The Nature of Negative Cooperativity in Alkaline Phosphatase

KINETIC PATTERNS CONTRARY TO THE FLIP-FLOP MODEL*

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The nature of strong negative cooperativity displayed by alkaline phosphatase from Escherichia coli was investigated by alternative substrate and product inhibition studies, and by catalytic rate constant (k_cat) measurements. To see whether the idled subunit in this dimeric enzyme plays a mechanistic role in the overall catalysis, the flip-flop model proposed by Lazdunski et al. (1971) Eur. J. Biochem. 20, 124) was examined. In this mechanism, the dephosphorylation of the phosphoryl enzyme intermediate at one subunit is supposedly facilitated by the binding of substrate to the second subunit. Kinetic theory predicts that for such a model, when an alternative substrate is present at fixed levels, double reciprocal plots with respect to the substrate should be linear. Our kinetic data, however, yielded linear competitive inhibition patterns. When the alternative substrate is present at constant ratios to the substrate, linear intersecting double reciprocal plots are predicted. Instead, parallel plots were obtained for three substrate-alternative substrate pairs. Nonlinear, noncompetitive inhibition by the product inorganic phosphate is predicted for the flip-flop model, but linear competitive inhibition was observed. In addition, the k_cat determined at pH 8, 25 °C in 0.1 M Tris-HCl is 27 s⁻¹, which agrees very well with the "off-rate" of inorganic phosphate, ~25 s⁻¹, determined by Hull et al. (1976) Biochemistry 15, 1547) using the NMR technique under identical conditions. All the kinetic experiments are consistent with a mechanism in which the idled subunit plays a discernible role. They also indicate that the flip-flop mechanism is not operative in the alkaline phosphatase system.

The dependence of k_cat of alkaline phosphatase on the concentration of Tris-HCl at 10 °C and 25 °C, pH 8, showed saturation phenomena. Analysis of the data revealed that an enzyme-Tris-HCl complex was involved, and that the observed k_cat could be resolved into two components corresponding to the product release rate constants for inorganic phosphate and Tris-phosphate.

An increasing number of enzymes, among them the alkaline phosphatase from Escherichia coli, have been reported to exhibit strong negative cooperativity, or half-of-the-sites reac-

tivity (1). Such a phenomenon poses an inevitable question: what is the advantage to be gained from this extreme form of negative cooperativity? With strong negative cooperativity, substrate activation is manifested only at very high substrate levels. In the normal substrate concentration range, the Michaelis-Menten type of kinetics prevails. From a kinetic standpoint, for a dimeric enzyme or an oligomeric enzyme functioning as a polymer, the net result of "half-of-the-sites catalysis" is impressive. When compared with a normal monomeric enzyme of equivalent kinetic parameters, it amounts to improving the affinity for a substrate by a factor of two (because the dimer initially has two chances to combine with the substrate), while reducing the maximum velocity by the same factor (because of the shutdown of half of the catalytic sites). From a regulatory standpoint, the Michaelis-Menten kinetics offers no additional control. Thus, the flip-flop model advanced by Lazdunski et al. (2, 3) on the basis of mechanistic considerations becomes a highly attractive explanation for the extreme case of negative cooperativity.

Alkaline phosphatase of E. coli is a dimeric enzyme consisting of two identical subunits (4). Strong negative cooperativity has been demonstrated for this enzyme in kinetic and binding studies performed at alkaline pH with the two binding constants differing by about 1000-fold (5). Symmetry in subunit arrangement is indicated by x-ray crystallography (6). Therefore, the negative cooperativity is presumably the consequence of a ligand-induced conformational change, rather than a pre-existing asymmetry in neighboring subunits. This enzyme catalyzes the nonspecific hydrolysis of phosphomonoesters and ³¹⁰ exchange from water into organic phosphate. The catalysis involves the formation of a covalent intermediate, the phosphoryl enzyme (7, 8). The flip-flop model of Lazdunski et al. (2, 3), when applied to alkaline phosphatase, contains several salient features. Strong anti-cooperativity is still required, such that on binding of the 1st molecule of substrate, the affinity of the other subunit for substrate is greatly decreased. Phosphorylation of the enzyme then leads to the regaining of substrate-binding capacity for the other subunit, and the binding energy is utilized to facilitate the dephosphorylation process. The two subunits on the dimeric enzyme thus complete the flip-flop cycle by serving alternately as the catalytic site. With this model Lazdunski et al. (2, 3) were able to confer a role to the second subunit, and still retain the Michaelis-Menten kinetics.

This report is an attempt to use E. coli alkaline phosphatase as a model to investigate the nature of strong negative cooperativity in oligomeric enzymes. A method using alternative substrates as a probe for the flip-flop hypothesis is presented. These studies on E. coli alkaline phosphatase, together with the product inhibition studies, reveal that the flip-flop mechanism, despite its attractiveness, does not seem to operate in this particular enzyme system.

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EXPERIMENTAL PROCEDURES

Materials

E. coli alkaline phosphatase was purchased from Worthington as a suspension in ammonium sulfate. The specific activity was 35 to 65 pmol of p-nitrophenyl phosphate formed/min/mg of protein when measured in 1 M Tris-HCl, pH 8.0, at 25°C, which was comparable to that observed with the pure crystalline enzyme (9). A highly purified sample of alkaline phosphatase was kindly provided by Dr. J. E. Coleman and J. F. Chlebowski of Yale University. [32P]AMP was a product of New England Nuclear. CMP was a product of Flast Laboratories, and p-nitrophenyl phosphate was from Calbiochem. AMP, 4-methylumbelliferyl phosphate, 4-bromo-2-hydroxy-3-naphthyl-0-anisidine phosphate, and 6-bromo-2-hydroxy-3-naphthyl-O-anisidine were products of Sigma. 4-Methylumbelliforene was obtained from Koch-Light Laboratories. All other chemicals were of analytical grade. CF-50 ultrafiltration membrane cones were a product of Amicon.

Methods

Enzyme Assays

Alkaline phosphatase was assayed by one of the following procedures, depending on the substrate employed.

Determination of Specific Activity—The enzyme was assayed at a p-nitrophenyl phosphate concentration of 1 mM in 1.0 M Tris-HCl buffer, pH 8.0, at 25°C, by following the hydrolysis of the substrate at 410 nm in a 1-cm lightpath, thermostated cuvette in a Cary 17 spectrophotometer (10). An extinction coefficient of ε360 nm = 15.2 was used for the calculation of the amount of product formed. The protein concentration was determined spectrophotometrically using an extinction coefficient ε360 of 0.71 (11).

Stopped Assay with NASBIP as Substrate—The reaction mixture containing varying concentrations of NASBIP in 50 mM Tris-HCl buffer, pH 8.0, in a final volume of 2.2 ml was incubated at 30°C. The reaction was stopped by addition of 0.5 ml of 2 M NaOH, 0.2 M EDTA solution. The fluorescent product was measured in a Hitachi Perkin-Elmer MFP-2A fluorometer thermostated at 10°C, using excitation and emission wavelengths of 405 nm and 515 nm, respectively. The amount of product formed was determined from a standard curve constructed with known concentrations of NASBI-OH, and was found to be linear as a function of assay time.

Continuous Assay with 4-MUP as Substrate—The fluorescent product 4-methylumbelliferyl phosphate produced on hydrolysis of 4-methylumbelliferyl phosphate was followed, by fluorescence measurements, using excitation and emission wavelengths of 370 nm and 450 nm, respectively. The buffer used was the same as described under "Stopped Assay with NASBIP as Substrate." The reaction temperature, however, was maintained at 5°C. After centrifugation for 10 min at 5,000 rpm in Amicon CF50 ultracentrifugation membrane cones the filtrated [32P]orthophosphate was counted in a Beckman LS-250 liquid scintillation counter. The relative velocity of the reaction was calculated as

\[
\frac{c.p.m. \text{ sample} - c.p.m. \text{ control}}{c.p.m. \text{ total} - c.p.m. \text{ control}} \times \text{ (time in minutes)}
\]

where the control sample was incubated without the addition of enzyme and the total sample was diluted with 1 ml of 0.5 M phosphate/ HCl without charcoal. The reaction time was adjusted such that about 5 to 7% of the AMP was hydrolyzed in each assay.

Purification of [32P]AMP

[32P]Orthophosphate was removed from [32P]AMP by adsorption of AMP to charcoal suspended in dilute HCl, pH 2.3. The suspension was centrifuged and the supernatant containing inorganic phosphate was discarded. After a second acid wash to remove any remaining phosphate, the AMP was eluted from the charcoal with 50% ethanol, 50% 0.85 M NH4Cl, and centrifuged. The supernatant containing AMP was lyophilized, then frozen, and stored at -80°C.

All glassware was acid-washed and rinsed in double distilled water to prevent contamination by phosphate.

Theory

The flip-flop model proposed by Lazdunski et al. (2) for alkaline phosphatase can be represented by Scheme 1.

In this scheme, S represents a phosphoester ROP, and E-P is the phosphorylated enzyme intermediate. Binding of S to E-P facilitates the dephosphorylation and results in the formation of SE which is kinetically indistinguishable from ES. (For each catalytic cycle, the enzyme "flip-flops" between ES and SE.) The direct hydrolysis of E-P to E (dashed arrow) is tacitly assumed to be negligible compared with the substrate-facilitated pathway of SE → ES. The initial rate equation derived according to Scheme 1 is given by

\[
v = \frac{K_1 k_2 k_4 S^2}{[k_1 k_2 + k_5 S + k_4 k_5 S](k_1 + k_2 + k_4 S)}
\]

Equation 1 reveals that the flip-flop mechanism gives rise to S and S' terms which can be canceled to yield the Michaelis-Menten type of kinetics described by Equation 2.

The second order terms occur because the flip-flop mechanism is analogous to a ping-pong mechanism in which both substrates are identical. It is clear, then, that in the presence of an alternative substrate S', the additional reaction pathways will generate noncancellation of second power terms. Nonhyperbolic kinetic behavior is expected if flip-flop catalysis is in operation. Scheme 2 is an illustration of the flip-flop model in the presence of two substrate analogs S and S' (R'-OP):

In Scheme 2, the substrate addition steps (enclosed by dashed lines) are assumed to be in rapid equilibration in order to simplify the mathematical expression of the resultant rate equation. This assumption is consistent with our transient kinetic studies (13) which indicate that substrate binding is extremely rapid. In addition, the general mathematical form is not affected by such an assumption.
The initial velocity equation derived by the method of Cha (14) for the measurement of a single product has the following expression:

\[ v = \frac{d(ROH)/dt}{E_0} = \frac{k_2 k_3 S / K_I}{k_0 + (k' + k_3) S / K_{I'} + (k_3 + k_0) S / K_I} \]

(3)

Equation 3 predicts nonhyperbolic kinetics when both \( S \) and \( S' \) participate in the reactions.

If the flip-flop mechanism is not operative, the substrate-binding steps involving the \( E' \) intermediate need not be considered, and Scheme 2 reduces to the usual mechanism proposed for alkaline phosphatase (16-18, Scheme 3):

![Scheme 3 diagram](image)

The kinetic parameters determined for various substrates in different experiments can also be compared to verify a given model.

### Results

#### Kinetic Studies with the Alternative Substrate Present at Fixed Levels—Fig. 1 shows the double reciprocal plots of initial rate studies obtained with \( E. coli \) alkaline phosphatase at six fixed levels (0 to 10.8 \( \mu M \)) of an alternative substrate (CMP). As in NASBIP the varied substrate. The fluorogenic NASBIP-OH was the only product measured, and CMP behaved, as anticipated, as a competitive inhibitor. The linear plots shown in Fig. 1, however, are contrary to the nonlinear plots predicted for the flip-flop model by Equation 3 and are consistent with the simple model described by Equation 4. That the inhibition by CMP is “linear competitive” is further supported by the linearity of the secondary plot of the apparent \( K_m \) (Kapp) versus CMP concentration shown in the inset to Fig. 1. Furthermore, a \( K_m \) of 1.2 \( \mu M \) for NASBIP and a \( K_m \) of 2.3 \( \mu M \) for CMP were obtained. The difference in these two values indicates that the observed linear plots are not due to the fact that the two alternative substrates happen to possess identical \( K_m \) and \( k_{cat} \) values.

#### Kinetic Studies with the Alternative Substrate Present at Constant Ratios to the Substrate—The experiment described in the foregoing section provides the first indication that the flip-flop model is not operative in the alkaline phosphatase system. There are several questions, however, that can be raised: (i) Is it possible that the double reciprocal plots appear linear because the predicted curvatures are not readily detectable? (ii) Does the observed difference between the \( K_m \) values of NASBIP and CMP represent a true difference or are they identical within experimental errors?

To answer these doubts, we apply the constant ratio substrate approach described under “Theory” to the study of several pairs of alternative substrates. With this procedure, both the flip-flop and the simple models generate linear double reciprocal plots, and the kinetic constants for the same substrates obtained in different experimental designs can be used to verify their accuracy.

Fig. 2 shows the results obtained at \( pH 8.0, 30^\circ C \), when \([\textsuperscript{32}P]\text{AMP} \) was the variable substrate.\(^2\) In one of the experiments, the alternative substrate CMP (solid circles) was present at a constant ratio to \( S \), i.e., \( S'/S = \alpha \). We can convert Equation 3 to the following reciprocal form:

\[ E_o = \frac{K'_{I'} S' + K'_{I} S}{K'_{I} + k_3' K'_{I} + k_0' K_3' + k_0' K_3' S' / K_{I'}} \]

(5)

Similarly, for Equation 4, we have:

\[ \frac{E_0}{v} = \frac{1}{K_2} + \frac{1}{k_3} + \frac{(k_0 + k_3) K_{I}}{k_0 K_{I}} + \frac{k_3}{k_0} \]

(6)

where \( k_{cat} = k_0 k_3 / (k_0 + k_3) \), \( K_m = K_0 k_3 / (k_0 + k_3) \), and \( K_m' = K_0' k_3' / (k_0' + k_3') \). Equation 5 predicts that the flip-flop mechanism will yield a family of intersecting lines when the \( S'/S \) ratio, \( \alpha \), is varied; whereas Equation 6 predicts that the simple model will yield a family of parallel lines at different \( \alpha \) values. The nonlinear or linear and intersecting or parallel double reciprocal plots constitute the bases for differentiating the flip-flop and simple models. It should be noted that the alternative substrate approach outlined above can be adapted to the study of other flip-flop or alternating catalytic site systems.

The kinetic parameters determined for various substrates in different experiments can also be compared to verify a given model.
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![Graph](image)

**FIG. 1.** Double reciprocal plots of the hydrolysis of NASBIP by *E. coli* alkaline phosphatase in the presence of the alternative substrate CMP at the following CMP concentrations: ○ (zero), ○ (1.1 µM), △ (2.2 µM), ○ (4.3 µM), □ (6.5 µM), ▽ (10.8 µM). The reaction was carried out in 50 mM Tris-HCl, pH 8.0, at 30°C. Velocity is expressed in arbitrary fluorescence units. Inset, *K*<sub>mac</sub> as a function of CMP concentration.

for AMP, 1.7 µM, show that the two substrate analogs have quite different Michaelis constants.

**Product Inhibition Studies**—The flip-flop model is tested further by product inhibition studies. With this model, the product Pi should be capable of binding both the free enzyme and the ES complex. It has been well established that Pi can combine with alkaline phosphatase to form a noncovalent complex and a covalent phosphoryl enzyme (7, 8). Binding of Pi to the ES complex is simply the reverse of the SE-P → ES step. The reaction pathways are shown in Scheme 4:

![Scheme 4](image)

The initial velocity equation has the following expression:

\[
\frac{v}{E_0} = \frac{d(ROH)/dt}{E_0} = \frac{k_3(k_2 + k_{-P})}{k_2(k_1 + k_{-P}) + [K(k_2 + k_{-P})]} \cdot \frac{k_3(k_2 + k_{-P})}{k_2(k_1 + k_{-P}) + [K(k_2 + k_{-P})]} \cdot S + (k_3 + k_2 + k_{-P})S^2
\]

This equation predicts nonhyperbolic kinetics and noncompetitive inhibition by Pi. With the simple model, however, linear double reciprocal plots and competitive inhibition by Pi are expected.

Fig. 4 shows the result of Pi inhibition at pH 8.3, 10°C, when 4-MUP is the varied substrate. Clearly, the inhibition patterns are in accordance with the simple model.

All the kinetic constants obtained in the alternative substrate and product inhibition studies are summarized in Table I.

**Catalytic Rate Constant as a Function of Temperature and Tris-HCl Concentration**—A distinctive feature of the alkaline phosphatase-catalyzed reaction is the relatively invariant *k*<sub>cat</sub> observed with various substrates. This indicates that a common rate-limiting step is involved, most likely the dephosphorylation of the phosphoryl enzyme or the dissociation of Pi. In addition, *k*<sub>cat</sub> for alkaline phosphatase has been shown to be affected by Tris concentration and ionic strength.

Since the Pi dissociation rate has been measured by Hull et al. (17) using the 31P NMR technique, it is important to compare it with the *k*<sub>cat</sub> determined from initial rate studies. To make this comparison of two parameters measured under different conditions meaningful, we determined *k*<sub>cat</sub> at 10°C and 25°C, pH 8.0, at Tris-HCl concentrations ranging from 0.005 to 1.0 M. The results obtained with 4-MUP as the substrate are presented in Fig. 5. It can be seen that the buffer concentration increases from 0.005 M to 1.0 M, but the velocity is not significantly affected. This suggests that the enzyme is not saturated with substrate and that the rate of reaction is limited by the rate of dephosphorylation of the phosphoryl enzyme. The activation energy calculated from the Arrhenius plot is 12.5 kcal/mol, which is similar to the value reported for other enzymes. The *k*<sub>cat</sub> values are also consistent with the Michaelis constant determined from initial rate studies.
The strong negative cooperativity or half-of-the-sites reactivity displayed by E. coli alkaline phosphatase and many other enzymes is a phenomenon whose biological significance is not yet understood. The flip-flop mechanism proposed by Lazdunski et al. (2, 3) to explain extreme negative cooperativity seems to be a persuasive one. There are two main segments for the flip-flop model, both of which are based on experiments with alkaline phosphatase: (i) The Michaelis-Menten type of kinetics predicted by the model is consistent with the kinetic behavior of alkaline phosphatase observed at substrate levels below the millimolar range. (ii) The rate of phosphate release from the phosphoryl enzyme at pH 5.0 is increased 10-fold in the presence of a competitive inhibitor, p-chloroanilidophosphonate (2). While these observations are consistent with the flip-flop model, they cannot be considered as cogent evidence. The kinetic model can be tested by various approaches, and the increased dephosphorylation rate can also be explained simply by the fact that the inhibitor competes with P_i for the free E, thereby preventing the reassociation of P_i with the enzyme to form E-P_i, the predominant species present at pH 5.0.

We first examined the flip-flop mechanism by introducing an alternative substrate into the reaction to create alternative pathways. As has been shown under "Theory", when the alternative substrate is held at constant levels, nonlinear double reciprocal plots are predicted for this mechanism. The linear primary and secondary plots shown in Fig. 1 fail to support the flip-flop model. Rather, it is in harmony with the simple model that has been proposed by other workers (18-19).

We next used the constant ratio alternative substrate approach to differentiate between the flip-flop and the simple models. The advantage of employing the constant ratio method is that linear double reciprocal plots are predicted for both models, yet the mechanisms can be distinguished by the parallel double reciprocal plots predicted for the simple model and the intersecting patterns predicted for the flip-flop model. The data shown in Figs. 2 and 3 demonstrate that parallel reciprocal plots are obtained with three different pairs of substrate-alternative-substrate, again indicating that the simple model, rather than the flip-flop model, better describes the alkaline phosphatase reaction.

The product inhibition studies with P_i shown in Fig. 4 further indicate that the flip-flop mechanism is not operative in E. coli alkaline phosphatase. The nonlinear noncompetitive inhibition expected from the flip-flop model (Equation 7) was not observed. It should be noted that in Scheme 4, the presence of P_i necessitates the addition of a pathway between E and E-P_i. This is because the tight binding of P_i to the enzyme and the formation of E-P_i from E and P_i, as evidenced by the 32p exchange reaction, dictate that this pathway be included when P_i is present. It should also be pointed out that in the flip-flop model proposed by Lazdunski et al. (2, 3), the E-P_i → E + P_i pathway is ignored, since the dephosphorylation is supposedly dependent on the energy provided by the binding of substrate to the second subunit. By doing so, they set another requirement for the flip-flop model; that is, dephosphorylation in the presence of substrate is at least 10 times faster than that in the absence of substrate. In other words, k_cat /K_m >> k_i.

Further support for the simple model is provided by the k_cat measurements. The data presented in Fig. 5 show that the k_cat determined from initial velocity studies agrees very well with the "off-rate" of P_i determined in the absence of substrate by the NMR technique under identical conditions; that is, k_cat =
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The flip-flop model is valid, it is required that $k_{\text{cat}}$ equal $k_{\text{cat}}$, the off-rate of phosphate from the SE-P complex. However, if $k_{a}$ and $k_{b}$ are of the same magnitude, there is no reason for the product release to proceed by the $k_{b}$ pathway, unless the $S$ which supposedly binds to the $E-P$ complex plays a crucial role in the hydrolysis of the phosphoryl enzyme intermediate.

But our transient kinetic studies (13) reveal that the presence of substrate has no influence on the hydrolysis of the phosphoryl enzyme. It can be concluded, therefore, that despite the attractiveness of the flip-flop model in conferring a mechanistic role to the idled subunit, it is not the mechanism operative in the E. coli alkaline phosphatase system. The negative cooperativity observed with this enzyme is in accord with the usual mechanism: upon substrate binding to the first subunit, the second subunit is reduced to a low affinity site. Although the possibility that an alternating catalytic site mechanism may be operative in some other enzyme systems cannot be ruled out, it is clear that the flip-flop model cannot be a general mechanism for oligomeric enzymes exhibiting strong negative cooperativity or half-of-the-sites reactivity.

The effect of Tris-HCl concentration on the $k_{\text{cat}}$ of alkaline phosphatase permits further analysis of the mechanism of reaction of the enzyme. As can be seen from Fig. 5, at both 10°C and 25°C, the $k_{\text{cat}}$ dependence on Tris-HCl concentration showed saturation phenomena, indicating that complex formation between Tris-HCl and the enzyme was involved. Since Tris has been shown to enhance the hydrolysis of the phosphoester through transphosphorylation to form Tris phosphate (21), a reaction scheme involving Tris binding to the enzyme can be constructed (Scheme 5).

![Scheme 5](image)

In this scheme, $T$ denotes total Tris, since it is in great excess, and $K_{T}$ is the dissociation constant for the $T$-E-P complex. According to this scheme, the $k_{\text{cat}}$ has the expression

$$k_{\text{cat}} = \frac{k_{H_{2}O} + k_{\text{Tris}}T/K_{T}}{1 + T/K_{T}}$$

Rearrangement of Equation 8 yields

The release of $P$, from the SE-P intermediate in the flip-flop model can be represented by two steps, the hydrolysis of the phosphoryl enzyme and the dissociation of product:

$$\text{SE-P} \rightarrow \text{SE-P}, \rightarrow \text{SE} + P$$

The SE-P complex (or the E-P complex in the simple model) is not included in the reaction schemes presented in this report because, in initial velocity kinetics, the addition of such a complex will not change the general mathematical form of the rate equation. When the rate of E-P hydrolysis is sufficiently greater than the rate of product dissociation as is the case at pH 8.0 (cf. Ref. 13), the overall process can be represented by the product dissociation step.

In Schemes 1 to 4, the involvement of Tris in the hydrolysis reaction is not shown lest the main kinetic features of the mechanisms be obscured by the complexity of the equations. By substituting $k_{a}$ or $k_{b}$ in Equations 1 to 7 with an expression having the form of Equation 8, one can obtain the appropriate rate equations showing the Tris effect. The basic characteristics of the equations are not changed by the substitution.

FIG. 6. Analysis of the effect of Tris-HCl on the alkaline phosphatase reaction. The data were taken from Fig. 3 and plotted according to Equation 9. The $k_{\text{H}_{2}O}$ used, 3 s$^{-1}$ at 10°C and 10 s$^{-1}$ at 25°C, were determined by extrapolation of $k_{\text{cat}}$ values given in Fig. 3 to zero Tris-HCl concentration. The $k_{\text{Tris}}$ values determined by this plot are 27 s$^{-1}$ at 10°C and 94 s$^{-1}$ at 25°C. The $K_{T}$ values so determined are 0.17 M at 10°C and 0.50 M at 25°C.

$$\frac{1}{k_{\text{cat}} - k_{H_{2}O}} = \frac{1}{k_{\text{Tris}} - k_{H_{2}O}} \left(1 + \frac{K_{T}}{\text{Tris}}\right)$$

The values of $k_{H_{2}O}$ at 10°C and 25°C, 3 s$^{-1}$ and 10 s$^{-1}$, respectively, are obtained from Fig. 5 by extrapolation of $k_{\text{cat}}$ at zero Tris-HCl concentration. The linear plots predicted by Equation 9 are shown in Fig. 6, which indicates that the Tris effect is consistent with the formation of an enzyme-Tris complex and that the $k_{\text{cat}}$ can be resolved into two components, $k_{\text{Tris}}$ and $k_{H_{2}O}$. From Fig. 6, it is determined that $k_{\text{Tris}} = 27$ s$^{-1}$ and $K_{T} = 0.17$ M at 10°C, and $k_{\text{Tris}} = 94$ s$^{-1}$ and $K_{T} = 0.50$ M at 25°C. In view of the fact that the rate of hydrolysis of the phosphoryl enzyme intermediate is much faster than the rate of phosphate release (13) at pH 8.0, the effect of Tris does not appear to be due to a nucleophilic function. Rather, it seems to be the enhanced rate of release of the product Tris-phosphate. It should be pointed out, however, that a major effect of the Tris-HCl buffer probably derives from the chloride ion. Dayan and Wilson (21) previously reported the effect of Tris on the activity of alkaline phosphatase, which also showed a saturation phenomenon. In their experiments, the ionic strength was held constant by the addition of NaCl, and Tris increased the velocity only by a factor of two. We have confirmed the observation of Dayan and Wilson (21). In addition, binding of chloride ion to E. coli alkaline phosphatase has been demonstrated by the $^{35}$Cl NMR technique (22). Therefore, the 9-fold increase in the rate of reaction by Tris-HCl reported here probably includes the contribution of both Tris and chloride ions.

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