Catalysis of the Hydrolysis of Phosphorylated Pyridines by Alkaline Phosphatase Has Little or No Dependence on the pK_a of the Leaving Group†

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ABSTRACT: Bacterial alkaline phosphatase is an active catalyst for the hydrolysis of N-phosphorylated pyridines, with values of the second-order rate constant k_{cat}/K_m in the range 0.4–1.2 × 10^6 M^{-1} s^{-1} at pH 8.0, 25 °C. There is little or no dependence of the rate on the pK_a of the leaving group; the value of β_{lg} is 0 ± 0.05, which may be compared with $\beta_{lg} = -1.0$ for the nonenzymic reaction. Phosphorylated pyridines do not have a free electron pair available for protonation or coordination of the leaving group. Therefore, this result means that the similar, small dependence on leaving group structure for the enzyme-catalyzed hydrolysis of phosphate esters [Hall, A. D., & Williams, A. (1986) *Biochemistry 25,* 4784–4790) does not provide evidence for general acid catalysis or electrophilic assistance of leaving group expulsion. The results are consistent with the hypothesis that productive binding of the substrate, which may involve a conformational change, is largely rate limiting for turnover of the enzyme at low substrate concentrations.

Alkaline phosphatase is a highly efficient catalyst for the hydrolysis of phosphate esters and other phosphate derivatives (McComb et al., 1979). Each monomer of the dimeric enzyme contains two Zn^{2+} ions, which are presumably involved in catalysis of the formation and hydrolysis of a phosphorylated serine hydroxyl group at the active site during turnover of the enzyme (Engström, 1961; Schwartz & Lipmann, 1961). The three-dimensional structure of the enzyme has been determined by X-ray crystallography (Sowadski et al., 1985; Kim & Wyckoff, 1991).

It is difficult to probe the catalytic mechanism of alkaline phosphatase by steady-state kinetics because k_{cat} represents either rate-limiting hydrolysis of the phosphoenzyme intermediate, at pH < 7, or rate-limiting dissociation of inorganic phosphate from the enzyme, at pH > 7 (Reid & Wilson, 1971; Bloch & Schlesinger, 1973; Bale et al., 1980). However, the second-order rate constant for reaction of the enzyme with substrate, k_{cat}/K_m, is a measure of the first irreversible step of the reaction and might provide information about the catalytic process. Hall and Williams (1986) have reported that the values of k_{cat}/K_m for the hydrolysis of a series of substituted phenyl phosphate monoesters show only a small dependence on the pK_a of the leaving group, with a value of $\beta_{lg} = -0.19$ from a Brønsted-type correlation of log k_{cat}/K_m against the pK_a of the leaving group. It has been suggested that general acid–base catalysis may contribute to the observed catalysis (Sowadski et al., 1985) and that a small dependence of the rate on the structure of the leaving group could be caused by protonation of the leaving oxygen atom by an acidic group or coordination with a metal ion at the active site of the enzyme in the transition state (Williams et al., 1973; Hall & Williams, 1986). This electrophilic assistance could facilitate leaving group departure and decrease the development of negative charge on the leaving group, so that only a small dependence of the rate on leaving group structure would be observed. An increase in β_{lg} from –1.2 to –0.7 in the presence of Zn^{2+} ions has been observed for the nonenzymatic hydrolysis of substituted salicyl phosphates (Steffens et al., 1975). However, other possible explanations for this small dependence on leaving group structure include an electrostatic interaction with a cationic group at the active site that offsets the development of negative charge on oxygen without coordination, a partially rate-limiting conformational change, or rate-limiting productive binding of substrate to the enzyme, which could be diffusion-controlled (Trentham & Gutfreund, 1968; Fernley & Walker, 1969; Hall & Williams, 1986).

We were interested in examining further the possibility that protonation of the leaving group in the transition state is responsible for the small dependence on leaving group structure, because this proton transfer provides a mechanism that could contribute to the catalysis that is brought about by the enzyme. For this reason, we have examined the values of k_{cat}/K_m for catalysis of the hydrolysis of a series of N-phosphorylated pyridines by alkaline phosphatase. These substrates, in contrast to phosphate esters and *N*-phenylphosphoramidates (Williams & Naylor, 1971; Snyder & Wilson, 1972), do not have lone pair electrons on the leaving group that are available to accept a proton from an acid catalyst or to coordinate with a metal ion in the transition state. Therefore, the dependence of k_{cat}/K_m on the structure of the leaving pyridine for the enzyme-catalyzed hydrolysis of these substrates provides a measure of the effect of leaving group structure on the rate of the catalyzed reaction in the absence of proton transfer or coordination with a metal ion.

MATERIALS AND METHODS

Materials. Alkaline phosphatase from *Escherichia coli,* type III-S from Sigma, was used without further purification. 4-Picoline, 3-picoline, 3,4-lutidine, and 3,5-lutidine were distilled, and 4-morpholinopyridine and 4-(dimethylamino)-pyridine were recrystallized prior to phosphorylation. Phosphorylated pyridines were prepared as described previously (Skoog & Jencks, 1984; Herschlag & Jencks, 1987); 4-morpholinopyridine was a gift from Mark Skoog. The disodium salt of 4-nitrophenyl phosphate was recrystallized prior to use. Phosphorus oxychloride (Fisher) and CHES buffer (Sigma) were used without additional purification.

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Methods. The hydrolysis of phosphorylated 3-picoline, phosphorylated 4-picoline, phosphorylated 3,4-lutidine, phosphorylated 3,5-lutidine, phosphorylated 4-morpholinopyridine, and phosphorylated 4-(dimethylamino)pyridine was followed spectrophotometrically at 270, 256, 270, 270, 303, and 303 nm, respectively. Reactions were carried out in 1- or 4-cm-path-length cuvettes and followed with a Perkin-Elmer 4E or a Zeiss PM-6 spectrophotometer. The enzyme was added to cuvettes containing 1.0 M CHES buffer adjusted to pH 8.00 or 10.00, to give a final concentration in the range 0.6–4.3 units/mL. The pH values were measured with a glass combination electrode containing saturated KCl.

The cuvettes were incubated at 25 °C for 15 min prior to initiation of the reaction by the rapid addition of the phosphorylated pyridine substrates to give a final concentration of 0.5–6.0 × 10^{-4} M. After the reactions had gone to completion (more than 7 half-times), 4-nitrophenyl phosphate was added to give a final concentration of 3.6 × 10^{-5} M and the release of 4-nitrophenol was monitored at 410 nm.

The enzyme-catalyzed hydrolysis of phosphorylated 4-morpholinopyridine was examined both in the presence of 0.4 mM inorganic phosphate (K_2HPO_4) and without the addition of inorganic phosphate (~30 nM inorganic phosphate was present at the initiation of all the reactions of phosphorylated pyridine substrates because of phosphate that remained from the original synthesis mixture). Reactions were carried out in the presence of 0.4 mM K_2HPO_4 in order to obtain k_{cat}/K_m conditions and to ensure that the concentration of inorganic phosphate stayed essentially constant throughout the entire reaction and the subsequent reaction of 4-nitrophenyl phosphate.

The synthesis of phosphorylated 3-picoline and 4-picoline was carried out with 3.5:1 and 1:1 molar equivalent ratios of $POCl_3$ to the pyridine nucleophile, in order to control for the effects of unreacted $PO_2Cl_2^-$ on the enzyme. $PO_2Cl_2^-$ is formed rapidly from $POCl_3$ in an aqueous solution (half-time < 0.1 s) and has a lifetime of ~3 min under the experimental conditions (Skoog & Jencks, 1984). All reactions were carried out with at least two substrate concentrations by using 1- and 4-cm cuvettes and in the presence of concentrations of enzyme that were sufficient to give an increase in rate of at least 50% compared with the nonenzymic reaction.

It was shown that the enzyme was stable over the entire course of the assay. The hydrolysis of phosphorylated morpholinopyridine by alkaline phosphatase was monitored, as described above, over 7 half-times. The same rate constant was then obtained when a second aliquot of phosphorylated morpholinopyridine was added to the cuvettes.

Pseudo-first-order rate constants for the hydrolytic reactions were obtained from semilogarithmic plots of the change in absorbance against time, which were linear for at least 3 half-times with all substrates. End points were determined by multiplying the change in absorbance over 7 half-times by 1.01 and subtracting this value from the initial absorbance.

The activity of alkaline phosphatase was assayed by measuring the release of 4-nitrophenol (ϵ = 1.62 × 10^4 M^{-1} cm^{-1}) spectrophotometrically at 410 nm under conditions similar to those of Garen and Levinthal (1960), with 1 M Tris buffer, pH = 8.00, and 1 mM 4-nitrophenyl phosphate as substrate. One unit is defined as the amount of enzyme that will release 1 μmol of 4-nitrophenol per minute at 25.0 °C.

RESULTS

Hydrolysis of Phosphorylated Pyridines. Figure 1 shows that the hydrolysis of phosphorylated 4-morpholinopyridine (●) and of 4-nitrophenyl phosphate (▲) catalyzed by alkaline phosphatase follows first-order kinetics at pH 8.0 in the presence of 0.4 mM inorganic phosphate. The reactions were carried out in the presence of a large excess of inorganic phosphate in order to obtain second-order rate constants, k_{app}, for reaction of the enzyme with different substrates and to avoid uncertainties from traces of inorganic phosphate that might contaminate the substrates and inhibit the enzyme by different amounts with different preparations of substrate. First-order plots similar to those shown in Figure 1 were found to be linear for more than $3t_{1/2}$ for all substrates examined. Values of k_{app} for the hydrolysis of a series of phosphorylated pyridines and of 4-nitrophenyl phosphate were obtained from the slopes of linear plots of a series of pseudo-first-order rate constants against enzyme concentration, as shown in Figure 2, and are summarized in Table I. The apparent second-order rate constants provide a measure of the relative activities of the different substrates, but are much smaller than the true values of k_{cat}/K_m because of the presence of inorganic phosphate, a competitive inhibitor of the enzyme (Applebury et al., 1970).

After the hydrolysis of the phosphorylated pyridine had proceeded to completion, an aliquot of 4-nitrophenyl phosphate

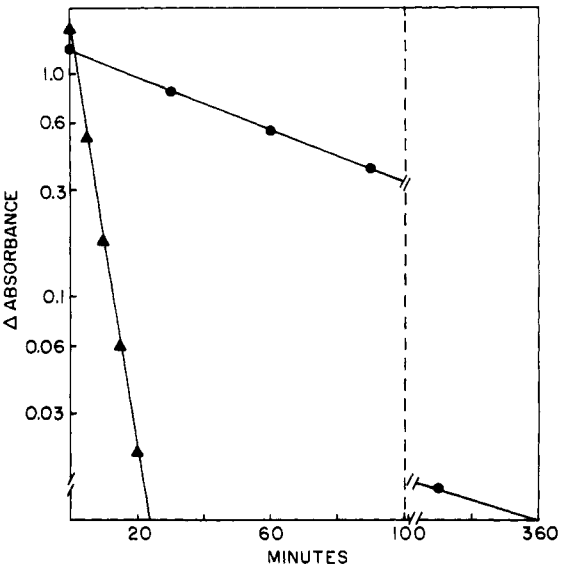

FIGURE 1: Semilogarithmic plots of the change in absorbance against time for the hydrolysis of phosphorylated morpholinopyridine (●) and 4-nitrophenyl phosphate (▲) at 25.0 °C, pH = 8.0, in the presence of 1.0 M CHES buffer, 0.4 mM inorganic phosphate, and 2.1 units/mL alkaline phosphatase.

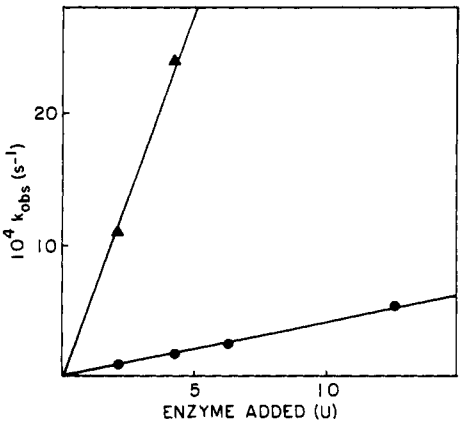

FIGURE 2: The dependence on enzyme concentration of the pseudo-first-order rate constants (k_{obs}) for the hydrolysis of phosphorylated morpholinopyridine (●) and 4-nitrophenyl phosphate (▲) catalyzed by alkaline phosphatase.

Table I: Apparent Second-Order Rate Constants (k_{app}) for the Enzymatic Hydrolysis of Substituted Phosphorylated Pyridines (pyr) and 4-Nitrophenyl Phosphate (pnpp), and Calculated Values of k_{cat}/K_m for the Hydrolysis of Substituted Phosphorylated Pyridines[a]

phosphorylated pyridine	pK_a[b]	$k_{app}^{pyr} \times 10^5$ (s^{-1} units^{-1})	$k_{app}^{pnpp} \times 10^4$ (s^{-1} units^{-1})	k_{cat}/K_m[c] (M^{-1} s^{-1})	$k_{nor} = k_{app}^{pyr}/k_{app}^{pnpp}$
4-(dimethylamino)pyridine[d]	10.06	5.7 ± 0.4	6.2 ± 0.6	4.3×10^6	0.093[d] 0.099[e] 0.120[f]
4-morpholinopyridine[d]	9.01	4.0 ± 0.3	6.0 ± 0.5	2.9×10^6	0.069[d] 0.067[e] 0.067[f]
3,4-lutidine[g]	6.79	5.3 ± 0.8	5.0 ± 0.9	5.1 ± 10^6	0.097
3,5-lutidine[g]	6.41	6 ± 1	6.4 ± 0.5	4.3×10^6	0.115
4-picoline[g]	6.33	6.0 ± 0.5	4.7 ± 0.6	5.9×10^6	0.108[g] 0.103[h]
3-picoline[g]	6.02	8 ± 2	4.4 ± 0.2	8.4×10^6	0.120

[a] At 25.0 ± 0.2 °C, pH = 8.0, in the presence of 1.0 M CHES buffer, unless noted otherwise. [b] Skoog and Jencks (1984). [c] Values of k_{cat}/K_m were calculated by multiplying $k_{app}^{pyr}/k_{app}^{pnpp}$ by $k_{cat}/K_m = 4.6 \times 10^7$ M^{-1} s^{-1} for 4-nitrophenyl phosphate (Snyder & Wilson, 1972). [d] In the presence of 0.4 mM inorganic phosphate. [e] pH 10.0, 0.4 mM inorganic phosphate. [f] 30 nM inorganic phosphate. [g] Solutions of these phosphorylated pyridines were prepared immediately before use, as described in Materials and Methods, with 3.5:1 molar ratio of $POCl_3$/pyridine. [h] Prepared with a 1:1 molar ratio of $POCl_3$/pyridine.

was added to the same reaction mixture and the first-order rate constant for hydrolysis of this substrate was determined by following the increase in absorbance at 410 nm. The values of k_{cat}/K_m for the phosphorylated pyridines in Table I were calculated from the ratios of the observed second-order rate constants for catalysis of the hydrolysis of the phosphorylated pyridines and 4-nitrophenyl phosphate. A value of $k_{cat}/K_m = 4.6 \times 10^7$ M^{-1} s^{-1} for 4-nitrophenyl phosphate was calculated from data of Snyder and Wilson (1972) that were obtained at 26 °C with an enzyme preparation that was free of contamination with inorganic phosphate; this value is slightly larger than $k_{cat}/K_m = 6.6 \times 10^6$ M^{-1} s^{-1} at 25 °C reported by Hall and Williams (1986). A rate constant of 2×10^7 M^{-1} s^{-1} has been reported by Chock (1980) for the hydrolysis of 4-methylumbelliferal phosphate at pH 8.3 and 10 °C. However, it should be noted that the dependence of the rate on the structure of the phosphorylated pyridine is independent of the value of k_{cat}/K_m that was used in these calculations.

The rate constants for the different substrates were also compared with a series of individual measurements as follows. The observed pseudo-first-order rate constant for nonenzymatic hydrolysis of the substrate, k_{HOH}, was subtracted from observed pseudo-first-order rate constants for the enzyme-catalyzed reactions that were at least 50% larger than the rate constants in the absence of enzyme. These rate constants were divided by the rate constants for the enzyme-catalyzed hydrolysis of 4-nitrophenyl phosphate, which were obtained with the same reaction mixtures, to give a normalized rate constant, k_{nor}, that is also reported in Table I. Several experiments that were carried out in the presence of 30 nM instead of 0.4 mM inorganic phosphate, or at pH 10.0 instead of pH 8.0, gave values of k_{nor} that do not differ significantly from those obtained under standard conditions (Table I). The absence of a significant difference in the values of k_{nor} that were obtained with phosphorylated 4-picoline that had been prepared with a 3.5-fold and a 1.1-fold excess of $POCl_3$ over 4-picoline shows that byproducts formed from the excess $POCl_3$ do not have a significant effect on the rate.

DISCUSSION

Phosphorylated Pyridines as Substrates. Alkaline phosphatase is an effective catalyst for the hydrolysis of phosphorylated pyridines, in spite of the fact that protonation of the leaving group is not possible with these substrates. The second-order rate constant for the enzyme-catalyzed hydrolysis of phosphorylated morpholinopyridine, with a leaving group of $pK_a = 9$, is only 15-fold smaller than that for 4-nitrophenyl phosphate, with a leaving group of $pK_a = 7$ (Table I). The second-order rate constant of 4.6×10^7 M^{-1} s^{-1} for the hydrolysis of 4-nitrophenyl phosphate by the enzyme is 10^{17} larger than the second-order rate constant for its reaction with water, while the corresponding rate increase for phosphorylated morpholinopyridine is a factor of 4×10^{13}. Most of this difference reflects the much faster nonenzymic hydrolysis of the phosphorylated pyridines. The decrease in k_{cat}/K_m of ~10-fold for the phosphorylated pyridines compared with 4-nitrophenyl phosphate (Table I) may be attributed to an electrostatic effect of the positive charge on the nitrogen atom of the phosphorylated pyridine, an unfavorable steric interaction of the active site with the two carbon atoms that are attached to the leaving nitrogen atom of the pyridine, a difference in the mechanism by which phosphoenzyme formation from the two substrates is catalyzed, or simply the different rates of productive binding of the two substrates to the active site of the enzyme.

A difference in the mechanism of catalysis is consistent with the suggestion of Hall and Williams (1986) that general acid catalysis contributes to the acceleration of the rate of phosphate ester hydrolysis that is brought about by the enzyme by protonating the oxygen atom of the leaving alcohol, in order to increase its leaving ability. However, the effective catalysis of the hydrolysis of phosphorylated pyridines shows that protonation of the leaving group is not a requirement for catalysis by the enzyme.

The Effect of Leaving Group Structure. The small increase in the second-order rate constants, k_{cat}/K_m, for catalysis of the hydrolysis of substituted phenyl phosphates by alkaline phosphatase with decreasing pK_a of the leaving group, with a value of $\beta_{lg} = -0.2$ (Hall & Williams, 1985, 1986), is in sharp contrast to the large dependence on the pK_a of the leaving group for the nonenzymic hydrolysis of substituted phenyl phosphate dianions, which follow a value of $\beta_{lg} = -1.2$ (Kirby & Varvoglis, 1967; Kirby & Jencks, 1965). It was suggested that the small dependence on pK_a in the enzyme-catalyzed reaction could arise from protonation of the leaving phenolate ion by an acidic group in the active site that decreases the negative charge on oxygen in the transition state, from electrophilic catalysis by a metal ion that has the same effect, or from a diffusion-controlled reaction of enzyme with substrate, followed by rapid hydrolysis (Hall & Williams, 1986). Protonation of the leaving group is believed to account for the rapid rate of hydrolysis and the small sensitivity to the

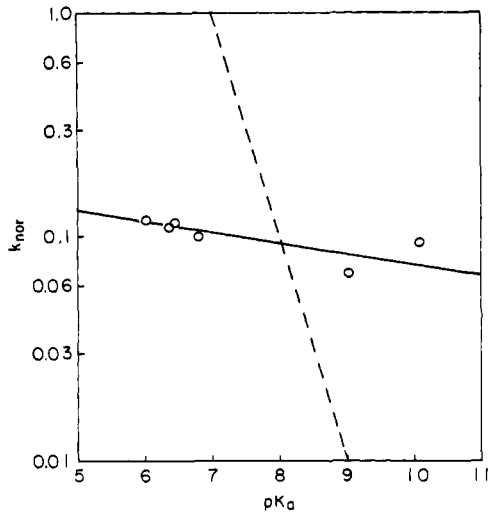

FIGURE 3: Brønsted-type plot of the mean values of k_{nor} for catalysis of the hydrolysis of a series of phosphorylated pyridines by alkaline phosphatase against pK_a of the parent pyridines, at 25.0 ± 0.2 °C in the presence of 1.0 M CHES buffer at pH 8.0. The dashed line has a slope of $\beta_{lg} = -1.0$.

structure of the leaving group in the nonenzymatic hydrolysis of the monoanions of phosphate esters and acyl phosphate monoanions (Butcher & Westheimer, 1955; Barnard et al., 1955; DiSabato & Jencks, 1961; Kirby & Varvoglis, 1967).

We have tested the hypothesis that protonation of the leaving group in the transition state accounts for the small value of $\beta_{lg} = -0.2$ in the hydrolysis of phosphate esters catalyzed by alkaline phosphatase by examining the dependence on leaving group structure of the hydrolysis rate of a series of phosphorylated pyridines with leaving groups of differing pK_a. Protonation of the leaving pyridine in the transition state for bond breaking is not possible with these substrates because the lone pair electrons of the pyridine nitrogen atom are partially bonded to phosphorus in the transition state for P–N cleavage and, in contrast to ester hydrolysis, there is no electron pair on the leaving atom that is available for protonation in the transition state. Therefore, if protonation of the leaving group accounts for the small dependence of k_{cat}/K_m on the pK_a of the leaving group for oxygen esters, a much larger dependence on the pK_a of the leaving group would be expected for the enzyme-catalyzed cleavage of phosphorylated pyridines.

The second-order rate constants, k_{cat}/K_m, for catalysis of the hydrolysis of phosphorylated pyridines by alkaline phosphatase show little, if any, dependence on the pK_a of the leaving pyridine (Table I). This small or negligible dependence of the rate of the enzyme-catalyzed reaction on the pK_a of the leaving group is in marked contrast to the large dependence on the nature of the leaving group in the uncatalyzed reaction, with $\beta_{lg} = -1.0$ (Skoog & Jencks, 1984). The ratios of k_{cat}/K_m for the enzyme-catalyzed hydrolysis of phosphorylated pyridines compared with 4-nitrophenyl phosphate, k_{nor}, are shown in a Brønsted-type plot in Figure 3. The data are consistent with the solid line, which has a slope of $\beta_{lg} = -0.05$. However, inspection of the structure of the substrates shows that the small decrease in k_{cat}/K_m is correlated with the bulk of the substituents on the leaving groups at least as well as with their pK_a, so that the basicity of the leaving group may have no effect on k_{cat}/K_m and the results are consistent with a slope of 0. There is abundant evidence that an increase in the bulk of substituents on the leaving alcohol causes a decrease in k_{cat}/K_m for the hydrolysis of the corresponding phosphate esters (Hall & Williams, 1986).

The similar structure–reactivity behavior for the hydrolysis of substituted phenyl phosphates and phosphorylated pyridines provides no support for the hypothesis that proton donation by an acidic group in the active site reduces the development of negative charge in a dissociative transition state. The crystal structure of the enzyme shows that there are no proton-donating groups on amino acid side chains near the active site (Sowadski et al., 1985).

Two possible explanations for the large difference in the behavior of the enzyme-catalyzed and the nonenzymic reactions are the following:

(1) There is a strong electrostatic interaction of a cationic group at the active site, presumably a metal ion, with the leaving group in the transition state, which does not involve coordination with lone pair electrons but offsets the decrease in positive charge on the leaving oxygen or nitrogen atom as the P–O or P–N bond breaks.

(2) There is little or no breaking of the P–N bond in the rate-limiting step of the enzyme-catalyzed reaction at low substrate concentration. It is known that k_{cat} for catalysis of phosphate ester hydrolysis represents either rate-limiting phosphoenzyme hydrolysis or dissociation of the E·P$_i$ complex, depending on the pH (Reid & Wilson, 1971; Gutfreund & Sykes, 1976; Chock, 1980), but it is unlikely that either of these steps is rate limiting for k_{cat}/K_m because the leaving group presumably dissociates rapidly from the phosphoenzyme, so that phosphoenzyme hydrolysis is irreversible.

One possible explanation of these results is that the transition state for bond cleavage in the enzyme-catalyzed reaction is grossly different from that in the nonenzymic reaction, with a large amount of bond formation and little bond breaking in an associative transition state. We believe that this explanation is unlikely for several reasons. First, an associative transition state for the nonenzymatic reaction of phosphate ester dianions is much higher in energy than the dissociative transition state, since it is not observed. Therefore, the enzyme would have to produce a considerably larger amount of catalysis to function by an associative mechanism. It has been shown that catalysis by Mg^{2+} or Ca^{2+} does not cause a significant increase in associative character of the transition state for the reactions of 4-nitrophenyl phosphate with water and substituted pyridines (Herschlag & Jencks, 1987). Second, Weiss and Cleland (1989) have concluded that the secondary ^{18}O isotope effect for the nonbridging oxygen atom supports a dissociative transition state for the hydrolysis reaction catalyzed by alkaline phosphatase.

It is possible that the reactions are partially or completely controlled by the rate of diffusional combination of the substrate with the enzyme. The second-order rate constants in the range $k_{cat}/K_m = 2–5 \times 10^7$ M^{-1} s^{-1} for catalysis of the hydrolysis of substituted phenyl phosphates (Snyder & Wilson, 1972; Chock, 1980) are somewhat lower than expected for a diffusion-controlled reaction, and the rate constants of $3–8 \times 10^6$ M^{-1} s^{-1} for the phosphorylated pyridines are even smaller. However, a rate constant of $2–3 \times 10^7$ M^{-1} s^{-1} for an enzyme-catalyzed reaction has been shown to be at least partially diffusion-controlled (Hardy & Kirsch, 1984). It is possible that only a fraction of the encounters of substrate with enzyme are productive, because a large fraction of the enzyme may exist in a conformation or ionization state in which binding cannot occur. It is also possible that the steric requirements for binding may require a particular orientation of the substrate. Inorganic phosphate binds snugly in a pocket that will barely accommodate it (Sowadski et al., 1985). It is known that the dissociation of inorganic phosphate is the rate-

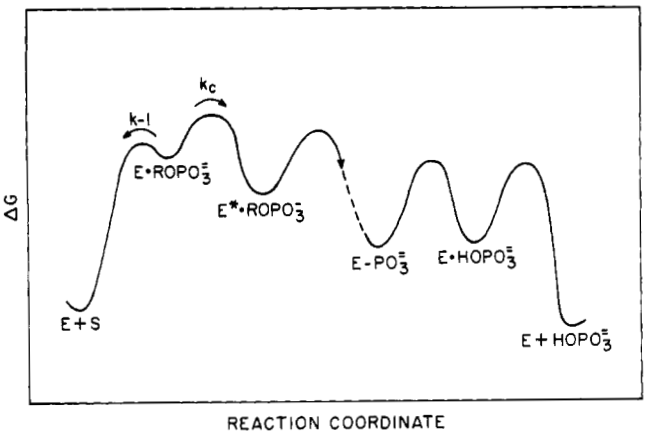

FIGURE 4: A hypothetical reaction coordinate diagram to illustrate how a conformational change, k_c, can be rate limiting for the second-order rate constant k_{cat}/K_m. At substrate saturation the rate-limiting step is phosphoenzyme hydrolysis or the dissociation of inorganic phosphate from the enzyme, which may involve a conformational change.

limiting step of the reaction in the presence of saturating substrate near neutral pH (Gutfreund & Sykes, 1976). Dissociation is the reverse of binding, so that it would not be surprising if binding were rate limiting in the forward direction.

Alternatively, a conformational change of the enzyme may be largely rate limiting for k_{cat}/K_m and account for the small dependence of the rate on substrate structure. Halford et al. (1969) have obtained evidence for a conformational change upon binding of 2-hydroxy-5-nitrophenylphosphonate to the enzyme at pH 8 and suggested that this step is kinetically significant for reactions of substrates. There also is evidence that a slow conformational change occurs upon the binding or dissociation of inorganic phosphate (Hall & Sykes, 1976; Chlebowski et al., 1979). A conformational change with the rate constant k_c will be rate limiting at low substrate concentration if k_{-c} is slower than k_p for phosphorylation of the enzyme by bound substrate (eq 1; Figure 4). The

$$E + X\text{-}PO_3^{2-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot XPO_3^{2-} \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} E^*\cdot XPO_3^{2-} \xrightarrow{k_p} E^*\text{-}PO_3^{2-} + HX \xrightarrow{k_h} E^*\cdot P_i \xrightarrow{k_d} E + P_i \quad (1)$$

phosphorylation step is irreversible under most conditions because the leaving group diffuses away from the enzyme and the rate-limiting step at substrate saturation is, therefore, the hydrolysis of phosphoenzyme (k_h; eq 1) or the dissociation of inorganic phosphate (k_d) (Reid & Wilson, 1971; Gutfreund & Sykes, 1976; Chock, 1980). It is likely that the dissociation of inorganic phosphate also involves a conformational change.

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