Alkaline Phosphatase Is an Almost Perfect Enzyme†

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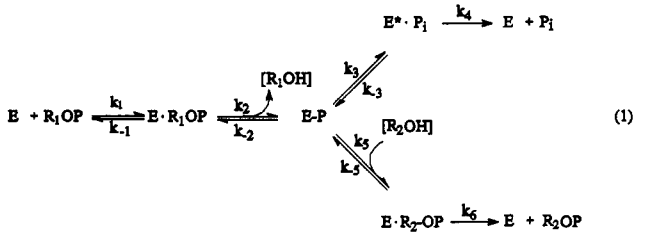
ABSTRACT: The second-order rate constant, k_{cat}/K_m, for catalysis of the hydrolysis of 4-nitrophenyl phosphate by alkaline phosphatase decreases with increasing viscosity in the presence of sucrose or arabinose, with a slope of $\partial[(k_{cat}/K_m)^0/(k_{cat}/K_m)]/\partial(\eta/\eta^0) = 1.4$ at pH 8.0, 25 °C. This is consistent with rate-limiting diffusional encounter of the substrate with active enzyme and indicates that alkaline phosphatase is a "perfect enzyme". However, the reported second-order rate constants of $k_{cat}/K_m = 6.6 \times 10^6$ to 4.6×10^7 M^{-1} s^{-1} are smaller than the diffusional limit; this shows that only ~0.1–1% of the diffusional encounters are productive. The first-order rate constant, k_{cat}, for rate-limiting hydrolysis of the phosphoenzyme intermediate at pH = 6 with saturating substrate concentration is independent of viscosity in aqueous sucrose solutions. This shows that sucrose does not destabilize the transition state for phosphoenzyme hydrolysis. However, at pH 8.0 product dissociation is rate limiting and k_{cat} decreases with increasing viscosity in the presence of sucrose, with slopes of $\partial(k^0/k_{obsd})/\partial(\eta/\eta^0) = 1.2$ in 0.04 M Mops buffer, 1.0 in 0.1 M Tris, and 1.2 in 0.67 M Tris buffer. This is consistent with rate-limiting diffusional separation of inorganic phosphate and of Tris phosphate from the enzyme. In contrast, glycerol causes a large decrease in k_{cat}/K_m at pH 8.0 and also decreases k_{cat} at pH 6. This shows that glycerol decreases the rate by a solvent effect on the catalytic activity of the enzyme, as well as by increasing the viscosity.

Alkaline phosphatase is a nonspecific phosphomonoesterase that is found in both procaryotes and eucaryotes. It is a dimeric metalloprotein that has two Zn^{2+} ions and a Mg^{2+} ion in each active site. Hydrolysis is brought about by nucleophilic catalysis with phosphorylation of a serine hydroxyl group, followed by hydrolysis of the serine phosphate ester and dissociation of inorganic phosphate (Weiss et al., 1988; Whyte, 1989). In the presence of saturating concentrations of active substrates, hydrolysis of the phosphoenzyme is rate limiting at pH ≤6 (k_3, eq 1),and dissociation of the noncovalent complex



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of enzyme with bound inorganic phosphate is rate limiting at pH ≥8 (k_4, eq 1) (Chlebowski & Coleman, 1972, 1974; Hull et al., 1976; Chlebowski et al., 1977; Gettins & Coleman, 1983). The phosphoenzyme also reacts readily with alcohols to form phosphate esters, which dissociate rapidly from the enzyme (k_5 and k_6, eq 1). Tris(hydroxymethyl)aminomethane is a good phosphoryl acceptor and causes a large increase in the maximal velocity because Tris phosphate dissociates rapidly, thereby bypassing the slow dissociation of inorganic phosphate from the enzyme (Trentham & Gutfreund, 1968).

The second-order rate constants, k_{cat}/K_m, for catalysis of the hydrolysis of phosphate monoesters and phosphorylated pyridines show little or no dependence on the pK_a of the leaving alcohol (Labow et al., 1993). This is in marked contrast to the nonenzymatic hydrolysis of phosphate monoester dianions, acyl phosphates, and phosphorylated pyridines. These compounds undergo hydrolysis with a very large dependence of the rate on the pK_a of the leaving group; values of β_{lg} are in the range −1.0 to −1.2 (Kirby & Varvoglis, 1967; Bourne & Williams, 1984; Skoog & Jencks, 1984). Therefore, the transition state for the rate-limiting step of the nonenzymatic hydrolysis reaction has no resemblance to that of the enzyme-catalyzed reaction. The small dependence of the rate on leaving group structure in the enzyme-catalyzed reaction might be ascribed to protonation of the leaving group of the bound substrate in the transition state, but this is not possible for phosphorylated pyridines, and the crystal structure of the enzyme shows there is no proton-donating group in the active site (Sowadski et al., 1985).

The simplest explanation for the absence of a significant effect of substituents in the leaving group on k_{cat}/K_m is that the enzyme-catalyzed reaction is diffusion controlled. However, the observed second-order rate constants in the range of $k_{cat}/K_m = 6.6 \times 10^6$ to 4.6×10^7 M^{-1} s^{-1} that have been reported for catalysis of the hydrolysis of phosphate esters (Snyder & Wilson, 1972; Hall & Williams, 1986) are smaller than the rate constants on the order of 10^9 M^{-1} s^{-1} that are expected for diffusion-controlled reactions. The observed rate of the enzyme-catalyzed reaction could be decreased if most of the enzyme molecules are not in an optimal conformation or ionization state for binding of substrate and catalysis under the conditions of the activity measurements, so that only ~0.1–1% of the collisions of substrate with the enzyme are productive.

We report here a study of the effect of viscosity on the second-order rate constants, k_{cat}/K_m, for catalysis of phosphate ester hydrolysis in aqueous solutions containing glycerol, sucrose or arabinose. The results are consistent with a diffusion-controlled reaction of alkaline phosphatase with phosphate esters.

MATERIALS AND METHODS

Materials. The following reagents were obtained from the indicated sources: Alkaline phosphatase, type III-S, and Mes

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buffer (Sigma), Tris (tris-(hydroxymethyl)aminomethane) base, ultra-pure sucrose and urea (Schwartz & Mann), spectroscopic grade glycerol (99% pure), tetrahydrofuran (99.5% pure, inhibitor free), 2-methoxyethanol (99% pure), acetonitrile (99% pure), methanol (99% pure), L-ascorbic acid (99% pure), D-arabinose (99% pure), and Mops buffer (Aldrich). 4-Nitrophenyl phosphate was a gift from B. Labow; it was recrystallized from ethanol–water and stored at −40 °C.

Methods. The relative viscosity, $\eta_{rel} = \eta/\eta^0$, of each reaction solution was measured with an Ostwald viscometer in a water bath at 25.0 ± 0.2 °C in the presence (η) and absence (η^0) of viscogen according to eq 2,

$$\eta/\eta^0 = (t/t^0)(\rho/\rho^*) \quad (2)$$

in which the viscosity of water is $\eta^* = 0.8904$, t is time and the density, ρ is $\rho^* = 0.9884$ g/mL for water at 25.0 °C. The values of η^0 for 0.67 M Tris, 0.1 M Tris, and 0.04 Mops buffers were found to be 1.21, 0.90, and 1.02, respectively. Densities were determined gravimetrically. Buffers of 0.1 and 0.67 M Tris, and of 0.04 M Mops were adjusted to pH 8.00, and 0.04 M Mes buffer was adjusted to pH 6.00.

Initial rates of hydrolysis of 4-nitrophenyl phosphate were followed at 410 nm with a Perkin-Elmer 4B UV/visible spectrophotometer. Glycerol, which is known to form a nearly ideal solution with water (Stokes & Robinson, 1966), was added directly to dry, tared cuvettes; other compounds and cosolvents were added to reaction cuvettes from concentrated solutions that were prepared volumetrically.

Inorganic phosphate was determined with the Lowry–Lopez (1946) method at 660 nm. A 0.60-mL aliquot of the reaction mixture was added to 2.28 mL of 2 M sodium acetate buffer, pH 4.0. Then 60 μL of 2 M acetic acid, 30 μL of 10% ammonium molybdate, and 30 μL of freshly prepared 10% ascorbic acid were added, the samples were incubated for 60 min at 50 °C and for 20 min at 25 °C, and the absorbance was measured at 660 nm.

Transphosphorylation from the phosphorylated enzyme to 3.26 M glycerol and 0.88 M sucrose was examined at pH 6.0 (0.04 M Mes buffer) and 8.0 (0.04 M Mops buffer) with 0.36 μM alkaline phosphatase and 1 mM 4-nitrophenyl phosphate at 25 °C. The formation of inorganic phosphate was determined at 80% and 100% reaction, and 4-nitrophenol release was followed spectrophotometrically at 410 nm by adding 50-μL aliquots of the reaction mixtures to 2.95 mL of 1 M NaOH (80% reaction) or 1 M Tris, pH 9.0 (100% reaction).

Second-order rate constants, k_{cat}/K_m, for catalysis of the cleavage of 4-nitrophenyl phosphate were determined in the presence of 0.67 M Tris buffer, pH 8.0, with 1 μM 4-nitrophenyl phosphate, which is 10-fold smaller than the K_m reported by Hall and Williams (1986). Tris reacts with the phosphoenzyme to give Tris phosphate and decreases the formation of inorganic phosphate, which inhibits the enzyme. All reactions followed first-order kinetics for several half-times. This shows that there is no significant saturation of the enzyme, which would give zero-order kinetics, and that product inhibition is not significant under the conditions of these experiments.

The value of k_{cat} at pH 6.0 was determined in the presence of 0.04 M Mes buffer with 1 mM 4-nitrophenyl phosphate and 11.4 units/mL of enzyme. Identical rates were obtained with 1, 2, and 3 mM 4-nitrophenyl phosphate. The values of k_{cat} were obtained by multiplying $\Delta A/\Delta t$ by [4-nitrophenol]/A_∞ and dividing by the concentration of enzyme. The concentration of enzyme was calculated from the activity at pH 8.0 in the presence of 1.0 M Tris buffer, as described by Hull et al. (1976) and Bradshaw et al. (1981).

The value of k_{cat} at pH 8.0 was determined in the presence of 0.1 mM 4-nitrophenyl phosphate and 0.04 M Mops, 0.1 M Tris, or 0.67 M Tris buffer, with 0.18, 0.06, or 0.08 units/mL of enzyme, respectively. This concentration of substrate is 10-fold larger than the K_m that was reported by Hall and Williams (1986). The reactions were followed for the initial 10% of reaction and k_{cat} was obtained by multiplying the initial slopes by [4-nitrophenol]/A_∞[enzyme].

RESULTS

The Effect of Cosolvents on k_{cat}/K_m at pH 8.0. Values of the second-order rate constants, k_{cat}/K_m, for catalysis of the cleavage of 1 μM 4-nitrophenyl phosphate by alkaline phosphatase were determined in the presence of various concentrations of sucrose, glycerol, methanol, 2-methoxyethanol, ethylene glycol, and acetonitrile. Pseudo-first-order rate constants for the disappearance of 4-nitrophenyl phosphate were determined in each of these media at a constant concentration of enzyme and were used to calculate the second-order rate constant.

Figure 1 shows that the effects of organic cosolvents fall into two distinct classes. The viscogens, sucrose and glycerol, bring about a monotonic decrease of k_{cat}/K_m with increasing concentration, while the other cosolvents have little or no effect on the rate at concentrations up to ~15 volume % and then cause a decrease of enzyme activity at higher concentrations. Partial specific volumes of sucrose (Lee & Timasheff, 1981) were used to determine the percent of volume occupied by sucrose at 25 °C in aqueous solution.

Figure 2 shows that there is a linear fractional decrease of k_{cat}/K_m in the presence of increasing concentrations or a arabinose, a viscogen. This is very different from the nonlinear decreases in rate that are observed with a polar organic solvent, acetonitrile, and a denaturing agent, urea, which do not cause significant increases in viscosity (<10% at concentrations up to 5 M). This nonlinear behavior is characteristic of the cooperativity that is expected for protein denaturation, while the linear decrease in the presence of arabinose is expected for a decrease in rate that is caused by increasing viscosity in a diffusion-controlled reaction. Figure 3 shows that the fractional decrease in the relative values of k_{cat}/K_m in the presence of increasing concentrations of sucrose is linear with increasing viscosity and is consistent with a slope of 1.4. These results are also consistent with a diffusion-controlled reaction of the enzyme with 4-nitrophenyl phosphate.

However, a much larger linear fractional decrease in rate, with a slope of 4.0, was found in the presence of glycerol at viscosities up to 2.3 (not shown). Therefore, glycerol must decrease the rate by a solvent effect on the conformation and activity of the enzyme or by binding at the active site, as well as by an increase of viscosity.

Effects of Cosolvents on k_{cat}. Figure 4 shows that at pH 6.0 there is no effect of sucrose on the first-order rate constant, $k(H) = 1.2\ s^{-1}$, for hydrolysis of the phosphoenzyme intermediate, which is rate limiting at pH 6 (Hull & Sykes, 1976). Thus, sucrose has no effect on the conformation of the enzyme that affects catalysis of the chemical reaction, i.e., the hydrolysis of the phosphorylated serine hydroxyl group at the active site. However, high concentrations of glycerol or acetonitrile were found to bring about a decrease in activity of approximately 50% in the presence of 25–30 volume % of

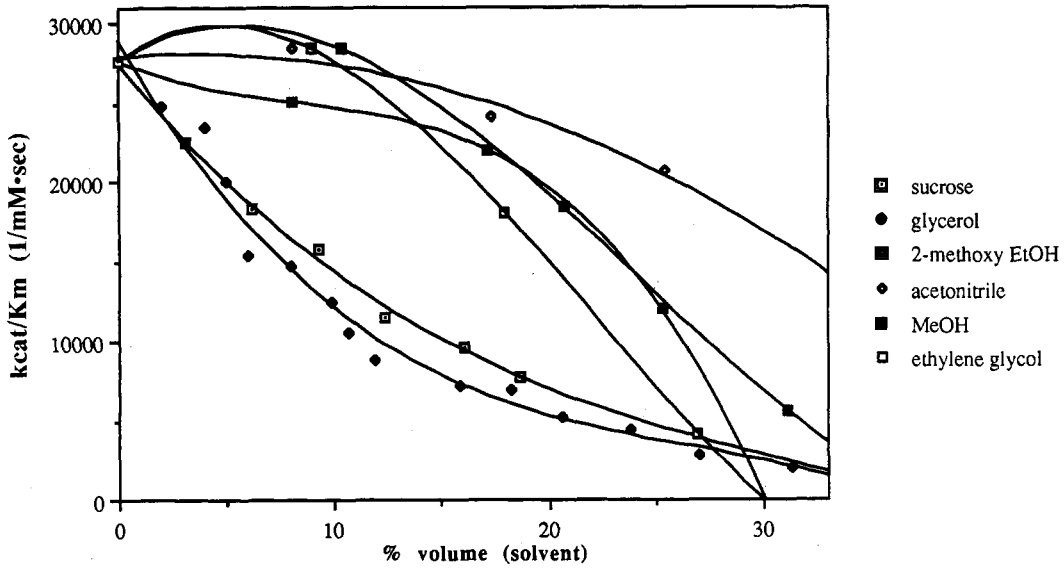

FIGURE 1: The effects of viscogens and cosolvents on the second-order rate constant, k_{cat}/K_m, for catalysis of the hydrolysis of 1 μM 4-nitrophenyl phosphate by alkaline phosphatase in the presence of glycerol, ◆; sucrose, ⊡; ethylene glycol, □; methanol, ■; 2-methoxyethanol, ▣; and acetonitrile, ◇, at 25 °C and pH 8.0 in the presence of 0.67 M Tris buffer.

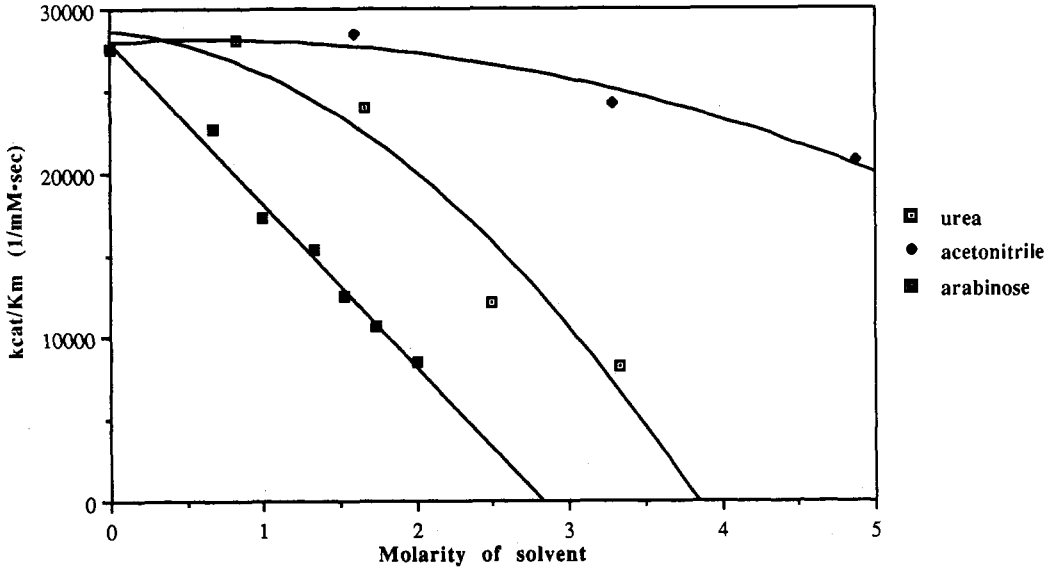

FIGURE 2: The dependence of k_{cat}/K_m for catalysis of the hydrolysis of 1 μM 4-nitrophenyl phosphate by alkaline phosphatase on the concentration of arabinose, ■, urea, ⊡, or acetonitrile (◆) at 25 °C and pH 8.0 in the presence of 0.67 M Tris buffer.

cosolvent at pH 6.0. This decrease presumably represents a solvent effect on the structure of the enzyme that decreases its catalytic activity.

At pH 8.0 the rate-limiting step for catalysis of the hydrolysis of phosphate esters in the presence of a saturating concentration of substrate is the release of product, i.e., the dissociation of inorganic phosphate from the enzyme. Figure 5 shows that the first-order rate constant for ester hydrolysis in 0.04 M Mops buffer at pH 8.0 in the presence of a saturating concentration of substrate and increasing concentrations of sucrose decreases with increasing viscosity, with a slope of $\partial(k^0/k)/(\eta/\eta^0) = 1.2$. The same slope was observed in the presence of 0.67 M Tris buffer and a slope of 1.0 was observed with 0.1 M Tris buffer. These results are consistent with diffusion-controlled dissociation of the product from the enzyme–product complex in the rate-limiting step at substrate saturation. If diffusion-controlled combination of the enzyme with substrate is rate limiting for the enzyme-catalyzed reaction in the forward direction, it is not surprising that dissociation of the product by diffusion-controlled separation of inorganic phosphate from the noncovalent enzyme•phosphate species is rate limiting in the reverse direction.

However, the hydroxyl groups of Tris can act as an acceptor for the phosphoryl group of the phosphoenzyme. This results in an increase in the maximal velocity of substrate turnover in the presence of Tris buffers because the phosphoenzyme intermediate can react with Tris, as well as with water, and thereby bypass the slow dissociation of inorganic phosphate from the enzyme–phosphate complex.

The observed maximal rate constant for the enzyme-catalyzed disappearance of 4-nitrophenyl phosphate in the presence of Tris or other alcohol acceptors, ROH, can be described by the rate law of eq a, or by eq b, which is obtained by dividing eq a by k_{hydrol}, the sum of the (pseudo) first-order rateby k_{hydrol}, the sum of the (pseudo) first-order rate constants

$$k_{obs} = k_{hydrol} + \sum k_{ROH}[ROH] \quad (a)$$

$$k_{obs}/k_{hydrol} = 1 + \sum k_{ROH}[ROH]/k_{hydrol} \quad (b)$$

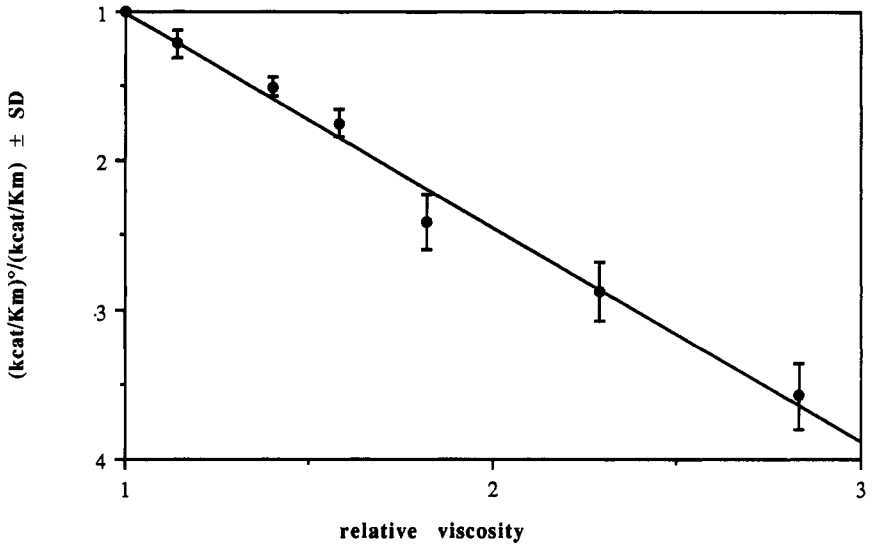

FIGURE 3: The dependence on viscosity of the second-order rate constant, k_{cat}/K_m, for catalysis of the hydrolysis of 1 μM 4-nitrophenyl phosphate by alkaline phosphatase in 0.67 M Tris buffer, at pH 8.0, in the presence of sucrose at 25 °C. The line has a slope of 1.4.

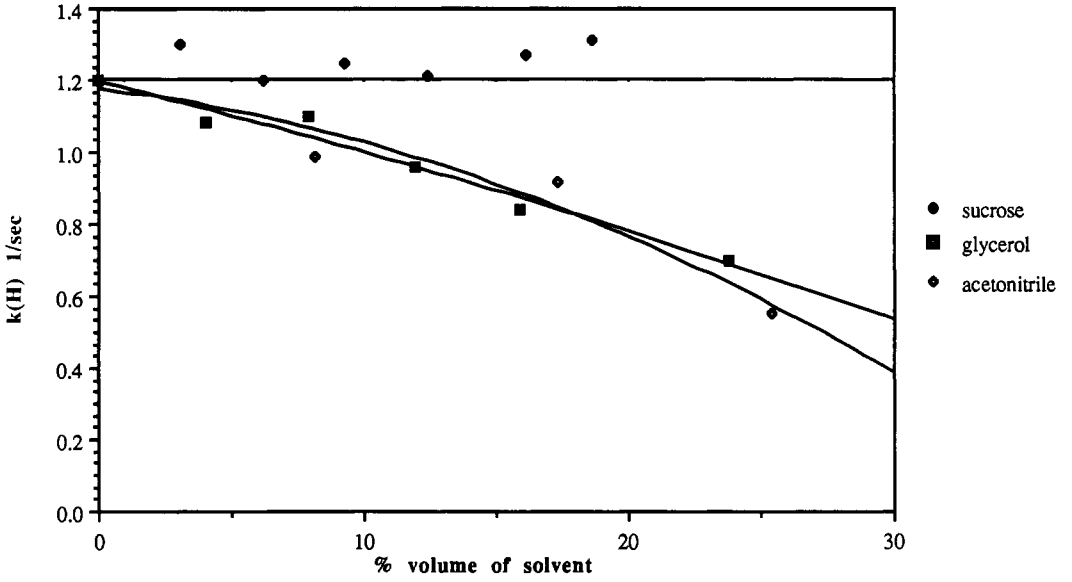

FIGURE 4: The dependence of the first-order rate constant, k(H), for catalysis of the hydrolysis of 4-nitrophenyl phosphate by alkaline phosphatase at substrate saturation (1 mM) in the presence of 0.04 M Mes buffer at pH 6.0 on the concentration of sucrose, ◆, acetonitrile, ◇, or glycerol, ■.

for reaction of the phosphorylated enzyme with water and alcohols to give inorganic phosphate and phosphorylated alcohols under the conditions of the experiment.

The ratio k_{ROH}/k_{hydrol} for Tris buffer at pH 8 has been shown to be 1.95 M^{-1} (Gettins et al., 1985). Correction of the observed rate constants for the reaction with Tris according to this ratio gives a rate constant for hydrolysis of 19 s^{-1} in the absence of viscogens, which is close to the observed rate constant of 17 s^{-1} for k_{cat} in Mops buffer. Thus, the reaction of phosphoenzyme with Tris provides an additional pathway, not an alternative pathway, for reaction of the phosphoenzyme intermediate under the conditions of these experiments.

Analogous measurements in Mops buffers at pH 8.0 in the presence of 3.26 M glycerol or 0.88 M sucrose gave ratios of k_{ROH}/k_{HOH} = 0.86 M^{-1} for reaction with glycerol and 0.34 M^- for reaction with sucrose.

If diffusional encounter of a phosphate ester with the enzyme is rate limiting for catalysis of phosphate ester cleavage by the enzyme at pH 8, then diffusion-controlled dissociation of a phosphate ester from the enzyme will be rate limiting when the phosphoenzyme reacts with an alcohol, according to the principle of microscopic reversibility. Therefore, if phosphate dianion, $HOPO_3^{2-}$, shows the same behavior as a phosphate ester dianion, its binding to the enzyme will be diffusion-controlled and its dissociation from the enzyme will also be diffusion-controlled.

A similar experiment with increasing concentrations of glycerol gave a larger slope of $\partial(k^0/k)/\partial(\eta/\eta^0) = 2.7 \pm 0.2$. This is analogous to the effect of glycerol on k_{cat}/K_m and suggests that glycerol decreases the rate by a solvent effect on the conformation and activity of the enzyme, as well as by slowing the diffusional separation of inorganic phosphate from the enzyme.

DISCUSSION

The addition of organic cosolvents to an aqueous solution of a protein generally destabilizes the protein. This destabilization can be described as an increase in the activity coefficient of the native protein, which can cause it to undergo denaturation, or to precipitate, as occurs in the fractionation

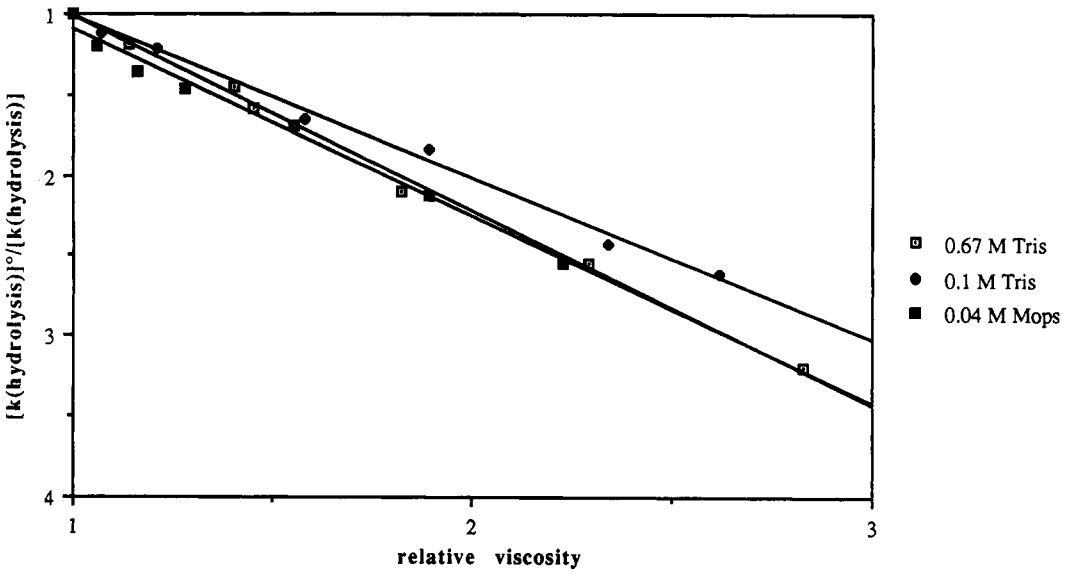

FIGURE 5: The dependence on viscosity of the first-order rate constant, k (hydrolysis), at pH 8.0 for catalysis of the hydrolysis of 4-nitrophenyl phosphate at substrate saturation in the presence of sucrose and 0.04 M Mops, ■; 0.1 M Tris, ◆; or 0.67 M Tris, ⊡; buffers at pH 8.0. The slopes of the lines are 1.2, 1.0, and 1.2, respectively.

of proteins on the addition of cosolvents at low temperature. Exposure to the organic cosolvent increases the activity coefficient of charged groups on the surface of the protein, which favors precipitation, and decreases the activity coefficient of the uncharged amino acid side chains in the interior of the protein; this favors exposure of these groups to the solvent and unfolding of the protein (Gordon & Jencks, 1963; Tanford, 1964; Robinson & Jencks, 1965; Timasheff, 1970; Lee & Timasheff, 1981).

However, the opposite behavior is observed with glycerol, sucrose, and other organic compounds with multiple hydroxyl groups. These compounds have an unfavorable interaction with the nonpolar side chains of the amino acids in an unfolded protein; they increase the activity coefficient of these groups and thereby stabilize the native, folded protein, in which the nonpolar side chains are not exposed to the solvent (Robinson & Jencks, 1965; Timasheff, 1970; Prakash et al., 1981). Therefore, the decrease in k_{cat}/K_m for catalysis of phosphate ester hydrolysis by alkaline phosphatase in the presence of these compounds is not likely to arise from denaturation of the protein.

The fractional decrease of the second-order rate constant, k_{cat}/K_m, for catalysis of the hydrolysis of phosphate esters by alkaline phosphatase in the presence of increasing concentrations of sucrose is linear with respect to increasing viscosity and follows a slope of 1.4 (Figure 2). The same slope of 1.4 was observed with arabinose as the viscogen. These results are consistent with catalysis by the enzyme in which the second-order rate constant k_{cat}/K_m represents productive combination of the substrate with the enzyme that is rate limiting for catlaysis and is diffusion controlled. The linear dependence of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ on the viscosity is expected for a diffusion-controlled reaction.

This linear dependence is very different from the highly cooperative behavior that is shown by organic cosolvents such as acetonitrile, methanol, and 2-methoxyethanol (Figure 1), which decrease the activity of an enzyme by disrupting the structure of the native protein (Timasheff, 1970). They have little or no effect on the enzyme at low concentrations and then cause a rapid decrease in activity at higher concentrations, as the enzyme unfolds. These compounds interact favorably with the side chains of nonpolar amino acids, which are generally located in the interior of the native protein, and facilitate exposure to the solvent of these amino acids in the denatured protein. In contrast, viscogens such as sucrose and glycerol stabilize the structure of the native protein because they interact unfavorably with nonpolar groups in the interior of a native protein that become exposed to the solvent when the protein is denatured, as described above (Gekko & Timasheff, 1981a,b; Lee & Timasheff, 1981).

The dependence of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ on viscosity in the presence of increasing concentrations of glycerol is also linear, but follows a slope of 4.0 that is much larger than is expected for a diffusion-controlled reaction. This shows that the relatively small glycerol molecule decreases the observed rate by binding directly to the active site or by a solvent effect that perturbs the structure and decreases the catalytic activity of the enzyme, as well as by an effect on viscosity. The latter conclusion is supported by the observation that the first-order rate constant, k(H), for hydrolysis of the phosphoenzyme at pH 6 is decreased in the presence of acetonitrile or glycerol, while the larger sucrose molecule has no effect on k(H).

The second-order rate constant of $k_{cat}/K_m = 4.6 \times 10^7$ M^{-1} s^{-1} for catalysis of the hydrolysis of 4-nitrophenyl phosphate by alkaline phosphatase at pH 8 (Snyder & Wilson, 1972) is $\sim$100 times smaller than the rate constants on the order of 5×10^9 M^{-1} s^{-1} that are observed for the diffusion-controlled encounter of small molecules in aqueous solution. However, it falls in the range of 10^6–10^8 M^{-1} s^{-1} that is generally observed for the binding of small molecules to enzymes (Eigen & Hammes, 1963). These relatively small rate constants suggest that most of the collisions between substrate molecules and the active site are not productive.

A barrier for binding could be provided by a requirement for loss of bound water from the active site before the substrate can bind productively. A water molecule has been observed between thermolysin and an inhibitor that is bound at the active site of the enzyme. This water molecule may be responsible for the slow binding of transition-state analogs that are potent inhibitors of this enzyme (Holden et al., 1987; Bartlett & Marlowe, 1987).

It is also possible that the substrate must collide with the active site in a particular orientation, or that the amino acid side chains that make up the active site must be fixed in a

favorable geometry at the moment of collision in order that an active enzyme-substrate complex can be formed. A large fraction of the catalysis that is brought about by enzymes arises from exact fixation of the substrate relative to catalytic groups at the active site. This brings about loss of translational and rotational entropy, which constitutes a large fraction of the Gibbs energy barrier for the chemical reaction and is important for the action of groups in the active site, such as acids, bases, and nucleophilic groups that bring about catalysis of the reaction by facilitating bond formation and cleavage (Jencks, 1975). This loss of entropy occurs when the substrate binds to the active site and could account for a decrease in the observed second-order rate constant below the maximum value for a diffusion-controlled reaction when most of the collisions of substrate with the enzyme are nonproductive because the orientation of the substrate relative to the active site is not optimal for productive binding (Jencks, 1975).

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