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 $0.4-1.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ 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 $\beta_a$ is $0 \pm 0.05$, which may be compared with $\beta_a = -1.0$ 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 small, similar 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 & Schiesinger, 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 $\beta_a = -0.19$ from a Brunsted-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 $\beta_a$ from -1.2 to -0.7 in the presence of Zn$^{2+}$ ions has been observed for the nonenzymatic hydrolysis of substituted salicyl phosphates (Steifens 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 & Gutfleid, 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 $N$-phenylphosphoromides (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 II-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 8.0 or 10.0, 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 (K₂HPO₄) and without the addition of inorganic phosphate (~30 mM 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 K₂HPO₄ in order to obtain kₐ₀/Kₘ 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 POCl₃ to the pyridine nucleophile, in order to control for the effects of unreacted PO₂Cl₂ on the enzyme. PO₂Cl₂ is formed rapidly from POCl₃ in an aqueous solution (half-time < 0.1 s) and has a lifetime of ~3 min under the experimental conditions (Skooq & 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 × 10⁵ M⁻¹ cm⁻¹) spectrophotometrically at 410 nm under conditions similar to those of Garen and Levinthal (1960), with 1 M Tris buffer, pH = 8.0, 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
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 \( \times \) 10^7 M\(^{-1}\) s\(^{-1}\) has been reported by Chock (1980) for the hydrolysis of 4-methylumbelliferyl 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, \( k_{H_2O} \), 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, \( k_{norm} \), 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 \( k_{norm} \) that do not differ significantly from those obtained under standard conditions (Table I). The absence of a significant difference in the values of \( k_{norm} \) 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 \( \times \) 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 \( \times \) 10^12. Most of this difference reflects the much faster nonenzymic hydrolysis of the phosphorylated pyridines. The decrease in \( k_{cat}/K_m \) of \( \sim \)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 phosphate-enzyme 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 \( \beta_K = -0.2 \) (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 diions, which follow a value of \( \beta_K = -1.2 \) (Kirby & Varovg, 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
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 $\beta_g = -0.2$ 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 $\beta_g = -1.0$ (Skoog & Jencks, 1984). The ratios of $k_{cat}/K_m$ for the enzyme-catalyzed hydrolysis of phosphorylated pyridines compared with 4-nitrophenyl phosphate, $k_{cat}/K_m$, are shown in a Bronsted-type plot in Figure 3. The data are consistent with the solid line, which has a slope of $\beta_g = -0.05$.

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 nonenzymatic 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-Pi complex, depending on the pH (Reid & Wilson, 1971; Guttfreund & 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 diacetals 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 diffusion 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 $3-8 \times 10^8$ M$^{-1}$ s$^{-1}$ for the phosphorylated pyridines are even smaller. However, a rate constant of $2-3 \times 10^8$ 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-
Specificity and Inhibition of Alkaline Phosphatase