CH_2CH_2O$-	16.1	$(4.2 \pm 0.5) \times 10^4$
HO-	15.7	1×10^5[c]

[a] From ref *72*. [b] k_{cat}/K_m is reported per active site; see the Methods for experimental details. The uncertainty is the standard deviation for $\geq$6 independent measurements. [c] k_{cat}/K_m for P_i was calculated from the value of k_{cat} for ^{18}O exchange of 0.1 s^{-1} per active site and the value of the K_d for P_i of 1 μM under similar reaction conditions (*20*, *122*, *123*).

A New Assay for Measuring Alkyl Phosphate Hydrolysis. To study enzymatic catalysis, it is essential to be able to monitor the chemical step. As AP-catalyzed reactions of aryl phosphates are not limited by the rate of the chemical step (*26*−*28*), we considered reactions of less reactive phosphate esters. Alkyl phosphates are inherently much less reactive than aryl phosphates, raising the possibility that their hydrolysis by AP could be limited by the chemical step. To avoid the problem of product inhibition described above and accurately determine the steady-state rate constant k_{cat}/K_m, it was necessary to develop a more sensitive assay.

We synthesized a number of primary alkyl phosphates with ^{32}P labels, taking advantage of the ready availability of ^{32}P-γ-ATP and the solvolysis reaction between ATP and alcohol nucleophiles (*50*). The great sensitivity of ^{32}P-detection has allowed AP-catalyzed reactions to be followed with just $10^{-12}-10^{-9}$ M alkyl phosphate substrate, far below the K_i for P_i of 10^{-6} M. Direct determination of the fraction of reaction, made possible by quantifying both substrate and product, and the ability to follow reactions to completion without build-up of inhibitory concentrations of product presumably gave improved accuracy in determination of the rate constants. Representative data are shown in Figure 2. To ensure that substrate is subsaturating under these conditions, the concentrations of the alkyl phosphate substrates were varied across a wide concentration range (Figure 2C). The observed rate constant (k_{obs}) is independent of the concentration of substrate, as expected for subsaturating concentrations of substrate (eqs 4−6). Thus, the reaction followed is described by the apparent second-order rate constant, k_{cat}/K_m (eq 16).

[6] This Brønsted dependence, with $\beta_{lg} = -1.0$, was obtained from reactions at 100 °C, and no correction was made for temperature [see also ref *127*]. A β_{lg} value of -0.64 has been determined at 39 °C for the reaction of phenolate with methyl aryl diesters (*80*). Correction for a nucleophile of p$K_a = 8$, as described in the text, yields a β_{lg}^{chem} value of -0.67 [$= -0.64 - (0.016 \times 2)$]. Use of this value would give $\beta_{lg}^{pred} = -0.08$ [$= 0.38 + (-0.67)$; eq 10] for the diester-like mechanism and would not change any of the conclusions reached herein.

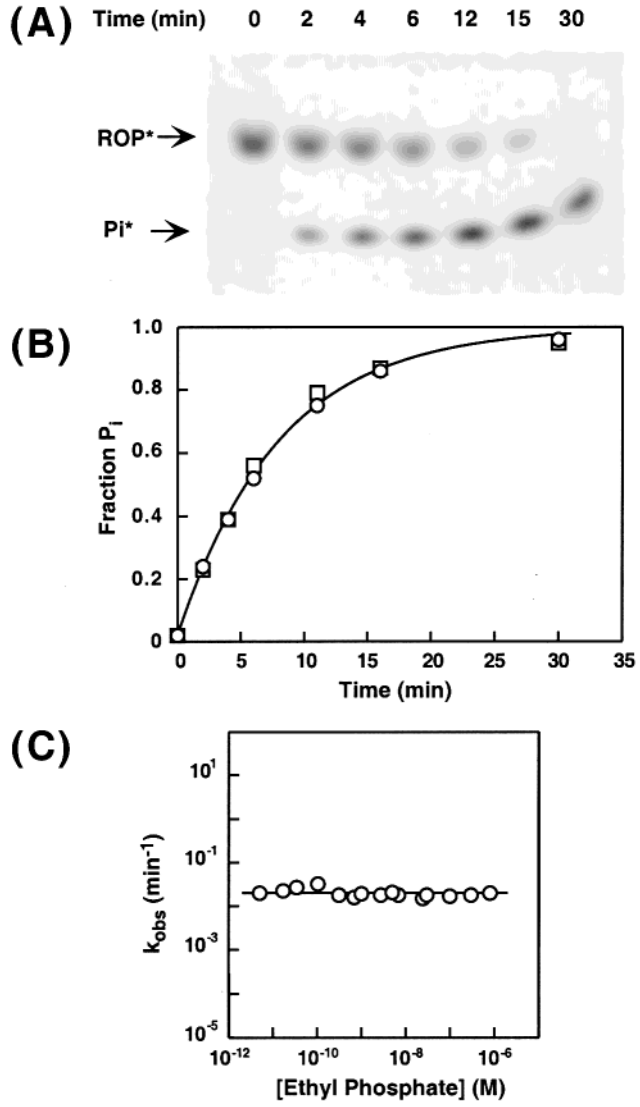
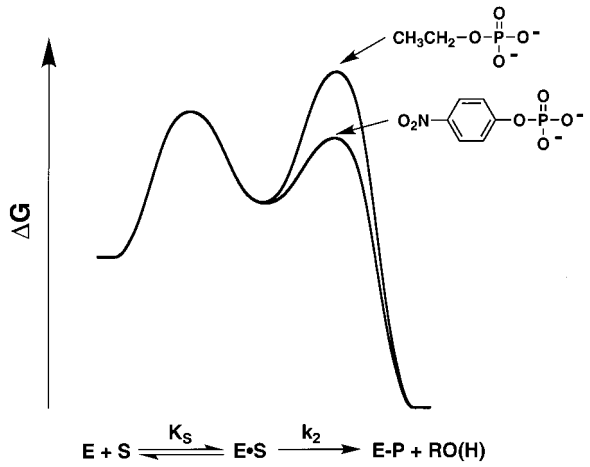


FIGURE 2: Radioactive assay for alkyl phosphate hydrolysis. (A) Time points for the hydrolysis of 2-methoxyethyl phosphate by AP were resolved via PAGE as described in Methods. The relative amounts of ^{32}P in the product (P_i*) and substrate (ROP*) bands were quantified with a Phosphorimager. (B) The reaction progress curve is given for the reaction shown in panel A and for a duplicate reaction. (C) The same rate constant is observed over a wide range of ethyl phosphate concentrations, as expected for k_{cat}/K_m conditions. The concentration of wild-type AP was 1.1 nM. Trace ^{32}P-labeled ethyl phosphate was used and unlabeled sodium ethyl phosphate was added to the indicated concentration (See Methods for details).

$$E + S \xrightarrow{k_{cat}/K_m} (E{\cdot}S)^{\ddagger} \rightarrow E + P_i \qquad (16)$$

For ethyl phosphate, the value of k_{cat}/K_m was 1.5×10^5 M^{-1} s^{-1} (Table 1), orders of magnitude higher than the previously published value of 350 M^{-1} s^{-1} (*30*). A likely explanation for the previous underestimate of k_{cat}/K_m is the presence of considerable concentrations of P_i formed during the initial phase of the reaction. Reaction of ethyl phosphate is ~100-fold slower than reaction of aryl phosphates (Table 1), consistent with a change in the rate-limiting step for alkyl phosphates. It is likely that the slower rate of reaction of ethyl phosphate and other alkyl phosphates is limited by the rate of the chemical step (Scheme 5). Indeed, the steep dependence of reaction rate on the pK_a of the alcohol leaving

Scheme 5



group described below strongly suggests that the chemical step is rate-limiting.

pH Dependence for k_{cat}/K_m. An early step in the basic characterization of a reaction is determination of the pH dependence. This can provide information about the protonation state of the active substrate and enzyme species and serves as a constraint in characterizing the reaction mechanism (*84*, *85*). Previous reports of the pH dependence for AP have employed aryl phosphate substrates (*15*, *21*). In light of the limitations of assays of aryl phosphate hydrolysis detailed above, we have reinvestigated the pH dependence of k_{cat}/K_m for hydrolysis of alkyl phosphates using the radioactive assay. Previously published pH−rate profiles for k_{cat}/K_m with PNPP as a substrate reported apparent pK_a values of 7.1 and 7.3 (*15*, *21*, *22*), far above the expected pK_a of ~5 for ionization of the substrate. This was interpreted as an additional enzymatic ionization. The results below strongly suggest that the previous result was incorrect, presumably for the same reasons discussed above.

The pH dependencies for k_{cat}/K_m with alkyl phosphates follow bell-shaped curves (Figure 3A,B). The solid line in Figure 3A illustrates that both the acidic and basic limbs for reaction of 2-fluoroethyl phosphate have a first-order dependence on the proton concentration, consistent with a single inactivating protonation at acidic pH and a single inactivating deprotonation at basic pH. For k_{cat}/K_m, these kinetic pK_a values could represent ionization of either free enzyme or substrate (*84*, *85*). The data described below support the model with ionization of the substrate to give the dianion (K_a^S) and two ionizations of the enzyme (K_a^{E1} and K_a^{E2}) as depicted in Scheme 6.

Establishing that Phosphate Monoester Dianions Are the Substrates in the AP Reaction. The acidic limb of the pH dependence for the AP-catalyzed hydrolysis of 2-fluoroethyl phosphate is best fit by a pK_a of 5.5 ± 0.1 (Figure 3A). The most likely candidate for this ionization is the substrate, as the second pK_a values of phosphate esters fall in this region. If this were true, the pH−rate profile would be expected to shift to lower pH for alkyl phosphates with lower pK_a values. To test this, we determined the pH dependence for k_{cat}/K_m with 2,2,2-trifluoroethyl phosphate and ethyl phosphate which differ in their respective pK_a values by almost a pH unit (Table 2). The k_{cat}/K_m values for trifluoroethyl phosphate

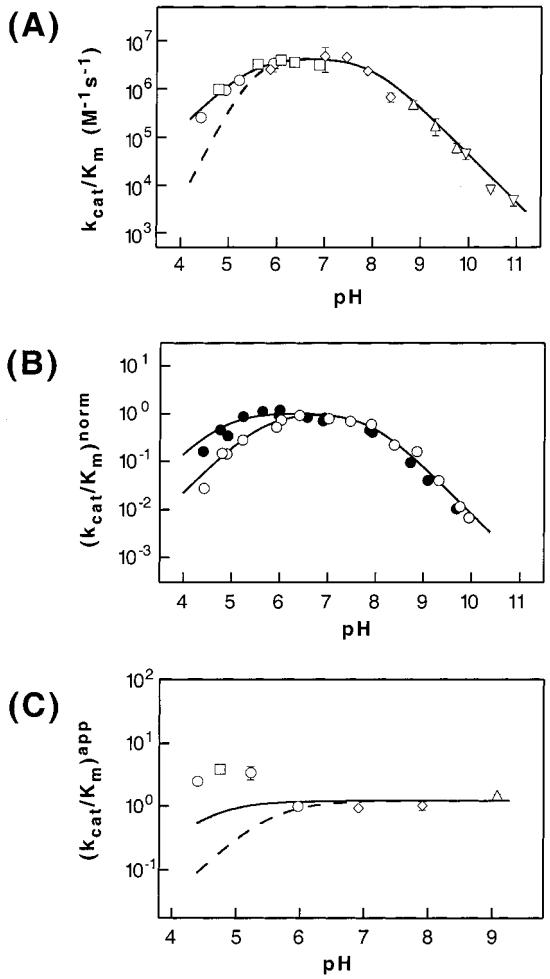


FIGURE 3: pH dependence for k_{cat}/K_m with alkyl phosphate substrates. The solid lines represent the best fit with two rate-controlling ionizations (eq 7). (A) The pH dependence for hydrolysis of 2-fluoroethyl phosphate yields pK_a values of 5.5 ± 0.2 and 8.0 ± 0.2. The dashed line illustrates the poor fit to a second-order dependence upon proton concentration predicted if a second group required for reaction also ionized with a pK_a of 5.5. Buffers were as follows: NaAcetate, ○; NaMES, □; NaMOPS, ◇; NaCHES, △; NaCAPS, ▽. See Methods for details. (B) The pH dependence for hydrolysis of 2,2,2-trifluoroethyl phosphate (●) and ethyl phosphate (○) are compared. To facilitate comparison, the values of k_{cat}/K_m were normalized by dividing by the maximum value of k_{cat}/K_m. The best fits with the model for two rate-controlling ionizations gave pK_a values of 5.0 ± 0.2 and 7.8 ± 0.2 for trifluoroethyl phosphate and 5.6 ± 0.1 and 7.9 ± 0.1 for ethyl phosphate. (C) The pH dependence for hydrolysis of DNPP in the presence of 1 mM tungstate (Symbols for different buffers are the same as panel A; see Methods for experimental details). The dashed line indicates the predicted pH dependence if an enzymatic group with a pK_a value of 5.5 must be deprotonated for full activity and the solid line describes the pK_a value of 4.5 that is expected for DNPP (*124*). Note that the pH independent reactivity of DNPP above pH 8 is consistent with an inactivating pK_a of 8 that affects binding of tungstate and DNPP to the same extent (see Methods).

(closed circles) and ethyl phosphate (open circles) across the pH range from ~4.5−10 were normalized and plotted together to facilitate comparison (Figure 3B). There is a clear shift of the pH−rate profile to lower pH for trifluoroethyl phosphate relative to ethyl phosphate, consistent with the lower pK_a value of trifluoroethyl phosphate (Table 2). This dependence of the kinetic pK_a upon the identity of the substrate suggests that protonation of the substrate is responsible for the decrease in activity at low pH.

Table 2: Summary of pH−Rate Profiles for Alkaline Phosphatase[a]

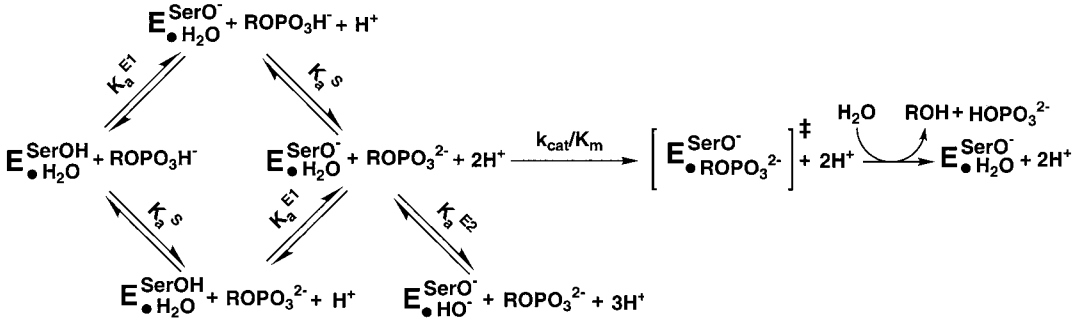
substrate/ inhibitor	solution[b]	enzymatic model 1[c]		enzymatic model 2[d]	
	pK_a	pK_1	pK_2	pK_S	pK_{E2}
ethyl phosphate	6.36 ± 0.02	5.6 ± 0.1	7.9 ± 0.1	6.4 ± 0.1	7.9 ± 0.1
2-fluoroethyl phosphate	6.03 ± 0.02	5.5 ± 0.2	8.0 ± 0.2	6.1 ± 0.1	8.0 ± 0.2
2,2,2-trifluoroethyl phosphate	5.62 ± 0.02	5.0 ± 0.2	7.8 ± 0.2	5.6 ± 0.1	8.0 ± 0.2
PNPP	4.96[e]	≤7[f]	7.9 ± 0.1[f]	≤7[f]	7.9 ± 0.1[f]
DNPP	4.5[g]	≤4.5	8.0[h]	4.3 ± 0.3	8.0[h]
p-nitrophenyl sulfate	≤4[i]	≤7[f]	7.9 ± 0.1[f]	≤7[f]	7.9 ± 0.1[f]
P_i	6.43 ± 0.05	5.5 ± 0.2	8.2 ± 0.2	6.4 ± 0.1	8.2 ± 0.1
tungstate	3[j]	≤4.5	8.0 ± 0.1	≤4.5	8.0 ± 0.1

[a] Compilation of kinetic and thermodynamic pK_a values from this work unless otherwise indicated. See Methods for experimental details. [b] The pK_a value for the first protonation of the indicated substrate or inhibitor, except for P_i, for which the pK_a for the second protonation is given. [c] The kinetic pK_a values were obtained from a simple model for two rate controlling ionizations, with pK_1 controlling the acidic limb, and pK_2 controlling the basic limb (eq 7). As described in the text, it was necessary to consider an additional enzymatic group that has a small stimulatory effect on the binding and reactivity of AP (model 2). [d] Summary of the best fits to the data using the model that includes an additional enzymatic pK_a with a small stimulatory effect. (See the text and the Supporting Information for the details of the model and the fits to the data.) The indicated values of pK_S, the pK_a assigned to the free substrate or inhibitor (Scheme 6), were determined using a fixed stimulatory pK_a of 5.4 and a second enzymatic pK_a of 8.0. (For simplicity the stimulatory pK_a was omitted in Scheme 6; the full scheme is shown in the Supporting Information.) Similarly, the indicated values of pK_{E2}, the pK_a assigned to the free enzyme (Scheme 6), were determined from fits to the data using a fixed stimulatory pK_a of 5.4 and the measured solution pK_a of the substrate or inhibitor. Allowing pK_S and pK_{E2} to vary simultaneously gave the same pK_a values within error (See the Supporting Information). [e] From ref *76*. [f] From ref *81*. [g] From ref *124*. [h] The pH independence up to pH 9 is consistent with the same pK_a of 8.0 affecting both tungstate binding and k_{cat}/K_m for DNPP to the same extent (Figure 3C; see Methods). [i] From ref *125*. [j] From ref *59*.

To further investigate the acidic limb of the pH−rate profile, we determined the pH dependence for 2,4-dinitrophenyl phosphate (DNPP), which has a pK_a value that is ~1 unit lower than trifluoroethyl phosphate and ~2 units lower than ethyl phosphate. If an enzymatic group were responsible for the acidic limb of the pH−rate profile, the pH dependence for k_{cat}/K_m with DNPP would be the same as for the alkyl phosphates. However, if AP preferentially binds and reacts with the dianion, then the pH−rate profile is expected to shift to even lower pH to reflect the lower pK_a of DNPP. The results are shown in Figure 3C. The dashed line shows the poor fit obtained if ionization of an enzymatic group with a pK_a value of 5.5 were responsible for the acidic limb, suggesting that the rate-controlling ionizations for reaction of the alkyl phosphates is not due to an enzymatic group.

The pH−rate profiles of Figure 3 suggest that the substrate binds and reacts as the dianion. Surprisingly though, the kinetic pK_a values observed for reaction of DNPP and for reaction of the alkyl phosphates do not match the pK_a values of these phosphate esters; each is shifted to a lower pK_a by ~0.5 units (Table 2). In addition, there is a 3-fold increase in k_{cat}/K_m at lower pH with DNPP as a substrate. The following controls showed that these discrepancies do not arise from experimental error or specific buffer effects. To

Scheme 6



ensure that the discrepancies did not simply arise from experimental uncertainty, multiple independent pH−rate profiles were obtained for each alkyl phosphate (see Methods). To allow direct comparison of the kinetic and substrate pK_a values, the substrate pK_a values were measured under the same conditions as used for the enzymatic reactions. These experiments confirmed that the kinetic pK_a does shift for different substrates and that the apparent pK_a and the actual substrate pK_a values do differ. Finally, to test whether buffer-specific effects were responsible for this discrepancy, the buffer concentration was varied across the entire pH range; the rate constants were independent of the concentration of buffer (see Methods for details).

We were therefore forced to consider an alternative explanation, that protonation of one or more enzymatic groups over this pH range exert small effects upon reactivity. An enzymatic group with a pK_a of ~5.4 that provides ~5-fold stimulation when protonated would quantitatively account for this upward deviation. This is required to account for the observed stimulation in the pH dependence for reaction of DNPP (Figure 3C). Furthermore, excellent fits are obtained for the pH−rate profiles for all of the alkyl and aryl phosphates, using a common stimulatory pK_a of 5.4 with a 3−6-fold effect, a common deactivating pK_a of 8.0 (see next section), and the independently measured pK_a value of each substrate (Table 2 and Supporting Information). This model and the fits to the data are described in detail in the Supporting Infomation. The model readily accounts for all of the data in Figure 3 and also accounts for the deviation observed in the pH profile for P_i inhibition (Figure 4A; see next section and Supporting Information).

The dependence of the pK_a for k_{cat}/K_m upon the identity of the substrate and the good agreement with the measured pK_a values for each substrate strongly suggest that the observed inflection in k_{cat}/K_m corresponds to the ionization of the phosphate ester substrate to form the dianion. These data provide experimental evidence for the widespread assumption that the dianion is the preferred substrate for AP (Scheme 6; K_a^S).

Evidence for the Anion of S102 (SerO⁻) as the Active Site Nucleophile. As the acidic limb fits a slope of one, corresponding to a single ionization, assignment of this pK_a to the substrate suggests that any enzymatic group that must be deprotonated for activity has a pK_a value of less than 5.5. If full activity were to require deprotonation of an enzymatic group with a pK_a value greater than 5.5, the acidic limb of the pH−rate profile would have a slope of two, to account for loss of a proton from both the substrate and the enzyme. The dashed line in Figure 3A illustrates that a poor fit is

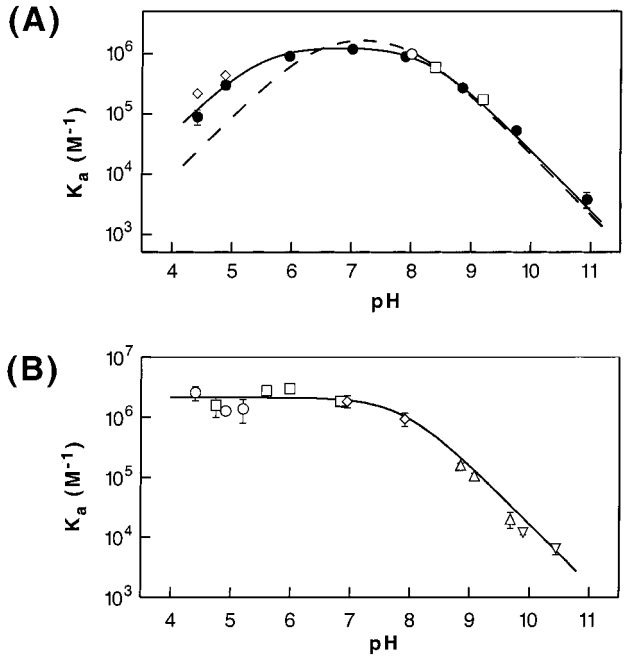


FIGURE 4: The pH dependence for phosphate (A) and tungstate binding (B). The association equilibrium constant, ($K_a = 1/K_d$) is plotted as a function of pH. Values of K_i were determined by inhibition of 2-fluoroethyl phosphate hydrolysis (pH 4.5−9) and inhibition of PNPP hydrolysis (pH 8−11) with [S] ≪ K_m, such that K_i is expected to be the K_d. The observed inhibition constants were the same for both substrates at a given pH, and the average value is reported. (A) Phosphate binding. The observed K_i values for P_i inhibition (◇) were corrected for the amount of covalent complex formed to obtain the value of K_d (●) for the noncovalent complex [see refs *7* and *126*)]. This correction was only significant at pH values less than 5, because the covalent species is much less stable than the noncovalent species at pH values ≥5 [*7*, *126*; unpublished results]. Previous measurements of K_i for P_i with low concentration of PNPP and similar ionic strength condition (□, ○) from refs 15 and 16, respectively, are in good agreement with the results obtained herein. Nonlinear-least-squares fit of the data to a model for two ionizations (eq 7) gave pK_a values of 5.5 ± 0.2 and 8.2 ± 0.2 (solid line). The dashed line shows the fit to a pK_a of 6.4, that corresponds to the pK_a of P_i free in solution (Table 2). (B) Tungstate binding. The different symbols represent the different buffers used, as described in Figure 2. The line represents the best fit of a model for a single ionization and yields a pK_a value of 8.0 ± 0.2.

obtained for this model and suggests that any essential deprotonated enzymatic groups have a pK_a value of less than 5.5. If S102 is deprotonated prior to phosphorylation (Scheme 6; K_a^{E1}), then the pK_a of this residue must be greatly perturbed relative to that of free serine. It has been suggested that the serine alkoxide is stabilized by interaction with Zn_{II} [Figure 1; *6*, *10*]. Although typical pK_a values for Zn-

coordinated water molecules are 8−10 (*72*), there is precedent for considerably larger perturbations of pK_a values in enzyme active sites. For example, pK_a values of 5.6, 6.2, and 6.8 have been assigned for Zn-coordinated waters at the active sites of β-lactamase (*86*), carboxypeptidase A (*87*), and carbonic anhydrase (*88*), respectively. Given the preponderance of positive charge within the AP active site, we favor the model in which the pK_a of S102 is ≤5.5 and the active form is the anion, $SerO^-$.

We further considered the alternative possibilities that the neutral serine is the nucleophile, or that the protonated and deprotonated serine are equally reactive. Reactions of phosphate monoesters are characterized by small values of β_{nuc}, so that the serine alkoxide is expected to be only slightly more intrinsically reactive than the serine alcohol and thus, mechanisms with neutral SerOH as the nucleophile must be considered (*50*, *66*, *67*, *89*−*91*). Although the pH dependence for k_{cat}/K_m does not alone provide sufficient information to infer the protonation state of S102, additional experimental observations support the assignment of $SerO^-$ as the nucleophile in the active form of AP.

We considered the possibility that the pK_a of S102 is 8.0, as reflected in the observed pH dependencies (Figures 3 and 4), and the protonated form of S102, SerOH, is the nucleophile. The pH dependence for the equilibrium between the covalent phosphoserine intermediate and noncovalently bound P_i can be used to discriminate between this model and that of Scheme 6, in which S102 has a pK_a of ≤5.5 and is active as the anion, $SerO^-$. The pH dependence of phosphate inhibition suggests that the inorganic phosphate dianion is the species that is bound by the enzyme (see "*pH Dependence for Binding of Phosphate and Tungstate*" below). It is also known that AP forms a covalent adduct with phosphate at low pH. Equations 17 and 18 depict the two possible equilibria for the formation of the covalent intermediate, with SerOH or $SerO^-$, respectively, as the predominant species present. If the bound form of the enzyme had a protonated serine nucleophile, then the internal equilibrium for formation of the covalent intermediate would be pH independent (eq 17). In contrast, if the P_i-bound form of the enzyme has a deprotonated serine nucleophile, then formation of the covalent intermediate results in the release of a hydroxide ion, and the equilibrium will favor the covalent intermediate at low pH, but not at high pH (eq 18). The results from labeling experiments at pH values from 5 to 8 show that the covalent intermediate increases in stability at low pH, consistent with eq 18 [*7* and refs therein, and unpublished results]. This experimental observation provides evidence against SerOH, with pK_a = 8, as the active nucleophile.[7]

$$E^{SerOH} + HPO_4^{2-} \rightleftharpoons E^{SerOH}_{\cdot HPO_4^{2-}} \rightleftharpoons E^{SerOPO_3^{2-}} + H_2O \quad (17)$$

$$E^{SerO^-} + HPO_4^{2-} \rightleftharpoons E^{SerO^-}_{\cdot HPO_4^{2-}} \rightleftharpoons E^{SerOPO_3^{2-}} + {}^-OH \quad (18)$$

[7] More complicated models were also considered in which the observed pH dependencies for reaction, P_i inhibition, and covalent adduct formation result from a more complex set of protonation states and equilibria. Evidence against such models comes from the observation by NMR of only a single ionic form of noncovalently bound P_i from pH 5−8 [*8* and references therein].

If the protonated and deprotonated serine nucleophile had the same reactivity, then this protonation event would not be reflected in the pH−rate profile, so that the pK_a value could not be obtained. Such a scenario would be surprising as active sites are typically composed of precise networks of interactions and the presence or absence of a proton on the nucleophile would induce rearrangement of active site hydrogen-bonding interactions. The activity of AP toward an alternative substrate also argues against this possibility. In previous work, it was shown that AP can catalyze the hydrolysis of phosphate diesters. This reaction follows the same pH dependence as reactions of phosphate monoesters (*92*). However, reactions of phosphate diesters in solution exhibit considerable dependence upon the pK_a of the nucleophile [e.g., $\beta_{nuc} = +0.31$ for attack of oxyanion nucleophiles upon methyl dinitrophenyl phosphate anion; *79*], whereas reactions of phosphate monoesters do not. The serine alkoxide would therefore be expected to be much more reactive than the neutral serine for this reaction, even if it were not for the phosphate monoester reaction. As no additional inflection is observed in the pH dependence for reaction of a phosphate diester at the AP active site, it is likely that S102 is deprotonated in the free enzyme throughout the experimentally accessible pH range of 5−11. This large perturbation in the pK_a of S102 suggests that the AP active site is well-suited to stabilize the serine alkoxide that is the nucleophile for phosphorylation of the enzyme (k_2; Scheme 1) and the leaving group for dephosphorylation of the enzyme (k_3; Scheme 1).

The apparent pK_a of 8.0 ± 0.2 that controls that basic limb of the pH−rate profile is the same for all three of the alkyl phosphates examined (Figure 3A,B). Higher pK_a values of 8.6 and 9.2 have been previously published for the pH dependence of k_{cat}/K_m with PNPP as substrate (*15*, *21*). However, by employing low concentrations of PNPP, to ensure that substrate is subsaturating and that no significant inhibition by P_i occurs, we have obtained the same pK_a value for the pH dependence of k_{cat}/K_m with PNPP as with the alkyl phosphates [7.9 ± 0.1; *81*]. Furthermore, the same pK_a value is observed for the pH dependence of two alternative AP substrates, a sulfate ester, *p*-nitrophenyl sulfate, and a phosphate diester, methyl 2,4-dinitrophenyl phosphate [Table 2; *81*, *92*]. The former is a poor substrate for AP and the product of the reaction, sulfate, does not bind to AP with appreciable affinity ($K_i \geq 1$ M, unpublished results), and hence product inhibition is not a concern. As discussed above, the serine nucleophile is unlikely to be responsible for this enzymatic ionization. The possible identity of this group with a pK_a of 8.0 ± 0.2 in the free enzyme (Scheme 6; K_a^{E2}) is discussed below.

pH Dependence for Binding of Phosphate and Tungstate. Basic limbs of pH−rate profiles are typically interpreted to implicate a general acid involved in the catalytic mechanism or as an active site group that prevents binding of substrate in the anionic form. Although general acid residues are employed by other phosphatases to protonate the leaving group [for review, see refs *66*], there is no structural evidence that AP has an acidic residue that could carry out this function. Rather, the leaving group is presumably stabilized by direct interaction with Zn_I with protonation of the leaving group occurring from solution (Figure 1). An alternative

model for the basic limb of the pH−rate profiles (Figure 3) is that loss of a proton blocks binding of substrate, thereby decreasing k_{cat}/K_m above the pK_a for the group. An active site group that blocks binding of the substrate might also block binding of P_i and tungstate, as they are analogues of phosphate esters. In addition to testing whether ionization of an enzymatic group effects binding, the pH dependence for binding of P_i provides information about the relative affinities of the different ionization states of P_i, and thus provides information about the active site environment.

Binding of P_i to AP is potentially complicated by the formation of the covalent phosphoserine intermediate. However, equilibrium experiments suggest that at pH values ≥7 the covalent complex is ≥100-fold less stable than the noncovalent complex, and hence the inhibition constant for P_i above pH 7 reflects the dissociation constant from the noncovalent complex [$K_i = K_d$; *7* and unpublished results]. The pH dependence for P_i inhibition was measured using both PNPP and 2-fluoroethyl phosphate and was identical for both substrates (Figure 4A). The logarithm of the association constant follows a bell-shaped pH dependence. This pH dependence reflects ionizations of the free enzyme and P_i inhibitor, analogous to the pH dependence for k_{cat}/K_m discussed above.

The basic limb has a pK_a of 8.2 ± 0.2 that is the same within error as that observed for k_{cat}/K_m. The same pK_a value is also observed for inhibition by tungstate (8.0 ± 0.1; Figure 4B). Observation of the same pK_a value for binding of inhibitors and for reaction of substrates (Table 2) favors a model in which this rate-controlling ionization prevents binding. The structure of the AP active site suggests several candidates for this group that must be protonated for binding. Both a Mg^{2+}-coordinated water and K328 interact with the bound phosphoryl group via a hydrogen bonding network (*6*, *11*, *83*). However, disruption of the Mg^{2+}-binding site by mutation of D153 does not appear to greatly affect phosphate binding (*11*). In addition, although mutation of K328 weakens phosphate inhibition, the observation of weaker binding of P_i to the K328A mutant at pH 10 than at pH 8 suggests that ionization of K328 is not responsible for the apparent pK_a of 8.0 in the wild-type enzyme (*83*, *93*). The side chain of R166 presumably interacts with substrates in its positively charged protonation state. However, the same inhibitory pK_a of 8.0 ± 0.2 is observed with the R166S mutant (unpublished results), indicating that R166 is not responsible for the inhibitory pK_a.

A model that could account for the inhibitory pK_a of 8.0 is that ionization of a zinc-coordinated water molecule, to yield Zn^{2+}·HO^-, blocks the active site and directly competes for coordination of substrate and P_i (Scheme 6; $E^{SerO^-}_{\cdot HO^-}$). Similar inhibitory pK_a values have been observed at high pH for the pH−rate profiles of other two metal ion enzymes, with pK_a values of 10 for an aminopeptidase (*94*) and 9.5 for the zinc-dependent β-lactamase (*86*). Competition by hydroxide ion may be a general characteristic of two metal ion catalysts. The close proximity of divalent metal ions could render it difficult to prevent the nonproductive binding of hydroxide. Direct physical probes will be required to test the validity of this model.

The acidic limb for P_i binding is best fit by a pK_a of 5.5 ± 0.1 (Figure 4A, solid line). The simplest model to explain the weaker binding of P_i below pH 6 is that the dianion binds more tightly than the monoanion. Consistent with tighter binding of the dianion, binding of tungstate, which has a much lower pK_a and thus remains a dianion across the experimentally accessible pH range, does not exhibit weaker binding below pH 6 (Figure 4B). However, the apparent pK_a for P_i inhibition differs from the pK_a of 6.4 for P_i under these conditions by one unit (Table 2). The dashed line shows the best fit to the data using the pK_a values of P_i (6.4) and of the enzyme (8.0) (Figure 4A). As discussed above for the reactivity of alkyl phosphates, the discrepancy between the experimental data and the simplest model appears to be caused by an additional protonation of the enzyme that contributes only slightly (~5-fold) to binding of P_i (Table 2 and Supporting Information). Thus, the data are consistent with electrostatic interactions with the highly charged active site that allow the dianion of P_i to bind more tightly than the monoanion.

It was previously suggested that P_i is preferentially bound as the trianion to AP (*8*). As the active site of AP contains a high density of positive charge, a stronger interaction with the trianion is an attractive possibility. However, the pH dependence for P_i inhibition provides no indication for tighter binding of the trianion (Figure 4A). The inhibitory deprotonation of the enzyme that is competitive with P_i binding is responsible for the log-linear decrease in the affinity constant with increasing pH. If the trianion bound substantially tighter than the dianion to the predominant enzyme species (presumably E^{SerO^-}), then the affinity constant would level off as the fraction of P_i in the trianionic form increased. Appreciable concentration of the trianion exists in solution at pH 11, as the pK_a of P_i is 11.2 under these conditions (*59*), but no such leveling off is observed at this pH (Figure 4A).

The observation that the dianion of P_i appears to bind as tightly or more tightly than the trianion of P_i raises the possibility that the proton of the P_i dianion contributes to binding. A dianionic phosphate ester substrate does not have a proton with which to donate a hydrogen bond, and P_i binds at least 10-fold more tightly than ethyl phosphate (K_d > 10 μM; data not shown). Several hydrogen bonding interactions in the AP active site could stabilize the dianion. Candidates include water molecules that are hydrogen bonded to K328 or Mg^{2+} (*6*, *11*, *83*). Similar preference for protonated forms of P_i has been seen for binding to the periplasmic phosphate binding protein, and this is thought to contribute to the substantial discrimination between P_i and sulfate (*95*, *96*). AP binds SO_4^{2-} at least 10^6-fold more weakly than $HOPO_3^{2-}$ (unpublished results).

It is also possible that the proton is transferred to S102 within the bound complex so that E^{SerOH}·PO_4^{3-} is the predominant species; i.e., E^{SerOH}·PO_4^{3-} is favored over E^{SerO^-}·HPO_4^{2-}. Such an internal proton transfer could not be detected from pH dependencies, as there is no net change in protonation state (*97*). Such a model would require extremely high affinity for the P_i trianion, on the order of picomolar or stronger. Further, the observation of fast AP-catalyzed ^{18}O exchange from bound phosphate, with the value of k_{cat}/K_m similar to that for ethyl phosphate (Table 2) suggests that there is at least a significant fraction of P_i bound as the dianion. This is because reaction of the trianion would require formation of an O^{2-} leaving group. Direct physical

approaches will be required to determine the position of the proton within the complex.

Strong binding of P_i under physiological conditions could provide an evolutionary advantage, perhaps contributing to a role in scavenging P_i. If P_i uptake by the cell has a limited maximal flux, free P_i that diffuses into the periplasm or is released from AP by the hydrolysis of phosphate esters could be lost. It can be estimated that 0.1−1 mM AP is present in the periplasm under growth conditions limited by P_i availability (*98*, *99*), well-above the K_d for P_i binding of ∼1 μM. Thus, AP can efficiently bind to P_i under physiological conditions, acting as a P_i buffer to prevent its loss from the periplasm until it can be bound by or transferred to other proteins and taken up by the P_i transport apparatus.

NATURE OF THE TRANSITION STATE FOR AP CATALYSIS

As transition state theory defines catalysis as stabilization of a reaction's transition state relative to its ground state, an important step in understanding enzymatic catalysis is characterization of the reaction's transition state (*100*, *101*). Depending upon the nature of the enzymatic transition state, specific interactions are expected to have different energetic consequences and different catalytic contributions (*35*, *50*, *102*).

LFERs, or Brønsted correlations, have been commonly employed in the characterization of nonenzymatic transition states. These relationships have also been applied to enzymatic reactions and can provide information about the nature of the transition state when the chemical step is rate-limiting and substituent-specific effects are minor [e.g., refs *26*, *103*−*107*]. As noted in the introduction, several previous studies have employed LFERs to study the transition state for AP, but have arrived at contradictory conclusions. AP-catalyzed hydrolysis of aryl *O*-phosphorothioates exhibits a steep leaving group dependence ($\beta_{lg} = -0.8$), consistent with a largely dissociative transition state (*26*). In contrast, the values of k_{cat}/K_m previously determined for alkyl phosphates gave a smaller value of β_{lg} of −0.2, most simply consistent with a more associative transition state (*30*, *54*). Studies with aryl phosphates, phosphorylated pyridines, and phosphoramidates gave β_{lg} values near zero, but could not provide information about the transition state because the chemical step is not rate-limiting for these substrates (*30*, *52*−*54*). Other studies did not include a sufficient number of homologous compounds to firmly establish the leaving group dependence (*32*, *108*). To address the validity of the earlier studies with alkyl phosphates and to investigate whether phosphorothioate and phosphate esters react via similar transition states at the AP active site, we have reinvestigated the leaving group dependence with a series of primary alkyl phosphates. After describing the experimental results and controls for steric effects, we compare the leaving group dependence for AP to the corresponding solution reactions.

Determination of β_{lg} for k_{cat}/K_m with Alkyl Phosphates. The alkyl phosphate esters investigated span a leaving group pK_a range of ∼4 units and a range of ∼10^3 in k_{cat}/K_m values (Table 1). The k_{cat}/K_m values obtained with the ^{32}P assay herein are 10^3−10^4-fold larger than those reported in the prior study (*30*, *54*). The low values of the previous k_{cat}/K_m determinations are consistent with severe product inhibition

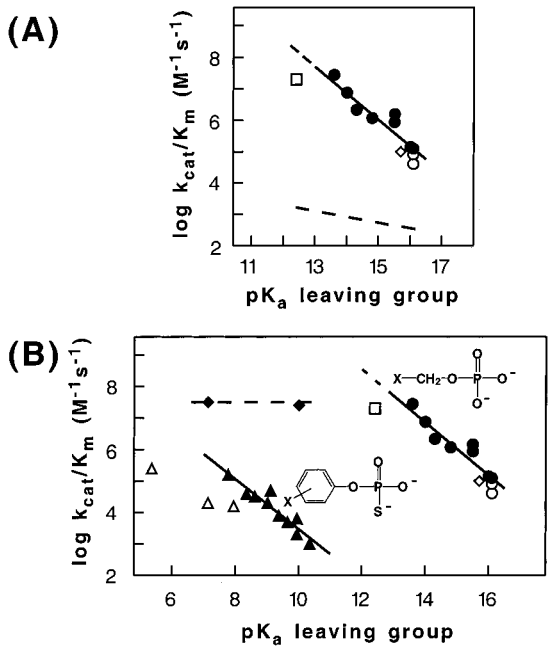


FIGURE 5: Leaving group dependence for reactions of AP. (A) The best fit to the values of log(k_{cat}/K_m) for a series of primary alkyl phosphates (●) yields a slope (β_{lg}) of −0.85 ± 0.1 (data from Table 1). Additional alkyl phosphates were not included in the fit (□, ○; see text for details). The rate constant, k_{cat}/K_d, for AP-catalyzed ^{18}O-exchange under similar conditions is from the literature (◇; see Table 1). The dashed line representing the data from the prior study is included for comparison (*30*). (B) The reactivity of alkyl phosphates from (A) and substrates belonging to other structural classes are compared. The values of log(k_{cat}/K_m) for a series of homologous aryl *O*-phosphorothiotes (▲), with a β_{lg} value of −0.77 ± 0.1 are from ref *26*; several aryl *O*-phosphorothioates were not included in this LFER [△; see ref *26* for details]. The values for two aryl phosphates, PNPP and phenyl phosphate (◆), are from Table 1; the chemical step is not rate-limiting for reactions of aryl phosphates (*26*−*28*).

that could have been caused by P_i contamination or accumulation over the course of the assays. A steep dependence of k_{cat}/K_m on the leaving group pK_a is observed with the improved assay (Figure 5A). This is in sharp contrast to the prior shallow leaving group dependence [Figure 5A, solid vs dashed line; *30*].

If specific interactions between the active site and particular leaving groups affect the reactivity of their cognate esters, the resulting LFER does not accurately report on the properties of the transition state. Thus, a large number of homologous compounds must be compared to construct a LFER that accurately reports on the enzymatic transition state. The 11 alkyl phosphates that we examined have primary alcohol leaving groups with at least one methylene group separating the substituents from the site of cleavage. Alkyl groups of varying chain length that have the same leaving group pK_a react at similar rates (Figure 5A, open circles). Propyl and butyl phosphate have leaving groups with pK_a values similar to ethyl phosphate, and they exhibit values of k_{cat}/K_m that are 0.85 and 0.57 that of ethyl phosphate, respectively (Table 1). Even isobutyl phosphate, which has a branched alkyl chain, exhibits a k_{cat}/K_m value that is only 4-fold lower (Table 1). Furthermore, P_i, with its hydroxide leaving group, has a reactivity similar to ethyl phosphate, an alkyl phosphate with a similar leaving group pK_a value (Table 1). These observations provide no indication of

alcohol leaving group interactions that substantially perturb the enzymatic reactivity.

The two most reactive alkyl phosphates examined, trifluoroethyl phosphate and propargyl phosphate, have values of k_{cat}/K_m that are similar to that for PNPP (Figure 5B). In addition, the k_{cat}/K_m value for trifluoroethyl phosphate deviates by 8-fold from the linear correlation extrapolated from the higher pK_a alkyl phosphate leaving groups (Figure 5A, open square). As k_{cat}/K_m for PNPP is limited by a binding step, we considered the possibility that these alkyl phosphates might also be limited by a binding step. Alternatively, some feature of trifluoroethyl phosphate or its interactions with the AP active site could be responsible for this deviation.

To address the possibility that k_{cat}/K_m for trifluoroethyl phosphate is not limited by the chemical step, we carried out viscosity effect experiments analogous to those reported previously for aryl phosphates and aryl phosphorothioates (*26*, *27*). The viscosity effects for trifluoroethyl phosphate and ethyl phosphate were the same within error (data not shown; see Methods for details), providing no evidence for a different rate-limiting step. Further evidence that the deviation of trifluoroethyl phosphate is due to a substituent specific effect, rather than a change in rate-limiting step, was obtained by examining the leaving group dependence of two AP mutants, R166S and R166K. These mutants exhibit values of k_{cat}/K_m that are $\sim 10^4$-fold less than that of wild-type AP [*47* and unpublished results]. Nevertheless, the k_{cat}/K_m value for trifluoroethyl phosphate with these mutants also deviates from the extrapolated line fit to the data for the other alkyl phosphates just as it does for wild-type AP (unpublished results). As the magnitude of k_{cat}/K_m with trifluoroethyl phosphate is greatly reduced for the R166 mutants, it is unlikely that the binding step is rate limiting. Unfavorable binding interactions with the trifluoroethyl group may be responsible for the deviation, but trifluoroethanol also deviates from other substituted alcohols in two nonenzymatic LFERs (*50*, *73*). Because of these consistent deviations, we have excluded the trifluoroethyl phosphate point from the analysis.

The Brønsted plot for k_{cat}/K_m with the alkyl phosphates gives a slope, or Brønsted value, of $\beta_{lg} = -0.85 \pm 0.1$ [$R^2 = 0.90$; Figure 5A; inclusion of trifluoroethyl phosphate yields a value of $\beta_{lg} = -0.72 \pm 0.1$ ($R^2 = 0.86$)]. Although the error determined by least-squares analysis is only ~ 0.1, the actual error in the β_{lg} parameter could be larger if undetected interactions with individual leaving groups were to bias the slope.

The steep leaving group dependence for hydrolysis of alkyl phosphates is the same within error as the leaving group dependence for hydrolysis of aryl *O*-phosphorothioates [$\beta_{lg} = -0.85$ and -0.77, respectively; *26*; Figure 5B]. There is considerable evidence that the nonenzymatic reactions of phosphorothioate and phosphate monoesters resemble one another (*26*, *109*, *110*), and the similar values of β_{lg} for the AP-catalyzed reactions of these compounds suggest that the enzymatic transition states are also similar. Furthermore, the observation of the same Brønsted correlations for two structurally distinct classes of substrates strengthens the conclusion that the AP-catalyzed reaction has a steep leaving group dependence.

Interpretation of β_{lg}. The AP-catalyzed hydrolysis of alkyl phosphates is highly sensitive to the electron withdrawing properties of the leaving group, with $\beta_{lg}^{obs} = -0.85$ (Figure 5A). This indicates substantial accumulation of negative charge on the leaving group in the transition state and provides evidence against a transition state at the associative extreme, as such a transition state would have little bond breaking and charge accumulation on the leaving group.[2] Additional analysis is required to evaluate to what extent the enzymatic transition state may be altered from the corresponding transition state in solution. As described below, the results of this analysis indicate that the observed value of β_{lg} for AP is consistent with a dissociative transition state, indistinguishable from the nonenzymatic transition state. Nevertheless, a better understanding of the active site environment and additional quantification of the effects of electrostatic interactions on observed LFERs will be required to further test this initial conclusion.

To compare the enzymatic reaction to the nonenzymatic reaction an analogous nonenzymatic reaction should be used. LFERs are sensitive to the pK_a of the nucleophile (*69*−*71*, *73*), so the enzymatic reaction, with its activated serine nucleophile, should be compared to a reference reaction with a nucleophile of the same pK_a. As described in the Methods ("*β_{lg} for the Solution Reaction Analogous to the AP Reaction*"), literature data allow such a correction to be made, giving a value of $\beta_{lg} = -1.1$ for the analogous nonenzymatic reaction (Scheme 2A,B).

Next, electrostatic interactions with active site groups that differ from interactions with water in the solution reaction must be considered. This is important because LFERs do not provide a direct readout of the extent of bonding. Rather, the approach uses a remote electrostatic sensor, a substituent positioned away from the site of bond cleavage and bond formation that gives differential electrostatic interactions in the ground state and transition state. The energetic difference sensed by the substituent depends on all of the differences between the ground state and transition state, not only the extent of bond cleavage in the transition state. For example, hydrolysis reactions of phosphate monoester monoanions follow a shallow leaving relationship [$\beta_{lg} = -0.3$; *68*]. This could be interpreted as indicative of only a small extent of P−O(R) bond cleavage in the transition state, but additional observations strongly suggest that there is actually a large extent of P−O(R) bond cleavage, with the shallow leaving dependence resulting from protonation of the leaving group oxygen in the transition state offsetting the charge that would otherwise accumulate from P−O(R) bond cleavage (*28*, *68*, *111*).

AP contains two Zn^{2+} ions and an Arg side chain in direct contact with the substrate (Figure 1). As noted in the introduction, there has been considerable speculation that phosphoryl transfer enzymes stabilize a more associative transition state than occurs for the nonenzymatic reaction because it was easier to imagine catalytic mechanisms for associative phosphoryl transfer and electrostatic interactions might mimic protonation of the phosphoryl oxygens (*30*, *33*−*46*).[2] Nevertheless, there is no convincing evidence that enzymes are able to effect such a change in the transition state for phosphoryl transfer, and there is considerable evidence against this proposal for several phosphoryl transfer enzymes (*26*, *49*, *66*, *91*, *103*, *112*−*121*). Associative enzymatic transition states would be expected to give a value

of β_{lg} less negative than that observed in solution. However, electrostatic interactions can affect β_{lg}, without changing the covalent bonding in the transition state. It is therefore critical to consider the active site interactions and effects of the local electrostatic environment on β_{lg}.[8]

For AP, we have followed k_{cat}/K_m, corresponding to reaction of free enzyme and substrate (eq 16). Thus, the observed value of β_{lg} includes the effects on binding (β_{bind}) and on reaction of the bound substrate (β_{lg}^{chem}; Scheme 3 and eq 10).[9] The positively charged zinc ions and arginine side chain at the AP active site are expected to make electrostatic interactions with the negatively charged phosphoryl group of the phosphate ester substrate. More strongly electron withdrawing substituents will decrease negative charge potential at the phosphoryl group of their cognate phosphate esters. These phosphate esters are therefore expected to bind less strongly to AP, relative to phosphate esters with less strongly electron withdrawing substituents. Weaker binding by phosphate esters with lower pK_a leaving groups will give a positive value for β_{bind}. Thus, the observed value of β_{lg} is expected to be less negative than the value that accounts for changes in bonding upon reaction of the bound substrate ($\beta_{lg}^{obs} > \beta_{lg}^{chem}$; eq 10). This effect is in the same direction as expected for an increase in associative character for the enzymatic reaction relative to the nonenzymatic reaction [β_{lg}^{obs} (enzymatic reaction) > β_{lg}^{chem} (nonenzymatic reaction); *47*]. Below we estimate the effects on binding in an effort to isolate the bond cleavage component of β_{lg}^{obs}.

The available solution data were used to estimate the effect of active site electrostatic interactions on the observed value of β_{lg}. A value of $\beta_{Zn\ bind}^{nonbridge} = 0.12$ has been observed for the association of Zn^{2+} with the nonbridging oxygen atoms of phosphate monoesters and substituted phosphonates (*74*−*76*), and a value of $\beta_{Zn\ bind}^{phenolate} = +0.36$ has been measured for binding of Zn^{2+} to phenolic oxygens in solution (*77*). These two binding interactions are roughly analogous to the binding interactions between the phosphate ester substrate and the two zinc ions in the AP active site (Scheme 4). These estimates give $\beta_{bind}^{\ddagger} = 0.4$ as a crude estimate for the electrostatic effect that is present in the enzymatic transition state but absent in the nonenzymatic transition state (see "*Definition of* $\beta_{bind}^{\ddagger}$ *and General Strategy for Analysis of the AP LFER*" in the Methods). We can then use eq 10 and the estimated value of $\beta_{lg}^{chem} = -1.1$ for the nonenzymatic reaction with an analogous nucleophile (see Methods "β_{lg} *for the Solution Reaction Analogous to the AP Reaction*") and $\beta_{bind}^{\ddagger} = 0.4$ to estimate the observed value of the enzymatic β_{lg} expected for a dissociative transition state that closely resembles that of the solution reaction. The value predicted for β_{lg}^{obs} of -0.7 (β_{lg}^{pred}; Scheme 2C) is indistinguishable from the observed value of -0.85 ± 0.1 (Scheme 2D; see "*Analysis to Evaluate a Dissociative Mechanism for the AP-Catalyzed Reaction*" in the Methods). In contrast, analogous considerations for more associative reactions resembling that of phosphate diesters or phosphate triesters in solution give less negative predicted values of $\beta_{lg}^{pred} = -0.3$ and $+0.1$, respectively (see Methods "β_{lg}^{obs} *for Reaction via a Diester- or Triester-Like Mechanism at the AP Active Site*").

Summary of LFER Analysis. The results described above demonstrate that the AP-catalyzed hydrolysis of phosphate monoesters proceeds much faster as the pK_a of the leaving group decreases (Figure 5), in contrast to some literature reports (*30*, *54*). This steep leaving group dependence suggests substantial accumulation of negative charge on the leaving group oxygen in the transition state, providing evidence against a largely associative transition state in the enzymatic reaction. Additional analysis reveals that the results are consistent with a dissociative transition state, with the same bonding as in the corresponding solution reaction. In contrast, the results are not consistent with a simple model for conversion of the bound substrate and reaction to resemble that of a phosphate diester or triester. Further evidence against such an increase in associative character for the enzymatic reaction comes from comparison of wild-type AP and a mutant lacking the active site arginine side chain that interacts with the oxygen atoms of the transferred phosphoryl group [Figure 1; *6*, *10*]. Such interactions have been suggested to increase associative character in enzymatic reactions (*30*, *33*−*46*). To test this idea, LFERs for the wild-type and AP mutants lacking R166 were compared. Such direct comparison circumvents the need to estimate the Brønsted coefficent for binding, as was required herein. The observation that removal of R166 does not render the β_{lg} value more negative strongly suggests that the interactions between the active site arginine and the nonbridging phosphoryl oxygens do not increase the associative character of the enzymatic transition state (*47*, *48*).

Nevertheless, the above results do not provide proof of the nature of the transition state for enzymatic phosphoryl transfer. Additional features of the active site environment could prevent β_{lg} from accurately reporting on covalent bonding in the transition state. Most importantly, we do not yet know how to quantitatively consider the effects of electrostatic interactions within active sites and the effects from the surrounding enzyme environment so that the values used to describe the electrostatic interactions in this analysis are necessarily crude estimates. Future challenges include obtaining more robust information about the nature of bound substrates, the magnitude of electrostatic interactions within

[8] A recent paper (*128*) criticized the conclusions from an earlier paper using the LFER approach (*26*). However, these criticisms did not correctly consider the ramifications of binding interactions. Williams and Chin (*128*) stated that the Brønsted relationship for equilibrium phosphoryl transfer needs to be considered to interpret β_{lg} values in terms of the extent of bond cleavage. This approach of obtaining the "effective charge" in the transition state from β_{lg}/β_{eq} is well-established (*63*−*65*, *71*). While powerful for a reaction that occurs in a single solvent environment, this approach does not hold for the AP reactions in which the substrate starts out in aqueous solution in the ground state but is bound within the active site environment in the transition state (i.e., k_{cat}/K_m). As noted in the text, the additional electrostatic interactions must then be considered. These effects were addressed in a previous paper (*26*) and are analyzed more extensively herein.

[9] The complication introduced by the additional binding step could in principle be avoided by following the reaction of the E·S complex directly. However, if rearrangements of the bound substrate are required prior to reaction, then Brønsted values for these rearrangements must be considered. More generally, analysis of k_{cat}/K_m avoids complications from nonproductive binding and allows rate constants to be determined that report on differences between the ground state of free enzyme and substrate and the E·S transition state that is being probed (*129*). For AP, structural analyses suggest rearrangements of bound substrates may be required for reaction (*6*, *9*, *10*). In addition, practical limitations have thus far prevented direct determination of the rate constants for the first chemical step in the AP-catalyzed hydrolysis of phosphate esters (*17*−*19*).

active sites, and more precise information about transition state bonding for both nonenzymatic and enzymatic phosphoryl transfer.

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SUPPORTING INFORMATION AVAILABLE

Description of the model to account for the discrepancy between the solution pK_a values and the pK_a values from the pH profiles, plots of the individual fits, and two summary tables of the parameters used in the fits. This material is available free of charge via the Internet at http://pubs.acs.org.

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