The Free Energy of Hydrolysis of the Phosphoryl-enzyme Intermediate in Alkaline Phosphatase Catalyzed Reactions*

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ABSTRACT: The kinetics of the hydrolysis of phosphate esters catalyzed by alkaline phosphatase (*Escherichia coli*) was studied in detail, including the effect of the addition of ethanolamine as a second phosphate acceptor, in order to evaluate the equilibrium constants for the reaction of inorganic phosphate with the enzyme. Two intermediates, a dissociable enzyme–phosphate complex (Michaelis complex), and a covalent phosphoryl-enzyme were assumed to be formed. The dissociation constants for the enzyme–phosphate complex are 1.2×10^{-4}, 7.6×10^{-6}, 2.3×10^{-6}, and 2.5×10^{-6} and the equilibrium constants for the hydrolysis of the phosphoryl-enzyme are 0.18×10^{-4}, 0.15×10^{-4}, 2.1×10^{-4}, and 1.5×10^{-4} at pH 5.5, 6.0, 7.0, and 8.0. These constants are for analytic concentrations and water at unit activity. Both intermediates are very stable with respect to enzyme plus inorganic phosphate at pH 5.5–8.0, the only pH range studied. However, the dissociable complex is much more stable than the phosphoryl-enzyme at pH 8.0 and 7.0, slightly more stable at pH 6.0 and considerably less stable at pH 5.5. The result is that in the equilibrium of the enzyme with inorganic phosphate, there is only 1 or 2% phosphoryl-enzyme at pH 8.0 and 7.0 but 35% at pH 6.0 and 90% at pH 5.5. These concentrations of phosphoryl-enzyme, although somewhat higher, are in remarkable agreement with the results of the numerous ^{32}P-labeling experiments of Engstrom, Agren, Schwartz, Lipmann, Pigretti, and Milstein. This suggests that the labeled phosphoprotein obtained by incubating alkaline phosphatase and inorganic phosphate is the phosphoryl-enzyme intermediate. The covalent phosphoryl-enzyme is about one million times more stable than simple phosphate esters probably because some of the binding interactions in the enzyme–phosphate complex are retained in the covalent intermediate.

The enzyme-catalyzed rate of oxygen exchange in inorganic phosphate was calculated for different pH's and it was found that the rate of exchange relative to the rate of hydrolysis of a phosphate ester should have the same value as the fraction of enzyme that exists as phosphoryl-enzyme. The calculated values agree with the meager published data for oxygen exchange.

A number of observations suggest that a phosphoryl-enzyme intermediate occurs during the hydrolysis of phosphate esters.

Foremost among these, is the remarkable finding that alkaline phosphatase preparations incubated at acid pH (≤ 6) with 10^{-3}–10^{-4} M ^{32}P-labeled P_i yielded labeled protein from which labeled serine phosphate could be obtained (Engstrom and Agren, 1958; Engstrom, 1961, 1962a,b, 1964; Schwartz and Lipmann, 1961; Schwartz, 1963; Milstein, 1964; Pigretti and Milstein, 1965). It has been assumed that only one specific serine residue is phosphorylated. Actually it is to be expected that many acceptor groups available in the solution will be phosphorylated since alkaline phosphatase is a nonspecific enzyme and can catalyze the phosphorylation of numerous acceptors. The extent of phosphorylation is dictated at equilibrium by the free energy of hydrolysis of the resulting phosphate ester and is independent of *any* enzymic mechanism. The unusual circumstance in the experiments referred to above is that the resulting phosphoprotein is inordinately stable, it is 10^6 times as stable as ordinary phosphate esters (Wilson and Dayan, 1965). However, since there is no special reason why a phosphoryl-enzyme intermediate should be so stable and indeed such an *a priori* suggestion might have been considered unattractive, it is evident that these labeling experiments by themselves bear no witness to the formation of a phosphoryl-enzyme intermediate.

Other observations (Aldridge *et al.*, 1964; Fernley and Walker, 1966; Fife, 1967; Williams, 1966; Ko and Kézdy, 1967; Barrett *et al.*, 1969; Gutfreund, 1967) are consistent with or indicate a phosphoryl-enzyme intermediate. The seeming lack of specificity of the enzyme can be explained by assuming that dephosphorylation is rate controlling.

If we agree that a phosphoryl-enzyme intermediate occurs, it would seem that the latter and the phosphoprotein obtained in the labeling experiments are the same. However, the identity of the two has not been established. There is, of course, always the possibility that the protein that is labeled is nonenzyme protein in the preparation. At the same time it is quite possible that alkaline phosphatase is a phosphoprotein regardless of whether it also forms a phosphoryl-enzyme intermediate. There are, of course, enzymes that are phosphoproteins; phosphorylase *a* comes readily to mind and it does not form a phosphoryl-enzyme intermediate.

The purpose of the present paper is to use a kinetic method (Epand and Wilson, 1964) to evaluate the equilibrium constant for the hydrolysis of the phosphoryl-enzyme intermediate at different pH's and also to compare these values with

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the values obtained for the phosphoprotein from the labeling experiments. Will the free energies of hydrolysis be consistent with the proposition that the phosphoryl-enzyme intermediate and the phosphoprotein are one?

This method will allow us also to evaluate the equilibrium constant for the dissociation of the noncovalent enzyme–phosphate complex and various kinetic parameters. Finally we shall be able to calculate the rate of oxygen exchange between P_i and water.

This method has already been applied for pH 8.0 (Wilson and Dayan, 1965) but lower pH's are more interesting for this purpose because no phosphoprotein forms at pH 8.0.

The information we seek concerns the equilibria between P_i and alkaline phosphatase (E)

$$E + P_i \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot P_i \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{–}P + H_2O \qquad (I)$$

The equilibrium constant for dissociation of the Michalis complex, $E\cdot P_i$, *i.e.*, the complex that does not involve the covalent bond is

$$\frac{(E)(P_i)}{(E\cdot P_i)} = \frac{k_2}{k_1} \qquad (1)$$

and the equilibrium constant for the hydrolysis of the phosphoryl-enzyme, E–P, to yield enzyme and P_i is

$$\frac{(E)(P_i)}{(E\text{–}P)} = \frac{k_2k_4}{k_1k_3} \equiv K_2 \qquad (2)$$

where k_4 is defined to contain the concentration of water. The activity of water is taken as 1 in dilute solution. Similarly

$$\frac{(E\text{–}P)}{(E\cdot P_i)} = \frac{k_3}{k_4} \qquad (3)$$

at equilibrium.

Phosphate competitively inhibits the hydrolysis of phosphate esters. The appropriate reaction is

$$\begin{array}{l} E + S \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} E\cdot S \xrightarrow{k_3'} E\text{–}P + P_1 \\ \qquad\qquad (+H_2O)\ k_4 \downarrow \quad \uparrow k_3\ (-H_2O) \\ \qquad\qquad E\cdot P_i \underset{k_1}{\overset{k_2}{\rightleftharpoons}} E + P_i \end{array} \qquad (II)$$

$$S{=}ROP{<}\begin{matrix} O \\ \| \\ OH \\ OH \end{matrix}$$

The steady-state solution has the Michaelis–Menten form for competitive inhibition

$$v = k_3'(E\cdot S) = \frac{k[E_0]}{1 + \dfrac{K_m}{(S)} + \dfrac{K_m(P_i)}{(S)K_i}} \qquad (4)$$

where

$$k = k_3' \Big/ \left(1 + \frac{k_3'}{k_4}\left[1 + \frac{k_3}{k_2} + \frac{k_4}{k_2}\right]\right) \qquad (5)$$

$$K_m = \frac{k_2' + k_3'}{k_1'} \Big/ \left(1 + \frac{k_3'}{k_4}\left[1 + \frac{k_3}{k_2} + \frac{k_4}{k_2}\right]\right) \qquad (6)$$

$$K_i = \frac{k_2}{k_1} \Big/ 1 + \frac{k_3}{k_4} \qquad (7)$$

The value of k_2 is probably very large compared with k_3 and k_4 so that the above expressions for k and K_m become rather less complicated. We note that K_i is not an equilibrium constant but is the Michaelis–Menten constant for phosphate acting as a substrate. By measuring both K_2 and K_i we can separate out k_2/k_1 and k_3/k_4 and thus we can evaluate the fraction of enzyme that exists as E–P and also the fraction that exists as $E\cdot P_i$ at any phosphate concentrate. The measurement of K_i involves simply using P_i as a competitive inhibitor in the hydrolysis of any substrate, but the measurement of K_2 is more involved.

Evaluation of K_2. To explain the kinetic method for evaluating K_2, let us consider the solvolysis of a substrate, S, to be specific 2-naphthyl phosphate, in the presence of an added phosphate acceptor, such as ethanolamine. This reaction is the same as for the hydrolysis of a substrate involving a covalent intermediate (Wilson *et al.*, 1950; Wilson, 1954) with the addition of a transphosphorylation pathway

$$\begin{array}{l} E + S \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} E\cdot S \xrightarrow{k_3'} E\text{–}P + P_1 \\ \qquad\qquad\quad ROH \swarrow_{k_5'} \quad H_2O \quad \searrow_{k_4'} \\ \qquad\qquad E + P_3 \qquad\quad E + P_i \end{array} \qquad (III)$$

where E is the free enzyme, $E\cdot S$ is the enzyme–substrate complex, E–P is the phosphoryl-enzyme, and ROH is ethanolamine. The products are: P_1, 2-naphthol; P_i, phosphate; and P_3, *O*-phosphorylethanolamine.

This reaction is formulated for negligible concentrations of P_1, P_i, and P_3 so that the reverse reactions need not be considered. Formation of P_3 in anticipation of our observations is shown as a second-order reaction, proportional to the concentration of ethanolamine. This reaction might have been written also to involve complexes between E and P_i, $E\cdot P_i$, and E and P_3, $E\cdot P_3$, as shown for P_i in reaction II, but for the purpose of deriving the kinetic equations to evaluate K_2 we require only the over-all constants represented by k_4' and k_5'.

The steady-state solution of the initial rate of solvolysis of substrate, $v = d(P_1)/dt = -d(S)/dt$, has the form of the Michaelis–Menten equation (Epand and Wilson, 1964)

$$v = \frac{k(E_0)}{1 + K_m/(S)} = \frac{V}{1 + K_m/(S)} \qquad (8)$$

where (E_0) is the total concentration of enzyme in all its forms. The catalytic rate constant, k, and the Michaelis constant, K_m, are

$$k = \frac{k_3'}{1 + k_3/[k_4' + k_5'(ROH)]} \qquad (9)$$

$$K_m = \frac{(k_2' + k_3')/k_1'}{1 + k_3'/[k_1' + k_5'(\mathrm{ROH})]} \quad (10)$$

When (ROH) is set equal to zero, the equations for k and K_m reduce to the form for hydrolysis without a second acceptor (Wilson and Cabib, 1956; Gutfreund and Sturtevant, 1956). We must distinguish between three different velocities

$$\frac{d(P_1)}{dt} = \frac{d(P_i)}{dt} + \frac{d(P_3)}{dt} \quad (11)$$

Examination of reaction III shows that the addition of a phosphate acceptor (ROH) will affect the rates of formation of 2-naphthol and phosphate in a manner depending upon the relationship of k_3' and k_4'. Consider only the extreme cases: (a) If k_3' is absolutely rate controlling, the addition of a phosphate acceptor will not change the rate of formation of 2-naphthol but will decrease the rate of formation of phosphate because the new pathway will lower the level of E–P. (b) If, on the other hand, k_4' is absolutely rate controlling, the rate of formation of 2-naphthol will increase in response to the new pathway, the level of E–P will not fall and the rate of formation of P_i will remain unchanged. The concentration of water has been included in k_4' and the ratio

$$k/K_m = k_1' \Big/ \left(1 + \frac{k_2'}{k_3'}\right)$$

is independent of k_4' and k_5' (ROH).

The ratio of the initial rates of formation of phosphate and *O*-phosphorylethanolamine, f, is an easily measured quantity.

$$f = k_4'/k_5'(\mathrm{ROH}) = \frac{d(P_i)/dt}{d(P_3)/dt} = \frac{d(P_i)/dt}{\frac{d(P_1)}{dt} - \frac{d(P_i)}{dt}} \quad (12)$$

The quantity f is expressed in terms of the differences in the rates of formation of 2-naphthol and P_i because it is easier to measure 2-naphthol than *O*-phosphorylethanolamine. From eq 8, 9, and 12 we obtain the general solution for the effect of adding the acceptor

$$k_3'(E_0) = \frac{V_0 V}{V_0 - f(V - V_0)} \quad (13)$$

$$k_4'(E_0) = \frac{V_0 V}{(1 + f)(V - V_0)} \quad (14)$$

$$k_5'(E_0) = \frac{V_0 V}{(1 + f)(V - V_0)f(\mathrm{ROH})} \quad (15)$$

where V_0 is the maximum velocity in terms of P_1 (*i.e.*, 2-naphthol) measured in the absence of ethanolamine and V is the maximum velocity in the presence of ethanolamine. The maximum value for V is

$$V = V_0\left(1 + \frac{1}{f}\right)$$

In general k_4', k_5', and k_3' can be evaluated and if one of these is very much larger than the other, this will show up in applying the equations. However, the large value will tend to be inaccurate depending upon the precision of the measurements. We will have to know k_5'. Now let us consider the reaction between enzyme and *very low* concentrations of *O*-phosphorylethanolamine.

$$E + \textit{O}\text{-phosphorylethanolamine} \underset{k_{-1}^0}{\overset{k_1^0}{\rightleftharpoons}} E\text{–}P + \text{ethanolamine} \quad (IV)$$

The equilibrium constant for this reaction is given by

$$K_1 = k_1^0/k_2^0 \quad (16)$$

At substrate levels much lower than K_m nearly all the enzyme is free, and the initial velocity approaches a second-order rate expression.

$$v = \lim_{(S),\, K_m \ll (S)} k(E_0)/1 + K_m/(S) = \frac{k}{K_m}(S)(E);\ \frac{k}{K_m} = \frac{V}{(E_0)K_m} \quad (17)$$

Therefore, the second-order rate constant, k_1^0, of reaction IV is the same as k/K_m, and inspection of reaction III shows that $k_2^0 = k_3'$.

Since V and K_m can be measured using *O*-phosphorylethanolamine as a substrate and $k_5'(E_0)$ can be obtained from eq 14, it is possible to calculate K_1. Although we have discussed a reaction between enzyme and *O*-phosphorylethanolamine at very low concentrations of *O*-phosphorylethanolamine, no experiment need be done at low concentrations.

The equilibrium constant, K_1, gives us the free-energy level of the phosphoryl-enzyme relative to *O*-phosphorylethanolamine. The equilibrium constant for the hydrolysis of the phosphoryl-enzyme, K_2, is given by K_3/K_1, where K_3 is the equilibrium constant for hydrolysis of *O*-phosphorylethanolamine and has already been determined (Dayan and Wilson, 1965).

The following experimental measurements are to be made at pH 5.5, 6, and 7; V, V_0, and f for solvolysis of 2-naphthyl phosphate; V_0 and K_m for *O*-phosphorylethanolamine; and K_i for phosphate. The K_m of *O*-phosphorylethanolamine was determined indirectly by using it as a competitive inhibitor in the hydrolysis of 2-naphthyl phosphate. This procedure was adopted because it is easier to measure low concentrations of 2-naphthol than low concentrations of phosphate. K_i for phosphate was measured in the same way.

Experimental Section

Chromatographically purified alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1; *Escherichia coli*) was obtained from Worthington Biochemical Corp. (BAPC-6170) as a suspension (10 mg/ml) in saturated ammonium sulfate. Enzyme dilutions were made with 0.1 M Tris–1 M NaCl at pH 8.0.

O-Phosphorylethanolamine and 2-naphthyl phosphate, purchased from Sigma Chemical Co., were used without further purification. 2-Naphthol from the same company was

recrystallized three times from alcohol–water and had a final melting point of 123°. The buffer used at pH 5.5 was 0.2 M acetate–1 M NaCl at pH 6.0 was 0.05 M Tris–0.05 M acetate–1 M NaCl and the buffer used at pH 7.0 was 0.1 M Tris–1 M NaCl. The pH values of the solutions were measured with a Corning pH meter, Model 12. All the experiments were run at 25°.

Fluorescence of 2-naphthol and 2-naphthyl phosphate was determined with an Aminco-Bowman spectrophotofluorometer at pH 6.0 and with a Farrand instrument at pH 5.5, standardized every half hour with a solution of quinine sulfate in 0.1 N H_2SO_4. Phosphate was determined according to the procedure described by Dryer *et al.* (1957), using a Beckman DU spectrophotometer for absorbancy measurements at 770 mμ.

2-Naphthyl phosphate was chosen as the substrate because 2-naphthol, the product formed by hydrolysis, can be measured fluorometrically. Fluorescence of 2-naphthol (pH 10.2 anion) was measured at its activation maximum, 345 mμ, and its fluorescent maximum, 420 mμ. At 420 mμ, interference by 2-naphthyl phosphate is negligible and the fluorescence of 2-naphtholate anion is proportional to its concentration. (Concentrations as low as 5×10^{-8} M could be accurately determined and lower concentrations with decreasing accuracy.)

V_0 and K_m for 2-Naphthyl Phosphate. The reaction was initiated by adding enzyme (0.033 μg at pH 5.5, 0.025 μg at pH 6, and 0.01 μg pH 7) to 1.0 ml of 2-naphthyl phosphate in buffer. The reaction was stopped after 4 min by adding 1.0 ml of 2 N NaOH and the fluorescence of 2-naphthol was immediately determined. Rates for six concentrations of substrate (2×10^{-8}–8×10^{-8} M at pH 5.5, 8×10^{-8} –8×10^{-7} M at pH 6, and 3×10^{-7}–3×10^{-6} M at pH 7) were obtained and the reciprocal plots of v^{-1} *vs.* $(S)^{-1}$, using the average value of (S) yielded straight lines with intercepts, $1/V_0$, and slopes, K_m/V_0. For the purpose of calculating the fraction of enzyme covalently bound to phosphate, the value of K_m does not have to be known accurately. We have only to know what values of (S) satisfy the condition $(S) \gg K_m$.

K_i for Phosphate. Initial rates of hydrolysis of 2-naphthyl phosphate, at a concentration (5×10^{-4} M at pH 5.5, 5×10^{-5} M at pH 6, and 3×10^{-4} M at pH 7) where $(S) \gg K_m$, were measured as before in the presence of six concentrations of phosphate (5×10^{-2}–3×10^{-1} M at pH 5.5 and 1.5×10^{-3}–1.5×10^{-2} M at pH 6 and 7). The total volume of the reaction mixture was 1.0 ml and the quantity of enzyme was 3.3 μg at pH 5.5, 2.5 μg at pH 6, and 0.40 μg at pH 7. Plots of v^{-1} *vs.* phosphate yielded straight lines intercepting at the ordinate, V_0^{-1}. The slope under these conditions is K_m (of 2-naphthyl phosphate)$/V_0K_i(S)$.

K_m for O-Phosphorylethanolamine. The K_m of *O*-phosphorylethanolamine was determined indirectly in an experiment in which it acts as a competitive inhibitor of 2-naphthyl phosphate hydrolysis. Initial rates of hydrolysis of 2-naphthyl phosphate, at a concentration (5×10^{-4} M at pH 5.5, 5×10^{-5} M at pH 6, and 3×10^{-4} M at pH 7) where $(S) \gg K_m$, were measured as before in the presence of six concentrations of *O*-phosphorylethanolamine (7.5×10^{-4}–3.8×10^{-3} M at pH 5.5, 7.6×10^{-5}–7.6×10^{-4} M at pH 6, and 5×10^{-4}–5×10^{-3} M at pH 7). The total volume of the reaction mixture was 1.0 ml and the concentration of enzyme was 3.3 μg at pH 5.5, 2.5 μg at pH 6, and 0.40 μg at pH 7. Plots of V^{-1} *vs.* *O*-phosphorylethanolamine yielded straight lines intercepting at the ordinate, V_0^{-1}. The slope, under these conditions, is K_m (of 2-naphthyl phosphate)$/V_0(S)K_m$ (of *O*-phosphorylethanolamine).

V_0 for 2-Naphthyl Phosphate and O-Phosphorylethanolamine. The maximum velocity, V_0, for the hydrolysis of 2-naphthyl phosphate and *O*-phosphorylethanolamine was determined by measuring the formation of phosphate at a substrate concentration (for 2-naphthyl phosphate, 8×10^{-4} M at pH 5.5, 2×10^{-3} M at pH 6, and 2.5×10^{-3} M at pH 7; for *O*-phosphorylethanolamine, 4×10^{-3} M at pH 5.5, 2×10^{-3} M at pH 6, and 8.8×10^{-3} M at pH 7) where $(S) \gg K_m$. The reaction was initiated by adding enzyme (20 μg at pH 5.5, 10 μg at pH 6, and 2 μg at pH 7) to 1.0 ml of the substrate in buffer. The reaction was stopped after 10 min by adding 1.0 ml of 5 N H_2SO_4, and phosphate was determined according to Dryer *et al.* (1957). Controls, in which enzyme was added after the acid, and phosphate standards were run simultaneously.

V, V_0, and f for the Hydrolysis of 2-Naphthyl Phosphate at pH 5.5, 6, and 7. Initial rates of formation of 2-naphthol and phosphate were measured at a concentration of 2-naphthyl phosphate (6.5×10^{-4} M at pH 5.5, 1×10^{-3} M at pH 6, and 2.5×10^{-3} M at pH 7) where $(S) \gg K_m$, in the presence of five concentrations of ethanolamine (about 0.10–0.60). The reaction was initiated by adding enzyme (3.3 μg at pH 5.5, 10 μg at pH 6, and 2 μg at pH 7) to 1.0 ml of substrate and ethanolamine in buffer and stopped after 10 min by adding 1.0 ml of 5 N H_2SO_4. A 50-μl aliquot was withdrawn for 2-naphthol determination in 2.0 ml of 1 N NaOH, and the remaining solution was analyzed for phosphate as before. Phosphate and 2-naphthol standards, and controls in which enzyme was added after the acid, were run concurrently.

Results

Equilibrium Constant for Hydrolysis of O-Phosphorylethanolamine. The value for this constant in terms of analytical

$$\text{O-phosphorylethanolamine} + H_2O \overset{K_3}{\rightleftharpoons} \text{ethanolamine} + P_i$$

concentrations and taking the activity of pure water as 1 was estimated to be 28.2 at pH 8.0 (Wilson and Dayan, 1965). This value is pH dependent since analytical concentrations are used. Our values for the apparent pK_a's of ethanolamine, 9.4, *O*-phosphorylethanolamine, 5.4, and phosphate, 6.3, at 25° in 1 M NaCl were used to calculate the values of K_3 at other pH. These values are given in Table III.

Equilibrium Constant for Reaction of O-Phosphorylethanolamine with Enzyme. K_m for 2-naphthyl phosphate and *O*-

$$E + \text{O-phosphorylethanolamine} \overset{K_1}{\rightleftharpoons} E\text{–P} + \text{ethanolamine}$$

phosphorylethanolamine and K_i for phosphate are given in Table I. The values of K_m for *O*-phosphorylethanolamine and K_i for phosphate are based upon the value of K_m for 2-naphthyl phosphate. However, the ratio of the K_m for *O*-phosphorylethanolamine and K_i does not depend upon the value used for K_m of 2-naphthyl phosphate in the calculation. Since it is this ratio that actually enters into our calculations of the amount of covalently bound phosphate, our conclusions do

TABLE I: Kinetic Parameters, 25°, 1 M NaCl.

Substrate or Inhibitor	K_m or K_i (M)	V_0 (μmoles/min mg of enzyme[a])
pH 5.5		
2-Naphthyl phosphate	1.3×10^{-5}	0.25
O-Phosphorylethanolamine	1.6×10^{-7}	0.23
Phosphate	1.5×10^{-3}	
pH 6.0		
2-Naphthyl phosphate	3.5×10^{-5}	0.82
O-Phosphorylethanolamine	2.3×10^{-7}	0.76
Phosphate	5.1×10^{-3}	
pH 7.0		
2-Naphthyl phosphate	2.7×10^{-5}	2.78
O-Phosphorylethanolamine	3.9×10^{-6}	2.70
Phosphate	2.3×10^{-3}	

[a] This enzyme preparation is about 40% pure.

TABLE II: Rates of Formation of 2-Naphthol and Phosphate as a Function of Ethanolamine Concentration, 25°, 1 M NaCl.

Ethanolamine (M)	Rate (μmoles/min mg of enzyme) 2-Naphthol	Phosphate	f[a]	f(ethanolamine)
		At pH 5.5		
0	0.28	0.27		
0.148	0.32	0.28	7.00	1.04
0.295	0.35	0.27	3.38	0.99
0.443	0.39	0.27	2.25	0.99
0.590	0.43	0.28	1.86	1.10
				Av 1.03
		At pH 6.0		
0	0.86	0.87		
0.114	0.96	0.85	9.70	1.11
0.229	1.08	0.86	4.14	0.95
0.343	1.19	0.86	2.72	0.94
0.457	1.30	0.87	2.02	0.92
0.572	1.35	0.88	1.81	1.04
		Av 0.87		Av 0.99
		At pH 7.0		
0	2.78	2.74		
0.119	3.33	2.83	5.16	0.62
0.238	3.84	2.85	2.62	0.62
0.356	4.26	2.90	1.89	0.67
0.475	5.06	2.70	1.23	0.58
0.594	5.55	2.75	1.01	0.60
		Av 2.79		Av 0.62

[a] The values of f were calculated using the average value for the rate of formation of phosphate.

TABLE III[a]

pH	K_3	$K_1 \times 10^5$	$K_2 \times 10^4$	$K_1 \times 10^6$	k_4/k_3	$k_2/k_1 \times 10^6$	(E–P)/(E$_0$)[b]
5.5	115	65	0.18	15.5	0.15	118	0.87
6.0	66	43	0.15	5.1	2.0	7.6	0.33
7.0	33	1.6	2.1	2.3	90	2.3	0.011
8.0[c]	28	1.9	1.5	2.5	59	2.5	0.017

[a] The equilibrium constants for the hydrolysis of *O*-phosphorylethanolamine, K_3, for the reaction of *O*-phosphorylethanolamine with alkaline phosphatase, K_1, for the hydrolysis of the phosphoryl-enzyme intermediate, K_2, for the hydrolytic conversion of covalently bound phosphate into noncovalently bound phosphate, k_4/k_3, for the dissociation of noncovalently bound phosphate, k_2/k_1, and the fraction of active sites containing covalently bound phosphate, (E–P)/(E$_0$), are given as a function of pH at 25°, 1 M NaCl. K_1 is not an equilibrium constant but is a kinetic constant determining the competitive inhibition produced by P_i and with appropriate good approximations it is the Michaelis constant for oxygen exchange. With these same approximations the column headed (E–P)/(E$_0$) gives the calculated ratio of the maximum velocity of oxygen exchange in P_i to the maximum velocity of hydrolysis of a good substrate. All equilibrium constants are given in terms of analytical concentrations and are therefore pH dependent. The activity of pure water is taken as one. Dimensions are given in the text to eliminate the possibility of ambiguity in the definition of the equilibrium constants. [b] Calculated for $(P_i) \gg K_1$. [c] Data taken from Wilson and Dayan (1965).

not depend upon an accurate measurement of the K_m of 2-naphthyl phosphate. This is fortunate because the measurement of these very low values of K_m is difficult.

The maximum velocity for hydrolysis of *O*-phosphorylethanolamine is slightly lower than that for 2-naphthyl phosphate (Table I). For our calculations we have taken them to be equal. Dephosphorylation was shown to be the rate-determining step in the hydrolysis of *p*-nitrophenyl phosphate at pH 8 (Dayan and Wilson, 1964; Wilson and Dayan, 1965). This is also true for hydrolysis of 2-naphthyl phosphate at pH 5.5, 6, and 7 since the constant rate of formation of phosphate but increasing rate of formation of 2-naphthol in the presence of increasing amounts of ethanolamine (Table II) immediately shows that dephosphorylation is rate controlling (see reaction 1).

Then, $V_0(E_0) = k = k_4$, and K_1, the equilibrium constant for the reaction of *O*-phosphorylethanolamine with enzyme becomes

$$K_1 = k_1^0/k_2^0 = k/k_5K_m = k_4/k_5K_m = \frac{f(\text{ethanolamine})}{K_m}$$

The values of K_1 in terms of total concentrations of reactants are given in Table III. Table II shows that f(ethanolamine) is a constant. The transfer of a phosphate group from

O-phosphorylethanolamine to the enzyme is very favorable. K_1 is dimensionless.

Equilibrium Constant for Hydrolysis of the Phosphoryl-enzyme. The equilibrium constant for hydrolysis of the phosphoryl-enzyme, K_2 (reaction I), is given by K_3/K_1. These results are written for an equilibrium expression in which the activity of water is 1 and for total concentrations and therefore are pH dependent. The equilibrium constants given in Table III indicate that the phosphoryl-enzyme is a stable intermediate with respect to free enzyme and free P_i at all pH's studied at standard concentrations, indeed even at 10^{-4} M P_i there is a good fraction of this intermediate. The dimensions of K_2 are in moles per liter.

Equilibrium Constant for Dissociation of the Enzyme–Phosphate Complex and the Equilibrium between E–P and E·P$_i$. The equilibrium constant for the dissociation of the enzyme–phosphate complex, k_2/k_1 (see reaction I), was obtained from the values of K_i and K_2 using the relationship $k_2/k_1 = K_2/[(K_2/K_i) - 1]$ (moles/l.)$^{-1}$. Similarly the equilibrium constant for the reaction of the covalent phosphoryl-enzyme, E–P, and water to form the enzyme–phosphate Michaelis complex, E·P_i, k_4/k_3 was obtained from the relationship $k_4/k_3 = (K_2/K_i) - 1$. This is the actual ratio of (E·P_i)/(E–P) that will be obtained when enzyme and P_i are incubated together and come to equilibrium.

The Amount of Phosphoryl-enzyme Intermediate. The fraction of active sites that exist as phosphoryl-enzyme intermediate when $(P_i) \gg K_i$ is given in Table III under the column headed (E–P)/(E_0). The values were obtained from the relationship (E–P)/(E_0) = K_i/K_2.

The full equation showing the dependence upon (P_i) is

$$(\text{E–P})/(\text{E}_0) = 1\Big/\left[\frac{K_2}{(\text{P}_i)} + \frac{K_2}{K_i}\right]$$

Discussion

The effect of ethanolamine on the enzyme-catalyzed hydrolysis of 2-naphthol phosphate is qualitatively the same at pH 7.0, 6.0, and 5.5; the rate of formation of 2-naphthol is increased but the rate of formation of phosphate is not changed. This indicates that dephosphorylation is the rate-controlling step and agrees with the conclusions of other workers (Fernley and Walker, 1966; Aldridge *et al.*, 1964; Trentham and Gutfreund, 1968). Previous studies using the same technique at pH 8.0 yielded the same results indicating that dephosphorylation is rate controlling at this pH also, but this conclusion does not agree with other workers. The large value of k'_3/k'_4 is probably responsible for the very low values of K_m (Table I) (see eq 6 or 10). Our results (Table III) show that both K_2 and k_2/k_1 are small, *i.e.*, both the phosphoryl-enzyme intermediate and the enzyme–phosphate complex are stable with respect to free enzyme and P_i. The reason why very little phosphoryl-enzyme forms at pH 8.0 and 7.0 is that the enzyme–phosphate complex is more stable than the phosphoryl-enzyme at these pH. At pH 6.0 the stabilities become nearly equal and at pH 5.5 the phosphoryl-enzyme intermediate becomes more stable than the enzyme–phosphate complex. Consequently, a sizeable fraction of phosphoryl-enzyme is formed at pH 6.0 and most of the enzyme is phosphoryl-enzyme at pH 5.5. Although somewhat higher, the kinetically predicted amounts of phosphoryl-enzyme intermediate are in remarkable agreement with the amounts of phosphoprotein obtained in the labeling experiments. The values obtained from labeling experiments were variable probably because of the difficulty involved in rapidly quenching the reaction. They were 1% at pH 8.0, 1–5% at pH 7.0, 12–30% at pH 6.0, and 29–45 and 100% at pH 5.5 (Engstrom and Agren, 1958; Engstrom, 1961, 1962a,b, 1964; Schwartz and Lipmann, 1961; Schwartz, 1963; Milstein, 1964; Pigretti and Milstein, 1965). Our treatment of the problem shows why equal labeling was obtained at 10^{-4} M P_i as at 10^{-3} M P_i. As long as $(P_i) \gg K_i$ all the enzyme is in the form of enzyme–phosphate complex plus phosphoryl-enzyme but the distribution between these intermediates is not affected by phosphate concentrations.

We have the result that the phosphoryl-enzyme intermediate is 10^6 times more stable than ordinary phosphate esters. Since this unusual situation is duplicated in the phosphoprotein obtained in labeling experiments (at pH 6.0 and 5.5 but not at pH 7.0 and 8.0), it is quite probable that the two are the same. (If the phosphoryl-enzyme intermediate and the phosphoprotein both had the usual stability of simple phosphate esters we should not be able to argue that they are probably the same substance.)

However, it must be noted that the rate of labeling as given by Aldridge *et al.* (1964) is at least two orders of magnitude too slow as compared with what should be obtained in the formation of the phosphoryl-enzyme intermediate.

We also have enough information to calculate the rate of oxygen exchange. The scheme for oxygen exchange in P_i is

$$\begin{array}{ccccc} \text{E} + \text{P}_i & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & \text{E}\cdot\text{P}_i & \xrightarrow{k_3} & \text{E–P} + \text{H}_2\text{O} \\ & & & & \quad k_3 \uparrow \downarrow k_4 \ \text{H}_2\text{O}^* \\ & & & & \text{E}\cdot\text{P}_i^* \xrightarrow{k_2} \text{E} + \text{P}i^* \end{array}$$

The rate of exchange has the Michaelis–Menten form

$$v = \frac{k(\text{E}_0)}{1 + \dfrac{K_m}{(\text{P}_i)}}$$

where

$$k = k_3\Big/\left[1 + \frac{k_3}{k_4}\left(1 + \frac{k_4}{k_2} + \frac{k_3}{k_2}\right)\right]$$

$$K_m = \frac{(k_2 + k_3)}{k_1}\Big/\left[1 + \frac{k_3}{k_4}\left(1 + \frac{k_4}{k_2} + \frac{k_3}{k_2}\right)\right]$$

In the reaction we have made the implicit assumption that the step E·P_i* → E–P removes *O whereas it is conceivable that during the lifetime of E·P_i* there might be a reorientation that would enable the removal of other oxygens. Thus the k_3 used here might be smaller perhaps by a factor of 4, than the value of k_3 used in the other part of the reaction and in other reactions.

If k_3 is small with respect to k_2, which is very probable, this question does not affect the results. It is quite probable that

both k_3 and k_4 are small with respect to k_2 and therefore the above equations can be simplified. With this approximation K_m becomes identical with K_1. Then the maximum velocity of oxygen exchange can be compared with the maximum velocity of substrate hydrolysis

$$\frac{k(\text{oxygen exchange})}{k(\text{hydrolysis of S})} = \frac{k_3/(1 + k_3/k_4)}{k_1} = \frac{K_1}{K_2} = \frac{(E\text{–}P)}{(E_0)}$$

The result is that the ratio of maximum velocities is the same as the fraction of phosphoryl-enzyme intermediate that is in equilibrium with enzyme and phosphate and has been given in Table III. Thus the prediction is that the maximum rate of oxygen exchange relative to the maximum rate of hydrolysis of a substrate will be only 1 or 2% at pH 7.0 and 8.0 but will increase to about 35% at pH 6.0 and reach 90% at pH 5.5.

The maximum velocities of oxygen exchange in P_i catalyzed by alkaline phosphatase in 1 M NaCl at 25° are calculated to be 0.08 μmole/min mg of enzyme at pH 7.0, 0.68 μmole/min mg of enzyme at pH 6.0, and 0.51 μmole/min mg of enzyme at pH 5.5 for 100% enzyme.

Under appropriate conditions oxygen exchange could be used to determine the free energy of hydrolysis of covalent intermediates. It is apparent that oxygen exchange of the type under discussion can occur reasonably rapidly only when the covalent enzyme intermediate is relatively stable. Schwartz (1963) has shown that the rate of oxygen exchange relative to the rate of hydrolysis of β-glycerol phosphate is 1–3% at pH 9 and 17–67% at pH 5.8. Our calculated values are consistent with these results.

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