The Nature of the Transition State for Enzyme-Catalyzed Phosphoryl Transfer. Hydrolysis of *O*-Aryl Phosphorothioates by Alkaline Phosphatase†

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ABSTRACT: There has been much speculation that enzymes change the nature of the transition state for phosphoryl transfer from the dissociative transition state observed in solution reactions to an associative transition state at the enzyme's active site. This proposal can be tested by comparing linear free energy relationships (LFERs) for nonenzymatic and enzymatic reactions, provided that the specificity of the enzyme's binding site does not perturb the dependence of rate on the intrinsic reactivity of a series of substrates. The shallow binding groove of *Escherichia coli* alkaline phosphatase (AP) and its wide specificity suggest that this enzyme may be suited for such an approach. A second requirement of this approach is that the actual chemical step is rate-limiting. Comparisons of the reactions of aryl phosphorothioates and aryl phosphates support the previous conclusion that a nonchemical step limits k_{cat}/K_M for reactions of aryl phosphates, but suggest that the chemical cleavage step is rate-limiting for the aryl phosphorothioates. We therefore determined the dependence of the rate of AP-catalyzed cleavage of a series of aryl phosphorothioates on the intrinsic reactivity of the substrates. The large negative values of $\beta_{leaving\ group} = -0.8$ for the enzymatic reaction (k_{cat}/K_M) and -1.1 for the nonenzymatic hydrolysis reaction suggest that there is considerable dissociative character in both the enzymatic and nonenzymatic transition states. Despite the wide specificity of AP, certain substrates deviate from the LFER, underscoring that extreme care is required in applying LFERs to enzymatic reactions. The large negative value of $\beta_{leaving\ group}$ suggests that AP can achieve substantial catalysis via a transition state with dissociative character.

In order to understand how an enzyme catalyzes a reaction, it is necessary to know what the transition state looks like. This is because transition state theory defines catalysis as stabilization of a reaction's transition state relative to its ground state (Wolfenden, 1972; Lienhard, 1973; Jencks, 1975). A basic question then arises: does an enzyme change the transition state from that observed in the corresponding solution reaction, or do enzymes greatly stabilize the solution transition state without significantly altering its nature?[1]

Linear free energy relationships (LFERs),[2] or Brønsted correlations, have provided much of the data characterizing solution transition states, and these relationships have been applied to several enzymatic systems (e.g., Nath & Rydon, 1954; Davis et al., 1988; Kirsch, 1972; for general review of LFERs see Lowry & Richardson, 1981; Jencks, 1987; Williams, 1992). For nonenzymatic reactions of monosubstituted phosphates, such as sugar phosphates, serine or tyrosine phosphates, and the γ-phosphoryl group of ATP, the transition state has been described as dissociative or metaphosphate-like because there is a large amount of bond breaking to the outgoing leaving group and only a small amount of bond formation to the incoming nucleophile (Figure 1A; Benkovic & Schray, 1978; Herschlag & Jencks, 1989a; Thatcher & Kluger, 1989; S. J. Admiraal & D. Herschlag, submitted). As discussed in Herschlag and Jencks (1987, 1990), it has often been suggested that interactions of nonbridging phosphoryl oxygen atoms with active site metal ions and positively charged side chains might render the transition state associative, more analogous to the transition state for reactions of phosphate triesters in solution (Figure 1B).

An intriguing result obtained with *Escherichia coli* alkaline phosphatase (AP) is that $\beta_{leaving\ group}$ for k_{cat}/K_M is -0.2 for a series of aryl phosphates (Chart 1, X = O; Hall & Williams, 1986). This is much less negative than the value for the corresponding solution hydrolysis reaction of $\beta_{leaving\ group} = -1.2$ (Kirby & Varvoglis, 1967),[3] raising the possibility that AP does indeed change the nature of the transition state for phosphoryl transfer, increasing its associative character.

An alternative explanation for the small value of $\beta_{leaving\ group}$ in the enzymatic reaction is that the chemical step was not monitored. The apparent second-order rate constant k_{cat}/K_M includes all reaction steps up to and including the first irreversible step, which for AP is dissociation of alcohol product. This kinetic parameter was used in the LFER for

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[1] It has often been noted that enzymes change the *pathway* of reaction from that observed in solution, for example, by employing covalent or general acid−base catalysis. The comparison between the "nature of the transition state" for the enzymatic and the nonenzymatic reactions is meant to involve analogous reactions, which follow the same reaction pathway, and refers to the extent of bond formation and bond breaking and the electron distribution in the transition states for analogous reactions.

[2] Abbreviations: AP, *Escherichia coli* alkaline phosphatase; LFER, linear free energy relationship; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES 2-(*N*-cyclohexylamino)ethanesulfonic acid; PNPP, 4-nitrophenyl phosphate; Tris, tris(hydroxymethyl)aminomethane.

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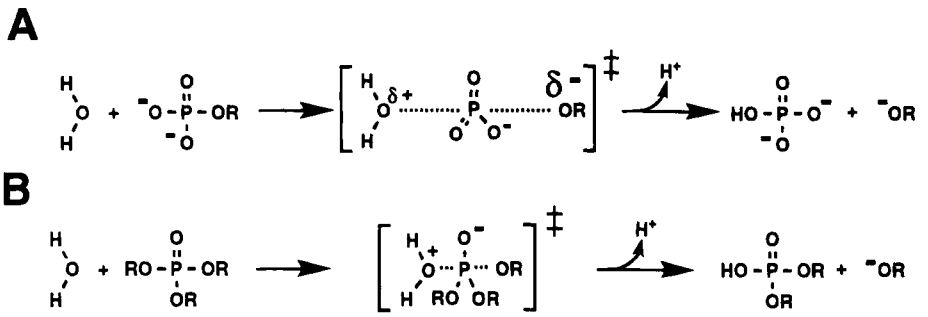
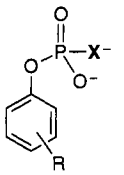


FIGURE 1: Dissociative (A) and associative (B) transition states for phosphoryl transfer reactions in solution. Reaction of a phosphate monoester dianion is used to illustrate the dissociative (or "exploded") transition state (A), and reaction of a phosphate triester is used to illustrate the associative (or "tight") transition state (B). The transition states have the same geometry but different bonding. The small amount of bonding to the incoming and outgoing groups in the dissociative transition state is depicted by the long bonds to the central phosphorus, and the decrease in charge on the PO_3 group being transferred is depicted by the second phosphorus−oxygen double bond.[4] In contrast, the large amount of bonding to the incoming and outgoing groups in the associative transition state is depicted by their proximity to the central phosphorus, and the buildup of charge in the PO_3R_2 group being transferred is depicted by the negative charge on the nonbridging phosphoryl oxygen in the transition state. It is crucial to recognize that the dissociative or associative nature of a transition state represents a continuum of possible transition state bonding schemes, and not simply two discrete alternatives.[5] For simplicity, the transferred phosphoryl group in the dissociative transition state is shown with two full double bonds and in the associative transition state with no double bond character and full negative charge.

Chart 1



X
S (*O*-aryl phosphorothioate)
O (*O*-aryl phosphate)

AP instead of k_{cat} because previous work had shown that k_{cat} is limited by release of inorganic phosphate (at pH >7) or hydrolysis of the phosphoenzyme intermediate (at pH <7) (Reid & Wilson, 1971a; Bloch & Schlesinger, 1973; Bale et al., 1980). However, it was subsequently suggested that binding of substrate or an associated conformational change is rate-limiting for k_{cat}/K_M (Labow et al., 1993), and data supporting this interpretation have recently been obtained from the viscosity dependence and absence of heavy atom isotope effects in the AP-catalyzed hydrolysis of the aryl phosphate PNPP (Hengge et al., 1994; Simopoulos & Jencks, 1994). Thus, the previous LFER did not provide information about the transition state for the chemical step, so that the nature of the transition state for AP-catalyzed phosphoryl transfer remains unresolved.

Despite the difficulties in following the chemical step, AP is an attractive candidate for the LFER approach. The location of the AP active site close to the surface of the enzyme, rather than in deep cleft or pocket, accounts for the ability of AP to efficiently cleave a wide array of phosphorylated substrates (Reid & Wilson, 1971b; Sowadski et al., 1985; Kim & Wyckoff, 1991). Thus, effects on binding and orientation within the active site that often obscure a simple dependence of reaction rate on intrinsic reactivity in enzymatic systems (Kirsch, 1972) may be minimized with AP.

It remained to find a way to follow the chemical step for AP. Previous studies had shown that sulfur substitution for a nonbridging phosphoryl oxygen atom decreases k_{cat}/K_M for AP, suggesting the possibility that the chemical cleavage step is rate-limiting for phosphorothioate ester reactions (Breslow & Katz, 1968; Mushak & Coleman, 1972; Chlebowski & Coleman, 1974). The results herein provide evidence that the cleavage step is indeed rate-limiting for the AP-catalyzed hydrolysis of substituted phenyl phosphorothioates (Chart 1, X = S). The large negative value of $\beta_{leaving\ group}$ obtained for these substrates is consistent with a dissociative transition state for the AP-catalyzed reaction, with little or no change from that for the corresponding solution reaction. The results have also revealed binding idiosyncracies that can obscure LFERs even with a widely specific enzyme such as AP. A large number of substrates within a structural class are required to apply the LFER approach to enzymatic catalysis.

EXPERIMENTAL PROCEDURES

Materials. AP from *E. coli* was purchased from Sigma (type III-L). Phenols were the best available commercial

[3] $\beta_{leaving\ group}$ is the slope of a LFER relating log k, where k is the rate constant for the reaction, and the pK_a for protonation of the leaving group, in this case a series of substituted phenoxides. A large negative value, as observed in the solution reaction, indicates that electron withdrawing substituents greatly stabilize the transition state, which is reflected in a large increase in rate with decreasing pK_a. This suggests that there is considerable development of negative charge on the leaving group in the transition state, and thus considerable bond cleavage (Figure 1A). Conversely, the small value of $\beta_{leaving\ group}$ observed in the enzymatic reaction indicates that substituents with increasing ability to stabilize charge development on the leaving group oxygen atom have only a small effect on the stability of the transition state (i.e., the reaction rate). This is consistent with a small amount of bond cleavage in the rate-limiting transition state, as in an associative transition state (Figure 1B). [See Hall and Williams (1986) and the text for alternative explanations.]

[4] It should be noted that the charge distribution and bonding of the PO_3^- species are not known (Rajca et al., 1987; Horn & Ahlrichs, 1990; Hengge et al., 1994).

[5] As different authors have adopted different terminologies, it is necessary to clearly define the nomenclature used herein: An "associative transition state" (or transition state that is "associative in nature") refers to a transition state that has an *increase* in the total bond order to the incoming and departing groups *relative to the reactant* and a "dissociative transition state" to a transition state that has a *decrease* in the total bond order to the incoming and departing groups. A "more associative transition state" (or "less dissociative transition state") is one in which the bond order to the incoming and departing groups is increased *relative to the reference transition state*. This transition can remain dissociative according to the above definition or, if the increase in associative character is large enough, the transition state can change to an "associative transition state". Thus, the following questions can be asked: Is the transition state for enzymatic phosphoryl transfer dissociative, like the solution reaction, or associative? If the transition state remains dissociative, is it less dissociative at an enzyme's active site than in solution?-

preparations (>98%) from Aldrich or Fluka. The disodium salt of PNPP (Aldrich) was recrystallized from methanol/water, and disodium phenyl phosphate (Sigma) was precipitated from methanol with diethyl ether. Water was double distilled from an all-glass apparatus.

Synthesis. Cyclohexylammonium salts of *O*-aryl phosphorothioates were prepared from thiophosphoryl chloride and the appropriate phenol via the *O*-aryl phosphorodichloridothioates (Tolkmith, 1958) with subsequent hydrolysis. A typical procedure, described for dicyclohexylammonium *O*-phenyl phosphothioate, was as follows. Dry, distilled pyridine (2.5 mL, 32 mmol) was added to freshly distilled thiophosphoryl chloride (9.7 mL, 96 mmol) and dichloromethane (2.6 mL). Phenol (3 g, 32 mmol) in anhydrous diethyl ether (15 mL) was then added dropwise with stirring at 0 °C. The mixture was allowed to warm to room temperature and stirred overnight. The resultant white slurry was filtered, the filtrate was evaporated *in vacuo*, light petroleum ether (20 mL) was added, the mixture was filtered, and the filtrate was evaporated again to yield the crude *O*-phenyl phosphorothiodichloridite (5.9 g, 26 mmol, 81%) as a yellow oil. Aqueous sodium hydroxide (2 N) (13 mL, 26 mmol) was added, and after the suspension was stirred at room temperature for 20 min, the solvent was removed under reduced pressure within 20 min. The residue was redissolved in diethyl ether (40 mL), filtered, and dried (Na_2SO_4). Cyclohexylamine (2.97 mL, 52 mmol) was added to the ether solution, resulting in precipitation of the desired cyclohexylammonium salt. The salts were recrystallized from anhydrous methanol or dissolved in methanol and precipitated by addition of diethyl ether. Yields ranged from 40% to 70% after purification. Structures were confirmed with ^{1}H-NMR and ^{31}P-NMR spectroscopy. Only one ^{31}P-NMR signal was observed (between 42.1 and 43.0 ppm for different esters), except for the 3,4-dinitro-, 3-cyano-, and 3,4-dimethyl-substituted compounds, which contained impurities with chemical shifts in the ^{31}P-NMR spectra between 4.5 and 0 ppm (<10%). These impurities, however, had no effect on reaction rates (see below). Purity was confirmed for several phosphorothioates with thin-layer chromatography [silica gel; acetonitrile−water, 88:12; staining for phosphates, see Seiler (1961); staining for thiophosphates, see Baumler and Rippstein (1961)].

Enzyme Kinetics. Reactions were performed in 0.45 M potassium/CHES buffer, pH 10.0, with 0−6 μM enzyme and 60−400 μM substrate, in the presence of 400 μM phosphate (potassium salt). Inorganic phosphate was included to ensure that the experiments were conducted under k_{cat}/K_M conditions. (This was confirmed, as described in Data Analysis below.) Inorganic phosphate is a strong competitive inhibitor of AP (Applebury et al., 1970). The K_i is ~6 μM (Butler-Ransohoff et al., 1992), so that 400 μM phosphate is expected to render the apparent K_M (K_M^{app}) ~70-fold larger than the intrinsic K_M. The K_M of 130 μM for 4-nitrophenyl thiophosphate (Breslow & Katz, 1968) is therefore expected to give $K_M^{app} \approx 9000$ μM, well above the concentration range of 60−400 μM phosphorothioate substrate used in these experiments. The presence of inorganic phosphate also minimizes deviations from first-order kinetics by product inhibition and complications arising from the possible contamination from inorganic phosphate in substrate solutions.

Kinetic runs were initiated by addition of the enzyme into a cuvette (path length 1 cm) at 25.0 ± 0.3 °C. Reactions were monitored using a UVIKON 9310/9410 spectrophotometer following the appearance of the phenolate chromophore at wavelengths between 279 and 413 nm, where the largest change in absorbance (ΔA) was observed (λ_{max}). (A large change in absorbance was also observed at ~240 nm; use of this wavelength to follow the reaction gave the same rate constants, but was less accurate, perhaps because of interference from protein absorbance.) Measurements for *O*-3,4-dimethyl phosphorothioate were carried out at pH 10.5, where a larger ΔA could be observed, and were corrected by ~20% to pH 10.0 by comparison with the *O*-phenyl phosphorothioate reaction under the same conditions at pH 10.0 and 10.5.

The concentration of active enzyme was determined from its activity using 1 mM PNPP as substrate in 1 M Tris, pH 8.0, as previously described (Garen & Levinthal, 1960). To show that the enzyme retained its activity over the whole course of kinetic runs, the enzyme was incubated under the reaction conditions without substrate. The rate of hydrolysis of added PNPP decreased by <15% even after 4 days, the duration of a run for the slowest reactions followed. In addition, good first-order kinetics were observed for all substrates, suggesting no loss of enzyme activity over the course of the run.

Addition of each of the substituted phosphorothioates (120−200 μM) had no significant effect (<10%) on the rate of PNPP hydrolysis, indicating that no inhibitors were present in stock solutions of phosphorothioates. The same conclusion was reached from the observation that the same pseudo-first-order rate constant for hydrolysis of the substituted phosphorothioates was obtained regardless of the starting concentration. For experiments to test the effect of the viscosity of the medium on k_{cat}/K_M, buffers were prepared with glycerol/water mixtures (0−20% glycerol, v/v). Experiments were carried out as above at 25 °C in 0.45 M potassium/CHES buffer, pH 10.0, in the presence of 400 μM inorganic phosphate. The enzyme concentration was 1 μM for the PNPP (57 μM) and phenyl phosphate (262 μM) reactions and was 8 μM for the *O*-3,4-dinitrophenyl phosphorothioate (110 μM), *O*-4-nitrophenyl phosphorothioate (60 μM), and *O*-phenyl phosphorothioate (177 μM) reactions.

Nonenzymatic Kinetics. Reactions in the absence of enzyme were carried out at 37 °C and ionic strength 1 M (KCl). Hydrolysis was followed at three different pH values (pH 9.9 in 50 mM CHES, and pH 10.5 or 11.2 in 50 mM CAPS) to ensure that the pH-independent hydrolysis rate was measured. Rate constants at the different pH's were equal within experimental error (<5% deviation), indicating that hydrolyses of the phosphorothioate dianions were followed. These slow reactions in the absence of enzyme were followed for an initial, linear period and analyzed as described previously (Kirby & Varvoglis, 1967).

Data Analysis. Enzymatic reactions and the reaction of *O*-3,4-dinitrophenyl thiophosphate in solution were followed for at least three half-lives; an end point was obtained after seven half-lives and multiplied by 1.01. Pseudo-first-order rate constants (k_{obsd}) were obtained from nonlinear least-squares fits (Kaleidagraph, Abelbeck Software) to an exponential curve. The fits were excellent (r >0.999) in all cases. A second-order rate constant k_2 was obtained by plotting k_{obsd}

Table 1: Second-Order Rate Constants (k_{cat}/K_M) for AP-Catalyzed Hydrolysis[a] and First-Order Rate Constants k_w for Nonenzymatic Hydrolysis[b] of Substituted *O*-Aryl Phosphorothioates

substituted phenol	pK_a[c]	λ_{max}[d] (nm)	ΔA[e]	no. of runs	k_{cat}/K_M (M^{-1} s^{-1} × 10^3)	k_w (s^{-1} × 10^{-7})
3,4-$(NO_2)_2$	5.36[f]	403	0.2	5	247 ± 8	313
4-NO_2	7.14	410	2.0	5	20.4 ± 0.6	2.23
4-Cl,3-(NO_2)	7.78[g]	403	0.10	5	148 ± 6	
4-CN	7.95	279	2.0	4	17.5 ± 0.4	
3-(NO_2)	8.35	303	0.05	5	36 ± 9	0.14[h]
3-CN	8.61	321	0.22	4	32.6 ± 0.4	
3-Cl	9.02	292	0.12	5	21.2 ± 0.4	
3-Br	9.11	293	0.29	5	47 ± 1	
4-Cl	9.38	298	0.30	5	7.4 ± 0.2	
3-MeO	9.65	285	0.25	5	5.4 ± 0.3	
4-F	9.95	293	0.15	4	5.7 ± 0.2	
parent	9.95	292	0.16	5	2.2 ± 0.2	
3,4-$(CH_3)_2$[i]	10.36	285	0.25	4	1.1 ± 0.2	
2,3,5-Cl_3	6.43[j]	303	0.2	3	54 ± 2	
2,4,5-Cl_3	6.72[j]	311	0.35	3	41.9 ± 0.01	
2,3,4-Cl_3	6.92[j]	310	0.45	5	18.5 ± 0.6	
2,3-Cl_2	7.71[j]	298	0.7	3	15.7 ± 2.9	
2,4-Cl_2	7.85[j]	306	0.5	4	10.7 ± 0.3	
2-Cl	8.48[j]	293	0.65	4	6.1 ± 0.3	

[a] At 25 °C, pH 10.0, in the presence of 0.45 M potassium/CHES buffer, unless noted otherwise. [b] At 37 °C, pH 9.9−11.2, ionic strength maintained at 1 M. [c] From Jencks and Regenstein (1976), unless otherwise noted. [d] λ_{max} is the wavelength above 260 nm that gave the largest change in absorbance upon reaction. [e] The observed change in absorbance at λ_{max} over the course of the reaction. [f] From Tull and Withers (1994). [g] Calculated from the increments of 4-Cl and 3-(NO_2) groups. [h] Followed at 418 nm. [i] Measured at pH 10.5 and corrected to pH 10.0 (see Experimental Procedures). [j] From Ba-Saif et al. (1986).

against enzyme concentration. Errors were calculated by a linear least-squares fit (Kaleidagraph).

The values of k_2 obtained from the slope of the dependence of k_{obsd} on AP concentration represent $(k_{cat}/K_M)^{app}$, the apparent second-order rate constant for the reaction between enzyme and substrate, based on the following: The disappearance of substrate followed a first-order decay, and the value of k_{obsd} did not change as the initial concentration of substrate was varied, showing that the reactions were first order in substrate. The value of k_{obsd} was linear over a range of AP concentrations (7-fold), indicating that the reaction was also first order in enzyme. The rate constant k_{cat}/K_M includes contributions from all steps up to and including the first irreversible step. As this is release of the phenol product for the AP reactions, dephospohorylation of the enzyme is not monitored. The only chemical step that contributes is phosphorylation of the enzyme, so that "chemical step" is used to refer to the phosphorylation step.

Values of k_{cat}/K_M in Table 1 were calculated from the $(k_{cat}/K_M)^{app}$ values obtained in the presence of inhibitory inorganic phosphate (see above) by assuming a value of 4.6×10^7 M^{-1} s^{-1} for PNPP, as described previously (Snyder & Wilson, 1972; Labow et al., 1993). The literature k_{cat}/K_M values in Figure 2 were also normalized to this value for PNPP. This absolute value does not affect any of the conclusions herein, as they are all based on the relative values of k_{cat}/K_M for different substrates.

RESULTS AND DISCUSSION

Previous results suggested that the AP-catalyzed cleavage of phosphate monoesters is limited by a nonchemical step

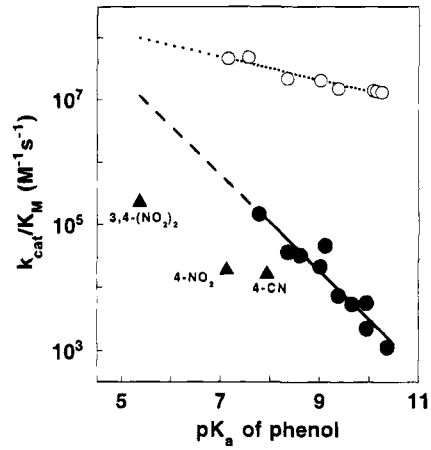


FIGURE 2: Dependence of AP-catalyzed hydrolysis of *O*-aryl phosphorothioates (● and ▲) and aryl phosphates (○) on leaving group ability. The solid line is a least-squares fit to the closed circles and has a slope of $\beta_{leaving\ group} = 0.77 \pm 0.09$ ($r = 0.956$). A linear least-squares fit to *all* of the points for *O*-aryl phosphorothioates (●, ▲) has a slope of $\beta_{leaving\ group} = 0.41 \pm 0.08$ with a considerably worse correlation coefficient ($r = 0.831$, line not shown). Possible origins for deviations of the substrates with large polar *para* substituents (▲) are discussed in the text. The data for reactions of aryl phosphorothioates are from Table 1, and the data for reactions of aryl phosphates with substituents in 3,4- and/or 5-position (○) are from Hall and Williams (1986) (see Experimental Procedures). The aryl phosphates give a slope of $\beta_{leaving\ group} = 0.19 \pm 0.02$.

(Labow et al., 1993; Hengge et al., 1994; Simopoulos & Jencks, 1994). We therefore wanted to slow down the chemical step to render it rate-limiting and allow determination of a LFER that reflects the dependence of the rate of enzymatic cleavage on the intrinsic reactivity of the substrate. A series of substituted phenyl phosphorothioates (Chart 1, X = S) were chosen based on previous work that showed a large decrease in k_{cat}/K_M for AP upon thio substitution (Breslow & Katz, 1968; Mushak & Coleman, 1972; Chlebowski & Coleman, 1974).

AP-Catalyzed and Nonenzymatic Hydrolysis of O-Aryl Thiophosphorothioates. The second-order rate constants, k_{cat}/K_M, for reactions of AP with a series of substituted phenyl phosphorothioates are listed in Table 1. The following strongly suggest that the chemical step limits k_{cat}/K_M for the phosphorothioate substrates, in contrast to the rate-limiting binding or conformational step for phosphate substrates (Labow et al., 1993; Hengge et al., 1994; Simopoulos & Jencks, 1994):

(i) Figure 2 confirms that the phosphorothioate reactions are much slower than those of the corresponding phosphate esters ($\sim 10^2-10^4$) and shows that the leaving group dependence is far more pronounced ($\beta_{leaving\ group} = -0.77 \pm 0.09$ and -0.19 ± 0.02 for the phosphorothioates (solid line) and phosphates (dotted line), respectively). These differences suggest that a different reaction step is rate-limiting for the phosphate and phosphorothioate substrates, and the steep dependence of the rate of the phosphorothioate reactions on intrinsic reactivity (i.e., $pK_{leaving\ group}$) suggests that the chemical step is rate-limiting for these compounds.

(ii) Viscosity effects have been applied to enzymatic reactions to determine whether a physical step (binding or conformational change) or the chemical step is rate-limiting (e.g., Brouwner & Kirsch, 1982; Hardy & Kirsch, 1984). The viscosity dependence of k_{cat}/K_M for the reaction of PNPP with AP provided evidence for a nonchemical rate-limiting

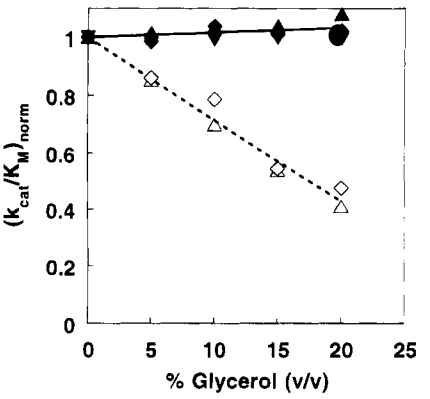


FIGURE 3: Comparison of viscosity effects on the AP-catalyzed hydrolysis of aryl phosphates (open symbols) and *O*-aryl phosphorothioates (closed symbols). Reactions of 4-nitrophenyl phosphate, △; phenyl phosphate, ◇; *O*-4-nitrophenyl phosphorothioate, ▲; *O*-phenyl phosphorothioate, ♦; and *O*-3,4-dinitrophenyl phosphorothioate, ▼, were performed under k_{cat}/K_M conditions as described in Experimental Procedures. Rate constants were normalized by dividing by the rate constants in the absence of glycerol (conditions: 25 °C, pH 10.0, 0.45 M potassium/CHES buffer, 400 μM inorganic phosphate).

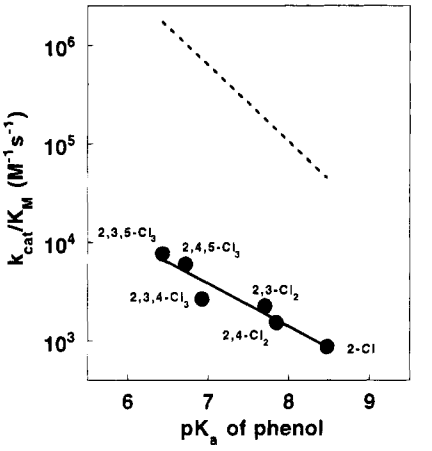


FIGURE 4: Dependence of AP-catalyzed hydrolysis of *O*-aryl phosphorothioates with 2-Cl substituents on leaving group ability. The solid line with a slope of $\beta_{leaving\ group} = 0.43 \pm 0.13$ ($r = 0.957$) is a least-squares fit to the data. The dashed line is Brønsted correlation from Figure 2 for 3- and/or 4-substituted *O*-aryl phosphorothioates ($\beta_{leaving\ group} = -0.77$). Data from Table 1 (conditions: 25 °C, pH 10.0, 0.45 M potassium/CHES buffer, 400 μM phosphate).

step (Simopoulos & Jencks, 1994). Different viscosity dependencies were observed for phosphorothioate and phosphate esters (Figure 3), suggesting that a different step is rate-limiting for these different classes of substrates. The occurrence of a viscosity effect for the phosphate ester reactions contrasted with the lack of a viscosity effect for the phosphorothioate ester reactions (Figure 3) provides further evidence for a rate-limiting binding or conformational step in the phosphate ester reactions and is consistent with a rate-limiting chemical step in the phosphorothioate ester reactions.

(iii) *Ortho* substituents decrease k_{cat}/K_M by ~100-fold for phosphorothioate substrates (Table 1, see also Figure 4 below), but have no significant effect for phosphate substrates (Hall & Williams, 1986), again suggesting that a different step is rate-limiting for these two classes of substrates.

The large negative value of $\beta_{leaving\ group} = -0.77$ for reactions of substituted aryl phosphorothioates (Figure 2) suggests that the enzymatic transition state has significant dissociative character. That is, electron withdrawing substituents provide a large rate enhancement, suggesting that the bond to the leaving group is largely broken in the transition state. Further, the following crude quantitative analysis suggests that a value of $\beta^{obsd}_{leaving\ group} \approx +0.2$ to -0.1 would be predicted if the AP-catalyzed reaction proceeded via an associative transition state similar to that for phosphate triesters. This value is much less negative than the observed value of $\beta_{leaving\ group} = -0.77$. Oxyanion nucleophiles react with phosphate triesters through an associative transition state, with $\beta_{leaving\ group}$ values of $-(0.3-0.6)$ in solution (Khan & Kirby, 1970; Ba-Saif et al., 1989). A $\beta_{leaving\ group}$ value even *less negative* than $-(0.3-0.6)$ would be expected for the AP-catalyzed reaction if it followed an associative, "triester-like" reaction pathway because $\beta^{obsd}_{leaving\ group}$ for the AP reaction was obtained for k_{cat}/K_M, which includes both the binding and chemical steps (Scheme 1). Thus, the observed value of $\beta_{leaving\ group}$ may include contributions from both steps:

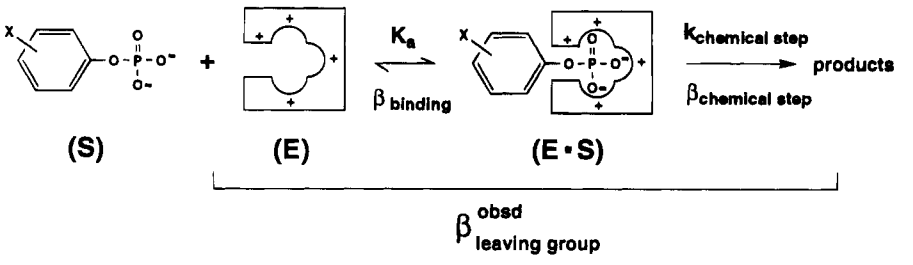
$$\beta_{leaving\ group} = \beta^{obsd}_{leaving\ group} = \beta_{binding} + \beta^{chemical\ step}_{leaving\ group} \quad (1)$$

$$\beta_{binding} = d(\log K_a)/d(pK_{leaving\ group}) \quad (2)$$

$$\beta^{chemical\ step}_{leaving\ group} = d(\log k_{chemical\ step})/d(pK_{leaving\ group}) \quad (3)$$

Because a phosphoryl group in the active site interacts with the positively charged Arg and Zn ions, electron withdrawing substituents on the leaving group could weaken these interactions. This would weaken binding of substrates with leaving groups of lower pK_a so that $\beta_{binding}$ is predicted to be positive. Thus, the observed value of $\beta_{leaving\ group}$ is predicted to represent a least negative limit for the value of $\beta^{chemical\ step}_{leaving\ group}$. *If* the bound monoester were converted into a triester-like species via the electrostatic interactions at the active site, a value of $\beta_{binding} \approx +0.5$ is predicted, from the $\beta_{equilibrium}$ value of +0.5 for conversion of phosphate monoesters to phosphate triesters (Bourne & Williams, 1984a; Ba-Saif & Williams, 1988; Williams, 1992). This value and the values of $\beta^{chemical\ step}_{leaving\ group} = -(0.3-0.6)$ for the corresponding solution reactions give predicted values for $\beta^{obsd}_{leaving\ group}$

Scheme 1



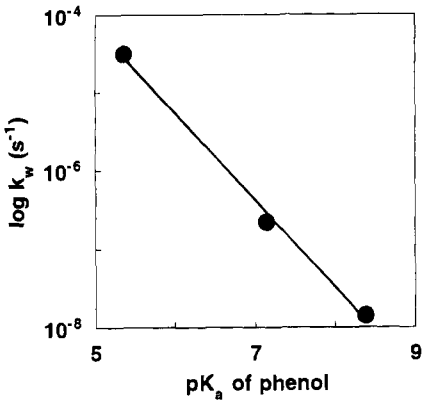


FIGURE 5: Dependence of the uncatalyzed hydrolysis of *O*-aryl phosphorothioate dianions on leaving group ability. The solid line with a slope of $\beta_{\text{leaving group}} = -1.11 \pm 0.07$ is a least-squares fit to the data. Data from Table 1 (conditions: 37 °C, pH 9.5−11.2).

of roughly +0.2 to −0.1 for a fully "triester-like", associative mechanism.

The value of $\beta_{\text{leaving group}}$ for the uncatalyzed hydrolysis of aryl phosphorothioate dianions was determined to allow comparison with the value of $\beta_{\text{leaving group}}$ for the AP-catalyzed reaction. A strong dependence on leaving group pK_a was observed, giving a $\beta_{\text{leaving group}}$ of -1.1 ± 0.09 (Figure 5). This is similar to the value of $\beta_{\text{leaving group}} = -1.2$ for hydrolysis of aryl phosphate dianions (Kirby & Varvoglis, 1967), suggesting a similar dissociative transition state obtains in the uncatalyzed phosphorothioate reactions (Figure 1A). The values of $\beta_{\text{leaving group}}$ of −0.77 and −1.1 for the AP-catalyzed and uncatalyzed reactions of aryl phosphorothioates are similar, further supporting the conclusion that there is significant dissociative character in the transition state for the AP-catalyzed reactions. Nevertheless, these values differ; there are several potential causes of the difference in these values:

(a) The alkoxide of Ser102 appears to be the nucleophile in the AP-catalyzed reaction, with Zn^{2+} coordination used to lower its pK_a to ~8; this is ~10 units higher than that for protonation of the water nucleophile that participates in the nonenzymatic reaction (p$K_a = -1.7$). LFERs in nonenzymatic reactions indicate that $\beta_{\text{leaving group}}$ decreases (i.e., becomes less negative) as the pK_a of the nucleophile increases, with a decrease of ~0.013 predicted for each increase in pK_a unit (Skoog & Jencks, 1984; Herschlag & Jencks, 1989b). This gives a correction for the $\beta_{\text{leaving group}}$ of the model reaction to the nucleophilicity of the enzymatic nucleophile of $\Delta\beta_{\text{leaving group}} \approx 0.013 \times 10 = 0.13$, rendering the $\beta_{\text{leaving group}}$ values for the enzymatic and nonenzymatic reactions more similar: $\beta^{\text{AP}}_{\text{leaving group}} = -0.77$ and $\beta^{\text{Soln,corrected}}_{\text{leaving group}} = -0.97$.

(b) It is possible that the remaining difference results from an actual change in the nature of the transition state relative to the solution reaction. However, *the data herein cannot be taken as support for such a conclusion*, as several other factors could render the observed value of $\beta_{\text{leaving group}}$ less negative. These include experimental uncertainty, direct effects of the aryl substituents on binding and positioning (see below), and indirect effects of the aryl substituents on binding via indirect electrostatic interactions with the active site metal ions [i.e., a value of $\beta_{\text{binding}} = +0.2$ (eq 1) could account for the difference in the observed values of $\beta^{\text{AP}}_{\text{leaving group}} = -0.77$ and $\beta^{\text{Soln,corrected}}_{\text{leaving group}} = -0.97$].

Deviations in and Limitations of LFERs in Enzymatic Reactions. Deviations observed in the LFER for reactions of AP with substituted phenyl phosphorothioates are analyzed below. From these deviations, general guidelines for the application of LFERs to enzymatic reactions are drawn, and these guidelines are used to examine LFERs previously obtained with AP and other phosphatases.

Three phosphorothioates, those with 3,4-$(NO_2)_2$, 4-NO_2, and 4-CN substituents, were omitted from the regression line in Figure 2 (triangles). These compounds, which all contain large polar substituents at the 4-position, are hydrolyzed more slowly than expected from their pK_a, relative to the other substrates. If included with the other compounds, a line with a much worse correlation coefficient is obtained (correlation coeff = 0.83 vs 0.96 with and without these substrates, respectively; Figure 2). The compounds that fall off of the line have the same viscosity effect as a compound on the line (Figure 3, *O*-phenyl phosphorothioate), suggesting that the deviations do not arise from a change to a rate-limiting binding or conformational step. The deviations may arise from unfavorable interactions with the hydrophobic surface of the enzyme.[6] Alternatively, slow reactions with the -NO_2 and -CN substituents at the *para* position could stem from resonance delocalization that decreases charge density on the phenolate oxygen and weakens interaction with the active site Zn^{2+}.

Aryl phosphorothioate substrates with 2-chloro substituents are hydrolyzed much slower than substrates lacking a 2-substituent (Figure 4, the solid vs dashed lines correspond to rate differences of 50−260-fold). Thus, none of the 2-substituted compounds follow the correlation for the compounds lacking this 2-substituent (Figure 4). Though deviations from steric effects are not uncommon, it was surprising that the value of $\beta_{\text{leaving group}}$ obtained from the series of 2-chloro-substituted aryl phosphorothioates was significantly less negative than that obtained from the series of compounds lacking the 2-substitution ($\beta_{\text{leaving group}} = -0.43$ and −0.77 for the 2-Cl and 2-H substrates, respectively, Figure 4, solid vs dashed lines). Comparisons of the following substrate pairs are revealing: Addition of a 3-Cl group to the parent phenyl phosphorothioate gave a 9.5-fold rate increase, whereas the same addition to 2-chlorophenyl substrate gave only a 2.6-fold increase and a *m*-chloro addition to the 2,4-dichlorophenyl substrate (i.e., 5-position) gave a similar 3.4-fold effect. Furthermore, addition of a 4-Cl group to the parent phenyl and 3-nitrophenyl substrates gave 3.3- and 4.1-fold rate increases, whereas the same addition to the 2-chlorophenyl and 2,3-dichlorophenyl substrates gave only 1.7- and 1.2-fold increases.

Thus, the rate effects from the same chloro substitutions are consistently smaller when an *o*-chloro group is present. Most simply, one would have expected the same effect from a 3-Cl or 4-Cl substituent, whether or not a 2-Cl substituent was present. One possible explanation is that the 3- and 4-Cl substituents are tolerated less well in the active site when there is already a Cl substituent at the 2-position, as the

[6] These same substituents at the *meta* position follow the correlation line in Figure 2. The *para* substituents may be especially sensitive due to the idiosyncratic nature of the binding site at this position or because there are fewer possible transition state orientations to accommodate *para* substituents than *meta* and *ortho* substituents. This is because rotation about the C1−O(aryl) bond repositions *meta* and *ortho* substituents within the binding site, but not *para* substiuents.

2-substituent could limit the conformations available for accommodating the 3- and 4-Cl substituents in the active site; this would diminish the rate enhancement expected from effects on intrinsic reactivity and lower the observed value of $\beta_{leaving\ group}$. An amplification of steric effects from one active site mutant upon addition of another active site mutation has been observed with certain dihydrofolate reductase mutants (Huang et al., 1994).

The deviations described above illustrate some of the problems encountered in constructing a LFER for an enzymatic reaction. The following guidelines may be useful in evaluating enzymatic LFERs.

(1) To learn about the nature of the reaction's transition state, the chemical step must of course be monitored.

(2) As in any linear free energy relationship, the compounds compared must be structurally related. The constraints will be more severe for enzymatic reactions than solution reactions, because of the relatively fixed nature of the enzymatic binding site. Thus, LFERs obtained for an AP mutant and a phosphotyrosyl protein phosphatase that include both aryl and alkyl phosphates and a LFER obtained for calcineurin that includes both amino acid phosphates and an aryl phosphate should be viewed with skepticism [see also #4 below (Martin et al., 1985; Zhang & Van Etten, 1991; Han & Coleman, 1995)]. Even a LFER with alkyl phosphates alone can be skewed by the inclusion of structurally disparate alcohol leaving groups (Han & Coleman, 1995).

(3) It is necessary to have many points in a LFER for an enzymatic reaction. Simply using substrates that are closely related structurally is insufficient because there may be idiosyncratic steric or electrostatic interactions with particular substituents that greatly perturb individual points. For example, it was concluded that the transition state for reaction of an AP mutant is associative based on a two point LFER, with PNPP and phenyl phosphate, that gave $\beta_{leaving\ group} = -0.36$ (Butler-Ransohoff et al., 1988). However, the negative deviations obtained herein with the *p*-nitro substituent suggest that this slope may be artificially low, so that the results do not provide evidence for an associative transition state.[7]

(4) Substrates that vary widely in substituent type and position should be investigated when constructing a LFER for an enzymatic reaction. A good correlation line provides one test of a LFER, but this is a necessary but not sufficient criteria for determining whether or not an enzymatic LFER accurately reflects the electronic changes in going from the ground state to the transition state. A simple case of this occurs if there is covariation of the pK_a values and another property of the substrates, for example, hydrophobicity. The slope of the LFER would then reflect contributions from hydrophobicity in addition to the contributions from the electronic changes in the reaction that one is trying to monitor. The following examples underscore this point.

(a) The LFER for general acid catalysis by externally added amines in the reaction of an aspartate aminotransferase mutant provides an elegant example of how such covariation can be dealt with (Toney & Kirsch, 1989). In this case both the volume of the added base and its pK_a appear to be important in reactivity. To sort this out, it was necessary to examine amines with similar pK_a values that varied in size, and amines with similar size that varied in pK_a. In contrast, the LFER from a series of 2-chloro-substituted phenyl phosphorothioates investigated herein (Figure 4) in which the pK_a was lowered by successive addition of chloro substituents does not adequately control for covariation. A similar problem may obtain in the LFER obtained for calcineurin, for which three of the four substrates used were tyrosyl, 3-fluorotyrosyl, and 2,3,5,6-tetrafluorotyrosyl phosphate. [The fourth substrate was PNPP, which is structurally dissimilar (Martin et al., 1985).]

(b) In a recent LFER constructed for k_{cat} for a mutant AP (Ser102Cys), phenyl phosphate, PNPP, and 2,4-dinitrophenyl phosphate were included with a several alkyl phosphates (Han & Coleman, 1995). In addition to the problem noted above of comparing compounds that are not closely related structurally (#2), the 4-NO_2 substituent and 2-substituents both may give inhibitory effects with AP (Figures 2 and 4). If the inhibitory effects increase as the pK_a of the leaving group decreases, the observed value of $\beta_{leaving\ group} = -0.3$ (Han & Coleman, 1995) represents only a least negative limit for the intrinsic $\beta_{leaving\ group}$. Similarly, if a LFER were obtained from the data herein with only phenyl, 4-nitrophenyl, and 2,3,5-trichlorophenyl phosphorothioate (Table 1), a slope of $\beta_{leaving\ group} = -0.38$ would have been obtained (plot not shown). The excellent correlation coefficient of 0.994 would have provided no indication of the inhibitory effects of these substituents. It cannot be concluded from such data that the transition state is primarily associative in character. Minimally, additional compounds need to be investigated.

(c) Finally, the variation of 10−100-fold in k_{cat} and k_{cat}/K_m for cleavage of alkyl phosphates of similar pK_a by a tyrosyl protein phosphatases suggests that structural differences between these substrates are important in determining the reaction rates, in addition to intrinsic reactivity (Zhang & Van Etten, 1991). These compounds should therefore be included in the same LFERs only with the utmost of caution.

(5) Additional features of the enzyme-catalyzed reaction and of the enzymatic active site can change the slopes of LFERs relative to those obtained in solution. Some of these features are as follows:

(a) A small value of $\beta_{leaving\ group}$ in a phosphatase reaction is consistent with a transition state that is primarily associative in character but is also consistent with general acid catalysis or electrostatic catalysis (Hall & Williams, 1986). If k_{cat}/K_m is followed, then electrostatic effects on binding can also come into play. As noted above, positiviely charged active site groups are predicted to render the observed $\beta_{leaving\ group}$ less negative ($\beta_{binding}$, Scheme 1 and eq 1).[8] Thus, a small value of $\beta_{leaving\ group}$ is not alone indicative of a predominantly associative transition state.

[7] Indeed, the similarity of the ratios of $(k_{cat}/K_M)^{PNPP}/(k_{cat}/K_M)^{phenyl\ phosphate}$ for the mutant APs (Arg166K and Arg166Q) to the ratio obtained herein for wild type AP with the corresponding phosphorothioates most simply suggests a similar dissociative transition state [ratio = 10−11 for the Arg166 mutants with the phosphate substrates (Butler-Ransohoff et al., 1988) and 9 for wild type AP with the phosphorothioate substrates (Table 1)].

[8] In some cases, this problem can be circumvented by following k_{cat}, when the chemical step limits k_{cat}, or by following single turnover reactions of the E·S complex. However, k_{cat} and k_{chem} can be affected by nonproductive binding, which arises from the possibility of multiple binding modes, and these values can be affected differently by different substituents. Multiple binding modes might be expected to be especially problematic for enzymes such as AP that have shallow hydrophobic binding sites.

(b) Conversely, values of $\beta_{\text{leaving group}}$ are rendered more negative in low dielectric media because electrostatic interactions are dampened less. Thus, it is conceivable that comparison of $\beta_{\text{leaving group}}$ for enzymatic and aqueous reactions could overestimate the extent of bond cleavage in the enzymatic reaction. However, the binding site of AP is on the enzyme's surface and is solvent accessible, so such an effect would appear to be unlikely for the AP reaction.

The wide variety of substituent types and substituent position for the LFER for cleavage of aryl phosphorothioates by AP herein allowed identification of substituents that appear to give inhibitory effects so that substrates with those substituents could be omitted from the LFER (Figures 2 and 4). This wide variation and the high correlation coefficient suggest that the large negative $\beta_{\text{leaving group}} \approx -0.8$ obtained (Figure 2) provides a reasonable estimate for the dependence of rate on leaving group ability. The simplest and most straightforward interpretation of this LFER is that the transition state for the enzymatic reaction is largely dissociative in nature, as discussed above. Nevertheless, these correlations cannot be proven to be free of steric and electrostatic influences on binding and positioning. In addition, it has not been ruled out that AP renders the transition state for reactions of phosphate esters associative in nature, while not significantly perturbing the transition state for the slower reacting phosphorothioate esters.

We would like to emphasize that the simplest model derived from these data is that the AP catalyzes its hydrolysis reactions via a dissociative transition state. We are aware of no data that contradict this model.

Nonenzymatic Thio Effects and Their Application to Enzymatic Reactions. Thio substitution *accelerates* the nonenzymatic hydrolysis of phosphate monoester dianions. A thio effect ($= k_w^O/k_w^S$) of $^1/_{12}$ was obtained for PNPP and the corresponding phosphorothioate (not shown), in agreement with the previously published value of $^1/_{10}$ (Domanico et al., 1986). In contrast to the rate increase upon thio substitution for the phosphate monoester reactions, reactions of phosphate triesters are slowed upon thio substitution (Ketelaar et al., 1952; Heath, 1956a,b; Cox & Ramsey, 1964; Fanni et al., 1986). The opposite effect of thio substitution for nonenzymatic reactions of phosphate mono- and triesters provides additional support for the view that the transition states for these reactions differ in their nature (see Figure 1 and Herschlag et al., 1991).

One might therefore consider using thio effects to probe the nature of transition states for enzymatic reactions, analogous to the use of isotope effects (Breslow & Katz, 1968). However, differences between the interactions of oxygen and sulfur with an enzyme, arising from differences in size, bond length, charge distribution, hydrogen bonding ability, and metal ion affinity, can obscure the differences arising from effects on intrinsic reactivity and therefore prevent the thio effect from revealing the nature of the transition state. Indeed thio effects have been observed in enzymatic reactions at positions that are not involved in the chemical reaction. This proves that enzymes can be sensitive to differences between phosphates and phosphorothioates that are distinct from their differences in intrinsic reactivity. The differential effects of *pro-*R_P and *pro-*S_P thio isomers of phosphate diesters observed in enzymatic, but not nonenzymatic reactions provide additional evidence for effects from factors other than intrinsic reactivity (see Knowles, 1980; Herschlag & Jencks, 1990; Almer & Stromberg, 1991; Herschlag et al., 1991, and references therein). Thus, the decreased rate for the AP-catalyzed reaction upon thio substitution (Breslow & Katz, 1968; Figure 2) does not provide evidence for an associative, triester-like transition state. Nevertheless, thio effects can be useful in distinguishing mechanistic possibilities in some enzymatic cases (Herschlag, 1994).

IMPLICATIONS AND CONCLUSIONS

A necessary step toward a complete understanding of enzymatic catalysis is obtaining an understanding of a reaction's transition state. Comparison of the transition states for the enyzme-catalyzed and uncatalyzed reactions can be informative about mechanisms of transition state stabilization.

Metaphosphate monoanion (PO_3^-) was first proposed as an intermediate in reactions of phosphate monoesters in 1955 (Barnard et al., 1955; Butcher & Westheimer, 1955; Kumamoto & Westheimer, 1955). Subsequent results from several types of physical organic experiments provided strong evidence for a transition state with dissociative character, as shown in Figure 1A (for review, see Benkovic & Schray, 1978; Herschlag & Jencks, 1989a; Thatcher & Kluger, 1989). Thus, the *transition state* is metaphosphate-like, though more recent work has provided evidence against the occurrence of a discrete metaphosphate *intermediate* (Bourne & Williams, 1984b; Skoog & Jencks, 1984; Herschlag & Jencks, 1989a). [Metaphosphate monoanion does not appear to have a significant lifetime in aqueous solution, even in the absence of strong nucleophiles (Herschlag & Jencks, 1989a).]

Even though the reactions of phosphate monoesters free in solution proceed via a dissociative, metaphosphate-like transition state, metal ion complexation with the nonbridging phosphoryl oxygen atoms could in principle render the transition state more associative. This might arise if the metal ion withdraws electrons from the phosphorus, thereby increasing its susceptibility to nucleophilic attack, as has been suggested on a number of occasions (e.g., Kirby & Jencks, 1965; Williams & Naylor, 1971; Benkovic & Schray, 1973; Hassett et al., 1982; Mildvan & Fry, 1987). This proposal has been tested in model systems: *Coordination by metal ions (Mg^{2+} or Ca^{2+}) caused no significant increase in the associative character of the transition state in nonenzymatic reactions* (Herschlag & Jencks, 1987; Herschlag & Jencks, 1989b; S. J. Admiraal and D. Herschlag, submitted).

Phosphoryl groups bound at enzyme active sites typically have extensive interactions with electron-deficient groups, including metal ions, positively charged side chains, and hydrogen bond donors. For example, the active site of AP contains two Zn ions and an Arg side chain, which have been suggested to have transition state interactions with the nonbridging phosphoryl oxygen atoms and could affect the nature of the transition state (Butler-Ransohoff et al., 1988; Kim & Wyckoff, 1991; Figure 6). However, the large negative value of $\beta_{\text{leaving group}} \approx -0.8$ obtained from a LFER for cleavage of aryl phosphorothioates catalyzed by AP suggests that the transition state remains predominantly dissociative in character. Thus, AP appears to be able to achieve substantial catalysis via a dissociative transition state.

Potential mechanisms for stabilization of a dissociative transition state include positioning of the reactants and stabilization of the buildup of negative charge on the leaving

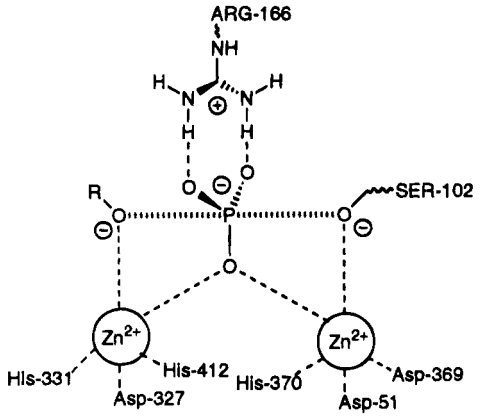


FIGURE 6: Proposed transition state interactions in the first step of the reaction catalyzed by AP. [Adapted from Steitz and Steitz (1993), with modifications.]

group (see Herschlag & Jencks, 1990). Nevertheless, a quantitative understanding of the catalysis by AP or other phosphoryl transfer enzymes is still lacking. In addition, it will be interesting to determine whether AP effects a small change in the nature of the transition state for phosphoryl transfer and whether any enzyme elicits a change to a transition state with predominant associative character.

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