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Correlation of Thermodynamic and Kinetic Properties of the Phosphoryl-Enzyme Formed with Alkaline Phosphatase†

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ABSTRACT: It has been proposed, but not established, that the phosphoryl protein of unusually high thermodynamic stability formed from the reaction of *Escherichia coli* alkaline phosphatase with inorganic phosphate is identical with the phosphoryl-enzyme intermediate formed during catalysis of phosphate ester hydrolysis. Proof of this identity is now derived from our observation that from pH 5.0 to 8.0 the ratio of the enzymatic rate for $H_2{}^{18}O$ exchange into P_i to that for nitrophenyl phosphate hydrolysis is about equal to the fraction of the enzyme which is covalently phosphorylated when the enzyme is equilibrated with P_i. This equality has been theoretically predicted [Levine, D., Reid, T. W., & Wilson, I. B. (1969) *Biochemistry 8*, 2374–2380] based upon a mechanism in which it is assumed that the phosphoryl-enzyme formed during catalysis of phosphate ester hydrolysis is identical with the phosphoryl-enzyme which forms by reaction with P_i. Evidence that substrate or substrate analogue binding to dimeric *E. coli* alkaline phosphatase influences binding and catalysis at a second active site and that catalysis occurs in a ternary complex is derived from our observations that (a) at pH 8.0, the k_{cat} for $H_2{}^{18}O$ exchange into P_i is increased from 0.12 s^{-1} to 0.17 s^{-1} by the noncompetitive binding of the substrate analogue 2-hydroxy-5-nitrobenzylphosphonate to an enzyme–P_i complex, (b) the K_m for *p*-nitrophenyl phosphate is decreased by 2-hydroxy-5-nitrobenzylphosphonate, and (c) the k_{cat} for $H_2{}^{18}O$ exchange into P_i at pH 7.8 is increased from 0.083 s^{-1} with 1.0 mM P_i to 0.12 s^{-1} with 50 mM P_i. Since, with 1 mM P_i, alkaline phosphatase would be expected to be fully saturated with a molecule of P_i bound at a high affinity site but not at a low affinity site, the change in rate when the P_i concentration is increased to 50 mM indicates that altered catalysis can occur in a ternary complex containing two P_i molecules bound to the dimeric enzyme.

It has not been established that the phosphoryl-enzyme formed during alkaline phosphatase catalyzed ester hydrolysis is identical with the isolable phosphoryl-enzyme derived from the reaction of enzyme with P_i at low pH (Engstrom & Agren, 1958; Engstrom, 1964; Schwartz, 1963; Pigretti & Milstein, 1965; Reid et al., 1969). This question has previously been analyzed by a study of the rate of dephosphorylation at high pH of the phosphoryl protein formed by the reaction of enzyme with P_i at low pH. It was found (Aldridge et al., 1964) that at pH 8.4 the rate of phosphoryl-enzyme hydrolysis is twice

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Table I: Alkaline Phosphatase Catalysis for *p*-Nitrophenyl Phosphate Hydrolysis and $H_2{}^{18}O$ Inorganic Phosphate Exchange[a]

pH	k_{cat} (exchange)[b] (s^{-1})	k_{cat} (hydrolysis) (s^{-1})	k_{cat} (exchange)/ k_{cat} (hydrolysis)	[E-P]/[E_0][c]
8.0	0.34 ± 0.02	13.6	0.026	0.017
6.8	0.31 ± 0.05	1.03	0.30	0.10
5.5	0.083 ± 0.005			
5.0	0.025 ± 0.004	0.024	1.01	1.0

[a] Reactions at 25 °C, with the ionic strength maintained at 1.1 with KCl. The NphP and P_i concentrations were 0.24 and 50 mM, respectively, in the hydrolysis and exchange studies. [b] Average of two rate measurements with 0.85 and 1.7 μM enzyme. [c] Determined by Reid et al. (1969) with the enzyme in 1.0 M NaCl. We interpolated the pH 6.8 value from results at pHs 7.0 and 6.5.

the steady-state rate for NphP[1] hydrolysis. This observation indicates that either the P_i-generated phosphoryl-enzyme is not identical with the intermediate in NphP hydrolysis or that dephosphorylation is not rate limiting with NphP. A subsequent study (Reid & Wilson, 1971a,b) showed that the kinetic properties of the phosphoryl-enzyme formed from P_i inexplicably depend upon how long the enzyme is incubated in a pH 7.0 buffer: the half-life for dephosphorylation is about 2 ms following a 2-h incubation, 11 ms after a 24-h incubation, and 3 s after 1 month. These results indicate that it is not possible to derive unambiguous conclusions from kinetic studies with the P_i-generated phosphoryl protein.

Wilson and co-workers (Levine et al., 1969; Reid & Wilson, 1971a,b) have proposed a method for determining whether the P_i-generated phosphoryl-enzyme is identical with the phosphoryl-enzyme formed during NphP hydrolysis. The method involved analysis of the kinetics for the reversible enzyme phosphorylation by P_i from study of the enzyme-catalyzed exchange of $H_2{}^{18}O$ into P_i. Correlation of the kinetics for the exchange reaction and hydrolysis of NphP, with the thermodynamics properties of the P_i-generated phosphoryl-protein, provides a test capable of determining whether the P_i-generated phosphoryl-enzyme is identical with the phosphoryl-enzyme formed during NphP hydrolysis. An experimental test of this proposal is described here.

Materials and Methods

2-Hydroxy-5-nitrobenzylphosphonate was synthesized as described previously (Halford et al., 1969): NMR (D_2O) δ 2.88 (d, J = 18 Hz) [Halford et al., 1969: δ 2.75 (d, J = 19 Hz)]. Anal. Calcd for $C_7H_5O_6NK_3P$ (mol wt 347.4): C, 24.20; H, 1.45; P, 8.92. Found: C, 24.25; H, 2.64; P, 8.93. Titration in 1.1 M KCl at 25 °C indicated three ionizable groups of equal neutralizing capacity with pK_a values of <3, 5.9, and 9.0 [Halford et al. (1969) report pK_as of <3, 6.3, and 9.4].

E. coli alkaline phosphatase (BAPC grade from Worthington) was dialyzed against 4×10^{-3} M $ZnCl_2$ in 1×10^{-4} M 2-(*N*-morpholino)ethanesulfonic acid at pH 6 in a Bio-Rad Biofiber Minibeaker, to remove ammonium sulfate and activate the enzyme. Subsequent exhaustive dialysis removed excess $ZnCl_2$. Activity was assayed spectrophotometrically at 25 °C, with 1.0 M Tris-HCl, pH 8.0, and 1×10^{-3} M NphP (obtained from Sigma). The specific activity for the different batches of enzyme used was 16–24 units/mg, where 1 unit represents 1×10^{-6} mol of substrate hydrolyzed per h. Results in Table I are corrected for variations in specific activity with different batches of enzyme. A molecular weight of 86 000 (Schlesinger & Barrett, 1965) and $E_{278}^{0.1\%} = 0.77$ (Rothman & Byrne, 1963) were used to determine the enzyme concentration. Enzyme assays were performed with a thermostated Zeiss PMQ II or Guilford 240 spectrophotometer, and spectra of the HNBP solutions were recorded with a Perkin-Elmer Coleman 124 instrument.

P_i–$H_2{}^{18}O$ exchange reactions were conducted in stoppered tubes under a toluene atmosphere at 25 °C, with an ionic strength of 1.1 maintained with KCl. The reactions were initiated by adding enzyme to a solution of P_i at the appropriate temperature. The pH was immediately recorded, and minor adjustments were made using either HCl or NaOH. Aliquots of the reaction mixture were removed at intervals and analyzed according to Applebury et al. (1970) in some of the reactions run with 0.050 M P_i and according to Boyer & Bryan (1967) in all other reactions. There was no difference between the rates obtained from the two methods of analysis. The content of ^{18}O of the purified inorganic phosphate was determined by the guanidine·HCl method (Boyer et al., 1961), using a Consolidated Electrodynamics 21-614 mass spectrometer. The percent excess ^{18}O was calculated according to eq 1 (Shain & Kirsch, 1968), where R represents the ratio

$$\% \text{ excess } {}^{18}O = [R/(2 + R)] \times 100 - 0.215 \quad (1)$$

of the magnitude of the peaks of m/e at 46 and 44. The natural isotope abundance of oxygen-18 in water was measured at 0.215%. When the percent ^{18}O is less than 2, the m/e peak at 48, resulting from $^{18}O{=}C{=}{}^{18}O$, is insignificant. When the percent ^{18}O was greater than 1.5, the ^{18}O content was calculated according to eq 2, in which 44, 46, and 48 refer to the

$$\% \text{ excess } {}^{18}O = \frac{(46) + 2(48)}{2(44 + 46 + 48)} \quad (2)$$

magnitude of the peaks at m/e 44, 46, and 48, respectively. The rate constants for exchange were calculated according to Boyer & Bryan (1967). When HNBP was included in oxygen exchange reaction mixtures, it was removed prior to isolation of the inorganic phosphate, by treatment with charcoal (Boyer & Bryan, 1967).

The alkaline phosphatase catalyzed hydrolysis of NphP at pH 8.0, 6.8, and 5.0 was followed by a quenching technique. One-milliliter aliquots of reaction mixtures containing 5×10^{-2} or 1×10^{-2} M Tris-acetate buffer, 1.1 M KCl, 2.4×10^{-4} M NphP, and 1.7×10^{-6} M enzyme were mixed with 2 mL of 0.5 M NaOH. This stopped the enzymic hydrolysis of the substrate, and the concentration of product *p*-nitrophenolate was determined from measurement of the absorbance at 400 nm. The enzyme was shown to be saturated with NphP, and the reactions followed zero-order kinetics. Rates were extrapolated to zero buffer concentration.

The activation of alkaline phosphatase by HNBP was examined by varying the concentration of NphP in the absence and presence of 2×10^{-4} M phosphonate. Hydrolysis of NphP was followed by observing the increase in absorbance at 400 nm, and the rates were computer fitted to the Michaelis–Menten equation (Hansen et al., 1967).

Results

Kinetics of NphP hydrolysis and exchange of $H_2{}^{18}O$ into inorganic phosphate were studied under identical reaction conditions; results obtained in typical oxygen exchange experiments are shown in Figure 1. At pH 8.0 k_{cat} for exchange

[1] Abbreviations used: HNBP, 2-hydroxy-5-nitrobenzylphosphonate; NphP, *p*-nitrophenyl phosphate.

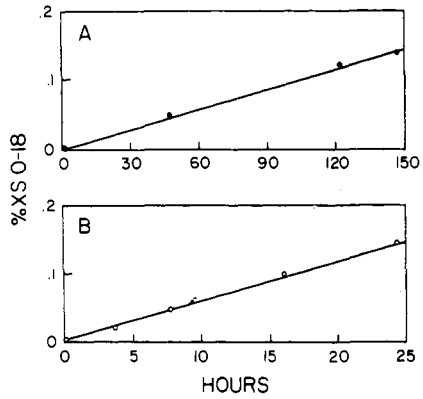

FIGURE 1: Alkaline phosphatase catalyzed exchange of phosphate and $H_2{}^{18}O$. (A) Reaction at pH 5.0, with 50 mM P_i, 1.7×10^{-6} M alkaline phosphatase, and 1.44% excess $H_2{}^{18}O$. The straight line is a theoretical line calculated for $k_{cat} = 0.022\ s^{-1}$. (B) Reaction at pH 8.0, with 50 mM P_i, 8.5×10^{-7} M alkaline phosphatase, and 1.36% excess $H_2{}^{18}O$. The straight line is a theoretical line calculated for $k_{cat} = 0.27\ s^{-1}$.

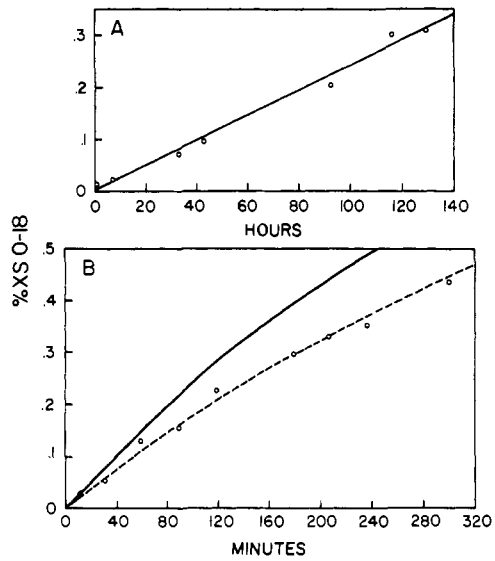

FIGURE 2: Effect of phosphate concentration on the rate of exchange of phosphate and $H_2{}^{18}O$. Reactions were at pH 7.8, with 1.0 M KCl, 0.01 M Tris-HCl, 1×10^{-6} M alkaline phosphatase, and 1.13% excess $H_2{}^{18}O$. (A) Reactions with 50 mM P_i; the solid line is a theoretical curve calculated for $k_{cat} = 0.12$. (B) Reaction with 1 mM P_i; the dashed line is a theoretical curve calculated for $k_{cat} = 0.0825\ s^{-1}$ and the solid line is for $k_{cat} = 0.12\ s^{-1}$.

of $H_2{}^{18}O$ into P_i is 2.6% of that for NphP hydrolysis (Table I). However, because the pH dependence for catalysis of the two reactions differs, the rates for NphP hydrolysis and oxygen exchange into P_i are identical at pH 5.0 (Table I).

The k_{cat} for enzyme-catalyzed exchange of $H_2{}^{18}O$ into P_i at 20 °C decreased from 0.12 s^{-1} with 50 mM P_i to 0.083 s^{-1} with 1 mM P_i (Figure 2). The solid line in Figure 2B is calculated by assuming that the rate constant with 1 mM P_i is equal to that determined with 50 mM substrate. The poor fit of the observed isotopic enrichment to this calculated curve indicates that the rate depends upon the substrate concentration in the 1–50 mM range.

Binding of the phosphonate derivative HNBP to alkaline phosphatase can be detected spectrophotometrically by an increase in absorbance at 430 nm (Halford, 1972). Addition of P_i to a solution containing the HNBP–enzyme complex generates a ternary complex composed of enzyme, HNBP,and P_i, which can be detected by difference spectroscopy (Halford, 1972). We have confirmed in spectrophotometric studies that

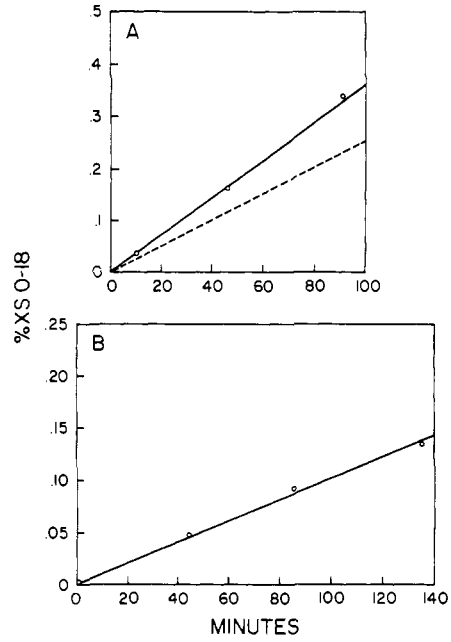

FIGURE 3: Effect of 250 μM HNBP on catalysis of exchange of phosphate and $H_2{}^{18}O$. Reactions at 25 °C, pH 8.0, μ = 1.1 (KCl), with 25 mM P_i, 0.1 M Tris-HCl, 13.5×10^{-6} M alkaline phosphatase, and 2.60% excess $H_2{}^{18}O$. (A) Reaction with HNBP; the solid line is a theoretical curve calculated for $k_{cat} = 0.17\ s^{-1}$ and the dashed line is for $k_{cat} = 0.12\ s^{-1}$. (B) Rate without added HNBP. Conditions as in (A), except that 1.03% excess $H_2{}^{18}O$ was used. The solid line is for $k_{cat} = 0.12\ s^{-1}$.

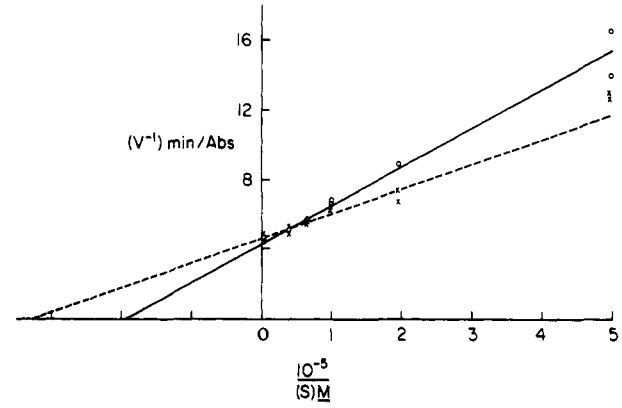

FIGURE 4: Effect of 200 μM HNBP on the alkaline phosphatase catalyzed hydrolysis of *p*-nitrophenyl phosphate. Reactions were at 25 °C, pH 8.0, μ = 1.1 (KCl), with 0.01 M Tris-acetate and 1.0×10^{-8} M alkaline phosphatase. Results from reactions with and without HNBP are represented by × and ○, respectively.

a ternary complex forms under conditions identical with those previously described, using 13.6 μM enzyme, 250 μM HNBP, and $H_2{}^{18}O$ (Caswell, 1976). This complex is catalytically active, since the rate constant for enzyme-catalyzed exchange of $H_2{}^{18}O$ into P_i is increased from 0.12 to 0.17 s^{-1} by 250 μM HNBP (Figure 3). The rate enhancement is much greater than the experimental uncertainty, as shown by the marked deviation of the experimental data from the theoretical line drawn by assuming that HNBP has no effect on the rate (dashed line in Figure 3A).

HNBP activation of alkaline phosphatase is also seen inthe enzymatic hydrolysis of NphP (Figure 4): with 0.2 mM HNBP, k_{cat}/K_m is 1.55 times that found in the absence of the phosphonate. The effect of HNBP is greatest at low NphP concentration (Figure 4). From the Lineweaver–Burk plot, it is calculated that in the presence of phosphonate k_{cat} equals $21.8 \pm 0.3\ s^{-1}$ and K_m equals 3.1 ± 0.03 μM; in the absence

Scheme I

path I (hydrolysis)

$$E + S \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_3'} E{-}P + ROH$$

$$k_4 \downarrow \uparrow k_3$$

$$E{\cdot}P_i \underset{k_1}{\overset{k_2}{\rightleftharpoons}} E + P_i$$

path II (exchange)

$$E + P_i \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}P_i \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{-}P + H_2O$$

$$(\pm H_2{}^{18}O)\ k_4 \downarrow \uparrow k_3$$

$$E{\cdot}P_i{}^* \xrightarrow{k_2} E + P_i{}^*$$

of HNBP, k_{cat} and K_m are 23.5 ± 0.3 s^{-1} and 5.2 ± 0.3 μM, respectively.

Discussion

Correlation of the Enzyme Catalysis with the Thermodynamic Stability of the Phosphoryl Protein. Levine et al. (1969) have investigated the question of whether the phosphoryl-enzyme intermediate formed during the alkaline phosphatase cleavage of esters is identical with the phosphoryl protein formed by reaction of the enzyme with P_i. This was done by using the kinetic parameters for enzymatic cleavage and synthesis of esters, calculated for the proposed reaction mechanism, to predict the thermodynamic stability of the P_i-generated phosphoryl protein. Although the correlation of predicted with measured thermodynamic properties was successful [see Table III of Levine et al. (1969)], their further attempt to predict the kinetic parameters for P_i–oxygen exchange and ester hydrolysis from the measured thermodynamic stability of the P_i-generated phosphorylation was not subsequently confirmed (see below). Further studies of the oxygen exchange were also not in agreement with the predictions of this kinetic scheme (see below). We have, therefore, studied the oxygen exchange and ester hydrolysis reactions to clarify this problem.

Levine et al. (1969) have analyzed the enzymatic hydrolysis of esters and the exchange of $H_2{}^{18}O$ into P_i as shown in Scheme I. In these reactions E·S is a Michaelis complex containing NphP, E–P is a phosphoryl-enzyme, and $E{\cdot}P_i{}^*$ is a Michaelis complex containing P_i which is enriched with oxygen-18. If the loss of P_i from $E{\cdot}P_i$ is faster than enzymatic phosphorylation by P_i (i.e., $k_2 > k_3$),[2] or phosphoryl-enzyme hydrolysis (i.e., $k_2 > k_4$),[3] and dephosphorylation is rate limiting in NphP hydrolysis ($k_3 > k_4$),[4] then $k_{cat} = k_4$ for NphP hydrolysis; these relationships between the rate constants are believed to be correct for reactions at low pH and are approximations at higher pH.[2-4] The k_{cat} for oxygen exchange was given as equal to $k_3/[1 + k_3/k_4]$. These rates were related to the thermodynamic stability of the phosphoryl-enzyme by

$$\frac{k_{cat}(\text{exchange})}{k_{cat}(\text{hydrolysis})} = \frac{[E{-}P]}{[E_0]} \qquad (3)$$

where $[E_0]$ is the total enzyme concentration and [E–P] is the amount of phosphoryl-enzyme present at equilibrium in a mixture of enzyme and P_i.

There is good agreement between the previously determined values of $[E{-}P]/[E_0]$ at pHs 8.0, 6.8, and 5.0 (Reid et al., 1969), given in the fifth column in Table I, and the ratio of the rates determined by us for NphP hydrolysis and exchange of $H_2{}^{18}O$ into P_i; the observed ratio for these reactions is given in the fourth column in Table I. The agreement is perfect at pH 5.5, where the phosphoryl protein is most stable and easily quantitated. At higher pHs the formation of the phosphoryl protein is decreased and less accurately determined. Our experimental confirmation of eq 3 proves that the phosphoryl protein formed from reaction of enzyme with P_i is identical with the phosphoryl-enzyme proposed from kinetic analysis of substrate hydrolysis.

In an earlier analysis of eq 3 based upon studies of 2-naphthyl phosphate and *O*-phosphorylethanolamine hydrolysis (Levine et al., 1969), the k_{cat} values for oxygen exchange into P_i catalyzed by alkaline phosphatase in 1 M NaCl at 25 °C were predicted to be 0.08, 0.68, and 0.51 μmol/min/mg of enzyme at pH 7.0, 6.0, and 5.5, respectively. This pH dependence was, however, not observed in a subsequent study (Applebury et al., 1970), where it was reported that catalysis is pH invariant in the pH range 5.0–8.0 (μ = 0.1). Our results (column 5, Table I) from studies at an ionic strength of 1.1 are also not in accord with the previously predicted pH dependence for the $H_2{}^{18}O$–P_i exchange. The basis for the discrepancy between the earlier calculated (Levine et al., 1969) and subsequently observed (Table I and Applebury et al., 1970) pH dependence is unknown.

The earlier results (Schwartz, 1963) from the study of the rates of enzyme-catalyzed $H_2{}^{18}O$ exchange into P_i and β-glycerol phosphate hydrolysis do not fit eq 3. However, the rates for the exchange of $H_2{}^{18}O$ into P_i (Schwartz, 1963) had an inexplicable nonlinear dependence on time and enzyme concentration, so that these data are not reliable.

Results obtained by Coleman and co-workers (Applebury et al., 1970; Chlebowski & Coleman, 1974) can be analyzed by eq 3. From data shown in Figure 4A of Chlebowski & Coleman (1974), the k_{cat}(exchange)/k_{cat}(hydrolysis of *p*-phenylazophenyl phosphate) values are approximately 0.18, 0.029, and 0.008 at pH 5.5, 6.5, and 8.0, respectively. At the corresponding pH values, $[E{-}P]/[E_0]$ (Figure 4 in Applebury et al., 1970) ratios are approximately 0.45, 0.12, and 0.01. The lack of agreement at pH 5.5 is inexplicable since pre-steady-state rate measurements (Chlebowski & Coleman, 1974)

[2] Bock & Cohn (1978) have determined, from analysis of the distribution of ^{18}O in P_i after enzymic catalysis of the ^{18}O exchange reaction, that $k_2 > k_3$ over the pH range 5–10, at low ionic strength. Unless the k_2/k_3 ratio is dramatically changed at the higher ionic strength used here, eq 3 will predict $[E{-}P]/E_0]$ from the rates for hydrolysis and exchange.

[3] From analysis of the ^{31}P NMR line width of the E·P complex at low ionic strength (Hull et al., 1976), k_2 (for the schemes given here) is 10–20 s^{-1} at pH 8 and 50 s^{-1} at pH 4; k_2 has been determined to be ~10 to 60 s^{-1} in other studies (Chlebowski et al., 1977; Chlebowski & Coleman, 1974; Otvos et al., 1979). Since k_4 is small at low pH (Hull et al., 1976), $k_2 > k_4$ under these conditions; therefore, eq 3 is expected to correctly describe the reactions at low pH. At high pH, k_2 and k_4 are about equal (Coleman & Chlebowski, 1979). As a result, the simple relationship given in eq 3, $[[E{-}P]/[E_0] = k_{ex}/k_{hyd} = (k_3)/(k_3 + k_4)]$, is replaced by $[E{-}P]/[E_0] = (nk_3)/(nk_3 + k_4)$, where $n = [1 + (k_3/k_2) + (k_4/k_2)]$. As a result, exact correlation of $[E{-}P]/[E_0]$ with the k_{ex}/k_{hyd} ratio is not expected; with k_2 equal to k_4, the deviation will be approximately twofold.

[4] The question whether dephosphorylation is rate limiting in NphP hydrolysis at alkaline pH is not resolved (see Reid & Wilson, 1971a,b). If dephosphorylation is not rate limiting, then eq 3 is changed to $[k_{cat}$(exchange)$]/[k_{cat}$(hydrolysis$] = (k_4/k_3')([E{-}P]/[E_0])$. It is, however, not possible to determine whether dephosphorylation is rate limiting at alkaline pH from the fit of the results to eq 3 because $[E{-}P]/[E_0]$ is very small at alkaline pH and is therefore subject to large error; also, if values for k_4 and k_3' do not appreciably differ, the deviation from eq 3 is not expected to be significant.

showed that dephosphorylation is rate limiting at this pH ($k_3' > k_4$), so that eq 3 is expected to be valid. At higher pHs deviation from eq 4 may be to some extent related to the inapplicability of some of the assumptions required in the derivation of eq 3 (see above and footnotes 2–4).

Catalysis in a Ternary Complex. We present here three experiments in which it is demonstrated that catalysis by alkaline phosphatase can occur in a ternary complex.

(a) HNBP decrease the K_m for NphP (Figure 4). This is consistent with a mechanism in which a binary complex composed of enzyme and HNBP has enchanced affinity for NphP. A ternary complex containing enzyme, HNBP, and NphP is formed in the presence of HNBP and saturating concentrations of NphP, and this species is catalytically active, as indicated by the equal k_{cat} in the presence and absence of HNBP (Figure 4).

(b) The k_{cat} for oxygen exchange into P_i is increased by HNBP (Figure 3); it has been previously demonstrated (Halford, 1972) that a ternary complex containing enzyme, P_i, and HNBP is formed under these reaction conditions. The increase in k_{cat} indicates that the ternary complex is catalytically active.

(c) Equilibrium binding studies show that P_i can bind to alkaline phosphatase at high and low affinity sites (see Coleman & Chlebowski, 1979); at the ionic strength used here the dissociation constants are 8.0 μM and >4 mM (Simpson & Vallee, 1970). Our observation that the rate of oxygen exchange is increased when the P_i concentration is increased from 1 mM, where only the high affinity binding site is saturated, to 50 mM where both sites are saturated provides evidence that (1) a ternary complex composed of enzyme and two P_i molecules is formed, (2) the ternary complex is catalytically active, and (3) the ternary complex has altered activity, as compared to a binary complex of enzyme and P_i.

In these three reactions it is not known whether the two ligands bind to the same or different subunits of the dimeric enzyme; this question will be difficult to answer. If binding is to different subunits, then the enhanced activity seen with ternary complexes containing the nonhydrolyzable phosphonate HNBP indicates that ligand binding at one subunit modifies activity of the other subunit. In the case of the enzyme complex containing two P_i molecules, this same factor may be responsible or, alternatively, both subunits can catalyze the exchange reaction.

Previous evidence that alkaline phosphatase forms a catalytically active ternary complex includes the observations that (a) the substrate analogue *p*-chloroanilidophosphonate produces a 10-fold increase in the rate of dephosphorylation of the phosphoryl-enzyme formed by reaction of enzyme with P_i at pH 5.0 (Lazdunski et al., 1971), (b) there is significant substrate activation at high concentration at high concentrations of NphP (Simpson & Vallee, 1970; Gottesman et al., 1969) and *p*-phenylazophenylphosphorothioate (Chlebowski & Coleman, 1974), and (c) binding of NphP to the enzyme is enhanced by imido diphosphate and the ternary complex composed of enzyme, imido diphosphate, and NphP is catalytically active (Kelly et al., 1974).

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