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Phosphorylated Intermediate of Alkaline Phosphatase†

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ABSTRACT: We have measured the phosphorylation of the subunits of alkaline phosphatase in the steady state with several substrates and at several pH values. Our results vary from 80% phosphorylation of *both* subunits at pH 7 to only 9% at pH 10. There is no evidence of anticooperativity. With the measurements of k_{cat}, we are able to evaluate rate constants in a minimal scheme. The results show that the main rate influencing steps are chemical dephosphorylation and dissociation of phosphate. The former predominates at pH 7.0 but declines in importance as the pH is raised. Our rate constants for dissociation of phosphate are in agreement with recent NMR studies.

Alkaline phosphates from *Escherichia coli* is a dimeric enzyme derived from a single cistron (Levinthal et al., 1962; Singer, 1961; Rothman & Byrne, 1963; Schlesinger & Levinthal, 1963; Levinthal et al., 1963). It is obtained as three major isozymes composed of three combinations of two slightly different subunits, A and B (Levinthal et al., 1962; Malamy & Horecker, 1964; Simpson et al., 1968; Lazdunski & Lazdunski, 1967; Singer et al., 1961; Schlesinger & Anderson, 1968; Levinthal et al., 1963). Isozyme I, the first to elute in chromatography from a DE-52 cellulose column has the composition A, A; isozyme II has the composition A, B; and isozyme III has B, B. Subunit B is derived from A by an epigenic modification, probably involving the removal of the N-terminal arginine amino acid residue (Kelley et al., 1973; Schlesinger et al., 1975). The enzyme binds 4 equiv of Zn^{2+} and 2 equiv of Mg^{2+} (Anderson et al., 1975).

The three isozymes can be displayed in polyacrylamide gel electrophoresis as the native enzyme and also as the apoenzyme, lacking Zn^{2+}, Mg^{2+}, and phosphate (McManaman & Wilson, 1978).

This enzyme follows Michaelis kinetics but shows substrate activation at high substrate concentrations, $\geq 10^{-3}$ M (Levinthal et al., 1962). This phenomenon has been the initial basis for the idea of anticooperativity between the subunits. Since then a number of papers have supported the idea of anitcooperativity. In this theory only one subunit is active at low substrate concentrations because the binding or reaction of substrate with the active site of one subunit greatly diminishes the affinity for substrate of the active site of the second subunit. This idea has been incorporated in the theory of a flip-flop mechanism (Lazdunski et al., 1971).

The reaction mechanism of this enzyme involves the formation of a covalent phosphoryl-enzyme intermediate (Morton, 1955; Agren et al., 1959; Engstrom & Agren, 1958; Engstrom, 1962; Schwartz & Lipmann, 1961; Barrett et al., 1969) analogous to the acetyl-enzyme intermediate of acetylcholinesterase and other enzymes that hydrolyze carboxylic esters (Wilson et al., 1950). The enzymic nucleophile is the hydroxyl group of a specific serine residue. Although kinetically labile, the phosphoryl-enzyme is amazingly stable (thermodynamically) as compared to other phosphate esters (Dayan & Wilson, 1963). Phosphate itself forms a very stable noncovalent complex with alkaline phosphatase, and the extra stability of the phosphoryl-enzyme can be attributed to the existence of noncovalent interactions similar to those that operate between phosphate and the enzyme in the noncovalent phosphate–enzyme complex.

The minimal scheme for the reaction mechanism is

$$\begin{array}{l} E + ROPO_3H_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot ROPO_3H_2 \xrightarrow{k_2} E\text{-}P + ROH \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad k_{-3} \Downarrow k_3 \pm H_2O \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad E\cdot P_i \xrightarrow{k_4} E + P_i \end{array} \qquad (1)$$

The step k_{-2} is omitted because [ROH] is almost zero in initial velocity measurements, and step k_{-4} is omitted for a similar reason. In this scheme, $E\cdot ROPO_3H_2$ represents the Michaelis–Mentin complex, E–P is the convalent phosphoryl-enzyme, $E\cdot P_i$ is the Michaelis–Menten complex between

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phosphate and enzyme, and P_i is inorganic phosphate. E stands for an enzyme subunit.

There has been considerable interest in finding out which steps are slow enough to be important rate-influencing steps. In a kinetic technique, Tris was used as a phosphate acceptor in a transphosphorylation experiment (Dayan et al., 1964). It was found that at a Tris concentration where equal quantities of P_i and Tris-phosphate were formed as products, the reaction rate was about doubled. This result suggested that dephosphorylation was the most important rate-influencing step.

This conclusion also explained the lack of specificity of this enzyme. However, if this were the case, a burst of *p*-nitrophenol should be obtained in a stop-flow experiment using *p*-nitrophenol phosphate as substrate, but none was obtained by some authors. However, a burst was obtained by others. Bloch & Schlesinger, (1973) explained why some observers got a burst and others did not. They showed that many enzyme preparations retain "endogenous" phosphate, which prevents the burst. Starting with the phosphoryl-enzyme (obtained by incubating enzyme and P_i in acid pH), the chemical dephosphorylation rate was measured using a rapid mixer–sampler (Aldridge et al., 1964). These studies showed that the dephosphorylation was too fast to be the rate-controlling step. Also, Reid & Wilson (1971) found that the steady-state level of phosphorylation during the hydrolysis of substrate was too low to allow for chemical dephosphorylation to be the slow step. Coleman suggested that dissociation of the phosphate–enzyme complex is the rate-controlling step in the hydrolytic mechanism, and recent NMR studies based on line broadening show that this step might be slow enough to determine the overall catalytic rate.

We have decided to reinvestigate both the problem of which steps are rate-influencing steps and the question of anticooperativity with the aid of a very useful technique that has recently been developed (McManaman, 1978; Cocivera et al., 1980). With this technique we can accurately measure the extent of phosphorylation of alkaline phosphatase without any danger of very large mistakes. In this method, the enzyme after short incubation with the substrate is shot into Cl_3AcOH–silicotungstic acid. The enzyme (we use isozyme I or III, which has only one type of subunit) is rapidly denatured and dissociated into subunits by this treatment. The subunits are then subject to gel electrophoresis. The phosphorylated subunit runs anodically faster and forms a separate band because it contains extra negative charges. After staining, both bands can be seen, and their relative amounts can be judged approximately, even by eye. We quantitate the bands by optical scanning and integration of the peaks.

Experimental Procedure

Isozymes of Alkaline Phosphatase. Escherichia coli strain C-90 obtained from Dr. R. Fall was grown under constant aeration for 14 h at 37 °C to an OD_{540} of 4.7. The growth medium was prepared according to Levinthal et al. (1962) slightly modified by increasing the amount of bactopeptone from 5 to 10%. The cells were harvested cold by centrifugation and washed three times with cold 0.01 M Tris (Sigma Chemical Co., St. Louis, MO) at pH 8.0. Alkaline phosphatase was harvested from cells by osmotic shock according to the procedure of Neu and Heppel (1965) and then pumped onto a 1.5 × 30 cm column of DE-52 cellulose (Whitman, Inc., Clifton, NJ) equilibrated with 0.01 M Tris, 10^{-3} M $MgCl_2$ (J. T. Baker Chemical Co., Phillipsburg, NJ, and 10^{-5} M $ZnSO_4$ (Fisher Scientific Co., Fair Lawn, NJ) at pH 7.5. The bound enzyme was washed with 1 L of this buffer solution and eluted using 0.12 M NaCl at pH 7.5. The active fractions were pooled, and, using an Amicon ultrafiltration system containing a PM-30 membrane, the enzyme was concentrated and equilibrated with 0.01 M Tris, 10^{-3} M $MgCl_2$, and 10^{-5} M $ZnSO_4$ at pH 7.2.

Isozymes of alkaline phosphatase were separated by DE-52 cellulose chromatography using a 1-L linear NaCl gradient (0 to 0.07 M) in the above buffer at pH 7.2. Five milliliter fractions were collected and assayed for enzyme. Those fractions containing enzyme were pooled and concentrated by ultrafiltration. The quantity of protein and the activity of the isozymes were determined according to Malamy & Horecker (1964). The numbering of the isozymes is based on their elution sequence; i.e., isozyme I is eluted first, at the lowest NaCl concentration. Each isozyme gave a single electrophoretic band. These bands move toward the anode at different rates; iosozyme III is the fastest. The specific enzyme activities (micromoles of substrate hydrolyzed per hour per milligram of protein) for isozymes I, II, and III were 3480, 4110, and 3280, respectively. For crystalline alkaline phosphatase, the specific activity is 3250 (Malamy & Horecker, 1964).

Substrates. All substrates were obtained from Sigma Chemical Company (St. Louis, MO) and used without further purification.

Steady-State Phosphorylation of Isozymes. Two approaches were used to determine the extent of phosphorylation of isozymes under steady-state conditions. Both gave similar results. In the first, which was used between pH 7.0 and 8.9, 0.25 mL of isozyme solution was added to 0.25 mL of substrate, while the solution was rapidly stirred with a vortex stirrer. After a reaction time of 1 or 2 s, the reaction was quenched by adding 1 mL of 10% trichloroacetic acid (Cl_3-AcOH) (Fisher Scientific Co., Fair Lawn, NJ) containing 4 mM silicotungstic acid and then vortex stirring. At pH 7.0, reaction times of 1, 2, and 4 s showed no difference in the extent of phosphorylation. The second method, which was used between pH 7.5 and 10.0, involved rapidly mixing 0.25 mL of substrate and 0.25 mL of enzyme by rapidly drawing the substrate solution into a spring-loaded syringe containing the enzyme solution. After the reaction times of 1 and 2 s, the reaction mixture was shot into 1 mL of (Cl_3AcOH–silicotungstic acid solution. The extent of phosphorylation was the same for the two reaction times. For both methods, the enzyme concentration was 3.7×10^{-7} M and the solutions contained 10^{-5} M $ZnSO_4$, 10^{-3} M $MgCl_2$, and 0.1 M NaCl unless otherwise specified. Up to pH 9.0, 0.01 M Tris was used to control the pH. The solution at pH 10.0 contained 0.01 M Tris and 0.01 M ethanolamine (Eastman Kodak Co., Rochester, NY).

The extent of phosphorylation was determined by polyacrylamide slab gel electrophoresis after the isozyme had been dissociated into its two subunits and precipitated by the Cl_3-AcOH solution. The precipitated protein was separated from the Cl_3AcOH solution by centrifugation. After the pellet was dissolved in 0.10 mL of 0.01 M Tris, 4 M urea, and 20% glycerol to which 0.01 mL of 1 N NaOH had been added, about 0.01 mL of solution containing 1 to 1.5 μg of protein was applied to a well of the gel (described below) and subjected to constant current (approximately 15 mA for eight wells) electrophoresis for 2 h, after which the gel was removed and strained with Coomassie Brilliant Blue R 250 (Ames Co., Elkhart, IN).

Two bands were observed: the faster moving phosphorylated subunit and the nonphosphorylated subunit. The relative

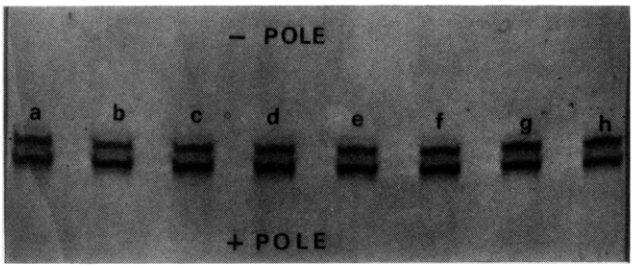

FIGURE 1: Polyacrylamide gel electrophoresis of colvalently phosphorylated (faster moving) and nonphosphorylated subunits of isozyme III of alkaline phosphatase obtained by reaction with phenyl phosphate at pH 7.5. In the absence of substrate, only one band is observed, the upper, slower moving band. The concentrations of phenyl phosphate in moles per liter were (a) 1.9×10^{-2}; (b) 9.4×10^{-3}; (c) 4.7×10^{-3}; (D) 9.4×10^{-4}; (e) 4.7×10^{-4}; (f) 2.3×10^{-4}; (g) 9.4×10^{-5}; and (h) 4.68×10^{-5}.

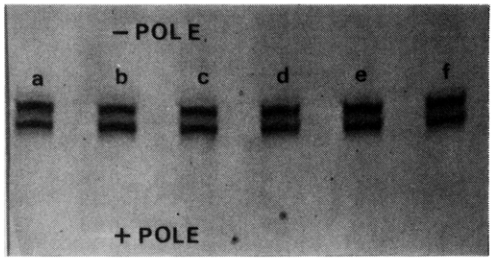

FIGURE 2: Polyacrylamide gel electrophoresis of covalently phosphorylated (faster moving, lower band) and nonphosphorylated subunits of isozymes III of alkaline phosphatase obtained by reaction with *p*-nitrophenyl phosphate at pH 8.0 and 25 °C. The concentrations of *p*-nitrophenyl phosphate in moles per liter were (a) 1.7×10^{-2}; (b) 8.6×10^{-3}; (c) 4.3×10^{-3}; (d) 8.6×10^{-4}; (e) 4.3×10^{-4}; and (f) 2.2×10^{-4}.

amount of protein in each band was determined photometrically at 560 nm using a Varian Model 635 spectrophotometer equipped with a gel scanner and a Spectra-Physics minigrator integrator.

The separating gel was formed from 5 parts of 30% acrylamide (Sigma) and 0.8% bis(acrylamide) (Sigma), 5 parts of 0.05 M *N,N,N′,N′*-tetramethylethylenediamine (Sigma) and 0.1 M citric acid (Baker) brought to pH 7.2 using solid Tris, 9 parts of 8.9 M urea, UltraPure (Schwarz/Mann, Orangeburg, NY), and 1 part of 0.5% ammonium persulfate (Bio-Rad Labs, Richmond, CA). The electrode buffer solution was 0.01 M boric acid at pH 7.2. Figures 1 and 2 illustrate gels for phosphorylation studies at pH 7.0 and 8.0, respectively. The bands move toward the bottom of the gel (positive pole). The faster moving band in each well is the covalently phosphorylated subunit of iosozyme III of alkaline phosphatase.

Kinetics. The enzyme-catalyzed hydrolysis of *p*-nitrophenyl phosphate (Sigma) was measured by the increase of *p*-nitrophenol, monitored at 400 nm using the Varian spectrophotometer at 25 ± 1 °C. At pH 7.0 and 7.5, the effect of substrate concentration on the rate was studied by adding 4 μL of enzyme solution to 3.0 mL of substrate solution containing 0.01 M Tris, 0.1 M NaCl, 10^{-5} M $ZnSO_4$, and 10^{-3} M $MgCl_2$. This procedure was reversed to study the effect of pH on the rate; i.e., 8 μL of substrate (to give 5×15^{-4} M *p*-nitrophenyl phosphate) was added to 3.0 mL of enzyme solution. In this manner, the rate was measured after incubating the enzyme for 2 and 24 h at each pH (stock isozyme solution at pH 7.2). The rate did not depend on the incubation time. To calculate the turnover rate, we have used the previously reported (Hinberg & Laidler, 1973) pH dependence for the extinction coefficient of *p*-nitrophenol. The molecular weight of the enzyme dimer was assumed to be 86 000 (Schlesinger & Barrett, 1965).

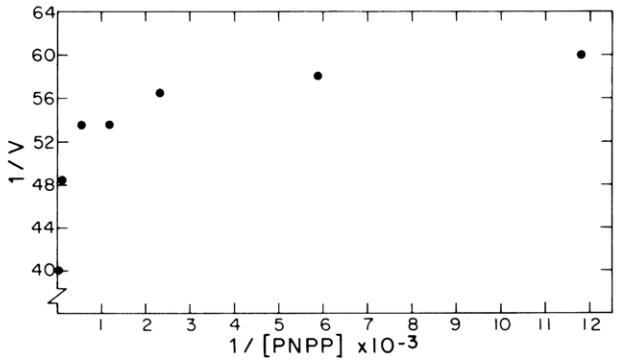

FIGURE 3: Plot of V^{-1} in units of absorbance change per minute vs. 1/[S] for catalysis of hydroylsis of π-nitrophenyl phosphate by isozyme III at pH 7.0 and 25 °C. The units for [S] are moles per liter.

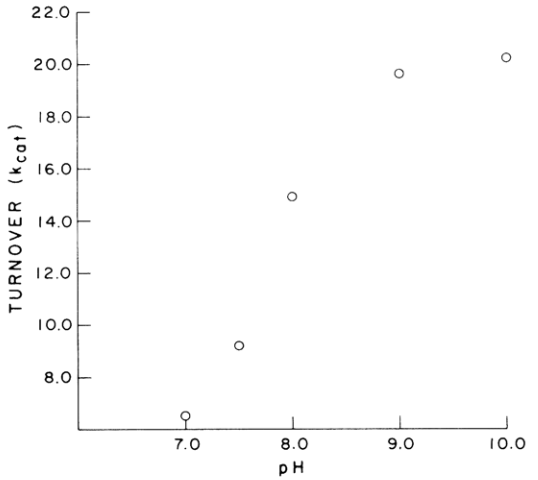

FIGURE 4: Plot of the turnover number (k_{cat}) in units of seconds^{-1} for the hydrolysis of *p*-nitrophenyl phosphate catalyzed by isozyme III vs. pH at 25 °C. Values are for one subunit of the dimer and were obtained from concentrations less than 5×10^{-4} M.

Results

Kinetics. Figure 3, which illustrates a plot of the reciprocal of the rate *V* in arbitrary units vs. the reciprocal of the concentration of the substrate *p*-nitrophenyl phosphate at pH 7.0 for isozyme III, shows that, as the substrate concentration exceeds 5×10^{-4} M, *V* departs from a slow approach to the extropolated maximum velocity, increases rapidly, and approaches a new maximum velocity which is about 40% higher. The same effect of substrate activation was observed above 5×10^{-4} M *p*-nitrophenyl phosphate at pH 7.5 using isozymes I and III. A similar effect has been observed previously for alkaline phosphatase, which was presumably a mixture of isozymes, in the same concentration range (>5×10^{-4} M substrate) at pH 7.8 and about the same ionic strength (Simpson & Vallee, 1970).

The rate is not altered by an increase in ionic strength in the range 0.1–0.5 M (NaCl) at pH 7.0 or by an increase in *p*-nitrophenol concentration in the 1×10^{-5} to 1×10^{-4} M range when 1.5×10^{-3} M *p*-nitrophenyl phosphate is used.

Figure 4 illustrates the pH dependence of the turnover number (moles of substrate/second, moles of enzyme subunits). Incubation of the enzyme at each pH for 2 and 24 h gave very nearly the same value, and the average of the two determinations is given in the figure. The value at pH 8.0 (obtained in the presence of 0.01 M Tris) is slightly less than half the value obtained in the presence of 1 M Tris, an effect that has been observed previously (Dayan et al., 1964; Chle-

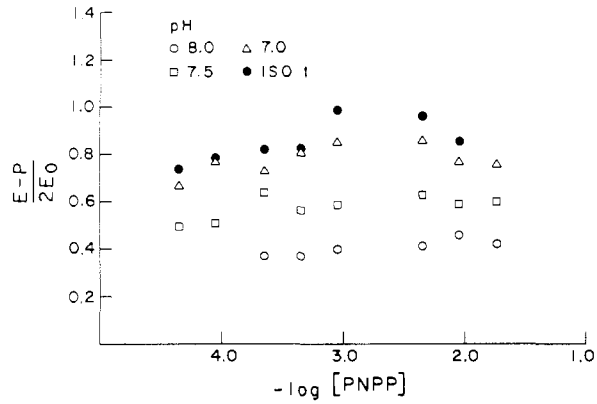

FIGURE 5: Fraction of phosphorylation of both subunits of isozyme III vs. the negative log of the concentration of *p*-nitrophenyl phosphate for the pH values indicated at 25 °C. Data for isozyme I at pH 7.0 are also included.

Table I: Effect of Ionic Strength and Concentration of *p*-Nitrophenol on $[E\text{-}P]/2[E]_0$ for Phosphorylation of Isozyme III by *p*-Nitrophenyl Phosphate at pH 7.0

[pnpp][a] (M × 10³)	[NaCl] (M)	[pnp][b] (M × 10⁵)	[E-P]/ 2[E]₀
1.0	0.10		0.84
	0.14		0.79
	0.30		0.78
	0.40		0.77
	0.50		0.80
1.8	0.10	1.1	0.81
		2.2	0.75
		3.3	0.75
		6.6	0.76
		11.0	0.75

[a] *p*-Nitrophenyl phosphate. [b] *p*-Nitrophenol.

bowski & Coleman, 1974). In addition, it is comparable to the value of 11.8 obtained previously (Chlebowski & Coleman, 1974).

Phosphorylation of Subunits by Substrate. In Figure 5 the fraction of covalently phosphorylated subunits ($[E\text{-}P]/2[E]_0$ is shown as a function of *p*-nitrophenyl phosphate at three pH values for isozyme III. Values for isozyme I at pH 7.0 are also presented. In all four cases, ($[E\text{-}P]/2[E]_0$) increases slightly and attains a constant value above about 10^{-4}, up to our highest concentration of 2×10^{-2} M. The maximum value, $([E\text{-}P]/2[E]_0)_{max}$, depends on pH, decreasing as the pH increases. At p.H 7.0, $([E\text{-}P]/2[E]_0)_{max}$ decreases somewhat at the highest concentration of *p*-nitrophenyl phosphate. The results in Table I indicate that this change cannot be due to an increase in ionic strength or the concentration of *p*-nitrophenol (as an impurity or product).

A plot of $([E\text{-}P]/2[E]_0)_{max}$ vs. pH is illustrated in Figure 6. For pH 7.0, 7.5, and 8.0, each point is an average of the $([E\text{-}P]2[E]_0)_{max}$ values over the substrate concentration range at which $([E\text{-}P]/2[E]_0)_{max}$ is essentially constant. For pH 9.0 and 10.0, the points represent the average of the values for two and three relatively high substrate concentrations, respectively. Figure 6 shows that $([E\text{-}P]/2[E]_0)_{max}$ decreases as the pH increases.

The above results were obtained under steady-state conditions for the intermediates. We made a few pres-steady-state phosphorylation measurements using a rapid mixing and quenching apparatus[1] at pH 7.0 and 8.0 using times of 10 and 5.0 ms. In both cases ($[E\text{-}P]/2[E]_0$) was relatively high but

[1] We thank Dr. H. Froede for the use of his apparatus.

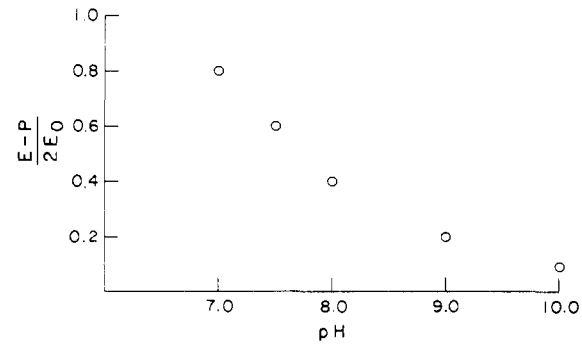

FIGURE 6: Fraction of phosphorylation of both subunits of isozyme III as a function of pH at 25 °C. Data points represent averages of the values for the various concentrations at which phosphorylation is constant.

Table II: Fraction of Phosphorylated Subunits of Isozyme III for Various Substrates at pH 7.5 and 25 °C[a]

[S][b] (M × 10⁴)	$[E\text{-}P]/2[E]_0$ pnpp	PP_i[c]	ADP[d]	GP[e]	PhP[f]
180	0.60	0.58	0.62	0.52	0.52
90	0.59	0.58	0.60	0.52	0.56
45	0.63	0.53	0.51	0.44	0.56
9.0	0.59	0.58	0.55	0.43	0.54
4.5	0.56	0.56	0.46	0.45	0.51
2.3	0.64	0.41	0.52	0.39	0.53
0.90	0.51	0.37	0.34	0.34	0.51
0.45	0.50	0.28	0.31	0.29	0.46

[a] All solutions contained 0.1 M NaCl, 10^{-5} M $ZnSO_4$, and 10^{-3} M $MgCl_2$. [b] These values are approximate. The actual concentration of each substrate was within ±10% of these values. [c] Pyrophosphate. [d] Adenosine diphosphate. [e] D-Glucose 6-phosphate. [f] Phenyl phosphate.

Table III: Fraction of Covalent Phosphorylation and Rate Constants[a] for Various Steps in the Mechanism Given in Equation 1

pH	$([E\text{-}P]^b/2[E]_0)_{max}$	k_{cat} (s^{-1})	k_4(min) (s^{-1})	k_3 (s^{-1})	k_{-3} (s^{-1})
7.0	0.80 ± 0.04	6.5 ± 0.2	32	8.3	0.8
7.5	0.60 ± 0.03	9.2 ± 0.0	23	16	1.2[c]
8.0	0.40 ± 0.03	14.9 ± 0.04	25	40	2.0[c]
9.0	0.20 ± 0.01	19.6 ± 0.6	25	98	<3
10.0	0.09 ± 0.01	20.2 ± 0.8	22	220	<6

[a] Values per subunit. [b] Averaged over the concentration range at which $[E\text{-}P]/2[E]_0$ appeared constant. Values are also averaged over several different experiments. Data for isozyme I are not included. [c] Obtained using extrapolated values for $[E\text{-}P]/2[E]_0$ from equilibrium phosphorylation by P_i. See ref 22. Although designated minimum values because k_3 was assumed to be much larger than k_{cat}, the values for k_4 are thought to be approximately correct.

substantially lower than the steady-state value. The turnover times at these pHs are longer than 40 ms (see Figure 4).

The effect of the nature of the substrate on the values for ($[E\text{-}P]/2[E]_0$) is illustrated in Table II, which lists ($[E\text{-}P]/2[E]_0$) as a function of concentration for five substrates. There are only small differences in $([E\text{-}P]/2[E]_0)_{max}$ for different substrates.

Discussion

It is worth pointing out some of the advantages of the method we use for measuring the steady-state level of phosphorylation over the usual method of using radioactive substrates (McManaman, 1978; Cocivera et al., 1980). We do not have to obtain complete recovery of the denatured or quenched enzyme, although we try to do so, because we

compare the optical absorbence of the two bands to obtain the relative amounts of phosphorylated and unphosphorylated subunits. The two subunits stain equally well. There is no chance of a large mistake because we can see the relative amounts by eye. Since we do not use radioactive substrates, we can make measurements with high substrate concentrations just as readily as with low substrate concentrations; we do not have to wash a precipitate of enzyme free from a 10 000-fold excess of radioactive substrate. We do not subtract a control value, which in other methods can be quite large.

Although one might entertain the possibility that a phosphorylation measurement of 60% of the subunits is really 50% when other methods are used, such a consideration would not be appropriate for our method of measurement. We measure both the phosphorylated subunits and the nonphosphorylated subunits. A value of 60% phosphorylation of subunits means that the band corresponding to the phosphorylated subunits was 50% darker than the band corresponding to the nonphosphorylated subunits, a difference which is unmistakably apparent to the eye.

On the other hand, our method is more time consuming, since it is more laborious to run gels and scan them than it is to count radioactive samples.

Before discussing the kinetic mechanism, we have to reach a conclusion as to whether marked negative cooperativity occurs. The evidence has been inconclusive, since conflicting results have been reported (Simpson & Vallee, 1970; Chlebowski & Coleman, 1974, 1976; Ko & Kezdy, 1967; Fernley & Walker, 1969; Trentham & Gutfreund, 1968; Chlebowski et al., 1976, 1977; Lazdunski et al., 1971; Bloch & Schlesinger, 1973, 1974; Hull & Sykes, 1976; Hull et al., 1976). Recently a restudy of equilibrium dialysis taking the endogenous phosphate into account has shown that the binding of alkaline phosphatase by the two sites is consistent with a single dissociation constant ($K_d = 1 \times 10^{-6}$ M) (Bloch & Bickar, 1978). Also, recent NMR evidence indicates that the equilibrium binding stoichiometry for P_i, which can be as high as two, depends on the number of metal ions added to the reconstituted enzyme (Otvos et al., 1979). The authors suggests that this dependence may account for conflicting NMR data reported previously (Chlebowski et al., 1976, 1977; Hull & Sykes, 1976; Hull et al., 1976).

Finally, a study of covalent phosphorylation of the enzyme using inorganic phosphate and making measurements by gel electrophoresis showed that the two subunits are active and act at least approximately independently (McManaman, 1978). Thus, newer evidence favors the independent participation of the subunits in binding P_i (alkaline pH) and in reacting with P_i to form the covalent phosphorylated species (acidic pH). As far as newer studies are concerned, there is no evidence for half-site reactivity or marked negative cooperativity of the enzyme in its interaction with P_i.

The idea of negative cooperatively of alkaline phosphatase stems from the observed substrate activation. This idea was supported by other studies. The idea of anticooperativity was supported by studies with radioactive substrates that showed a stepped dependence of steady-state covalent phosphorylation on substrate concentration in which one and finally two subunits were phosphorylated (Lazdunski et al., 1971).

Our results over the same concentration range differ from the study just discussed (Figure 5). We do not observe The stepped function of phosphorylated subunits vs. concentration that would be expected if there were marked (but not absolute) anticooperativity. We can immediately rule out the possibility of absolute anticooperativity because more than half of the subunits are phosphorylated at pH 7.5 and 7.0.

At pH 7.0 both subunits strongly participate in the catalysis at substrate concnetrations below 5×10^{-4} M, even though this is the concentration at which substrate activation "sets in". This circumstance also holds for pH 7.5. At higher pHs, phosphorylation is less than 50%; therefore, we cannot say immediately that both subunits are functioning. However, it is still true that at pH 8.0 (and at pH 9 and 10, but with only 2 and 3 points, respectively) as well as pH 7.0 and 7.5°, there is no increase in phosphorylation above 10^{-3} M substrate concentration, as might be expected if the second subunit were beginning to participate in the catalysis.

This discussion of our results shows that the phenomenon of substrate activation, which formed the basis for the idea of anitcooperativity, does not involve anticooperativity.

Because our results indicate there is no anticooperativity, we have written the minimal kinetic scheme (eq 1) in terms of independent subunits. This scheme does not account for substrate activation and cannot apply at very high substrate concentrations. The scheme does not explicitly indicate any conformational changes, so that if any slow ones should occur their effect on the rate will be reflected in the values of the constants that are represented in the scheme. The particular constant involved will then be an overall constant representing the rate of the conformational change as well as the process explicitly presented. For example, if $E{\cdot}P_i$ should undergo a conformational change before dissociating, then k_4 indicated in the scheme would turn out to be the reciprocal of the sum of the reciprocal of the rate constant for the dissociation and the reciprocal of the rate constant for the conformational change.

The scheme leads to the following pertinent equations:

$$K_m = \frac{(k_{-1} + k_2)/k_1}{1 + \frac{k_2}{k_4}\left(1 + \frac{k_{-3}}{k_3} + \frac{k_4}{k_3}\right)} \tag{2}$$

$$k_{cat} = \frac{k_2}{1 + \frac{k_2}{k_4}\left(1 + \frac{k_{-3}}{k_3} + \frac{k_4}{k_3}\right)} \tag{3}$$

and

$$\frac{[E-P]}{2[E]_0} = \frac{\frac{k_2}{k_4}\left(\frac{k_{-3}}{k_3} + \frac{k_4}{k_3}\right)}{\left[1 + \frac{k_2}{k_4}\left(1 + \frac{k_{-3}}{k_3} + \frac{k_4}{k_3}\right)\right]\left(1 + \frac{k_m}{[S]}\right)} \tag{4}$$

We have represented the total concentration of subunits in all its forms as $2[E]_0$, with $[E]_0$ as the enzyme concentration. We have done this to emphasize that we are treating both subunits as equally and independently active. The largest possible value for $([E-P]/2[E]_0)$ is one. For $[S] \gg K_m$, we get

$$\left(\frac{[E-P]}{2[E]_0}\right)_{max} = \frac{\frac{k_2}{k_4}\left(\frac{k_{-3}}{k_3} + \frac{k_4}{k_3}\right)}{\left[1 + \frac{k_2}{k_4}\left(1 + \frac{k_{-3}}{k_3} + \frac{k_4}{k_3}\right)\right]} \tag{5}$$

We use eq 3 and 5 and measure k_{cat} and $(E-P]/2[E]_0)_{max}$ in the paper. We also know or measure k_{-3}/k_3 from measurements of equilibrium phosphorylation using high concentrations of P_i. The value of this quotient is small. We are still left with one more unknown than we have equations. This turns out

not to be too serious because we do have more information. The equations used to calculate the individual rate constants are

$$k_4 = \frac{k_{cat}}{1 - \frac{k_{cat}}{k_2} - \left(\frac{[E-P]}{2[E]_0}\right)_{max}} \quad (6)$$

and

$$k_3 = \frac{1}{\frac{([E-P]/2[E]_0)_{max}}{k_{cat}} - \frac{k_{-3}/k_3}{k_4}} \quad (7)$$

We can calculate a minimum value for k_4 by assuming that k_2 is much larger than k_{cat} (at least 100 times larger). It turns out that for our values of k_{-3}/k_3, which are always quite small, and using even the minimum value of k_4, the second term in the denominator of eq 7 is small compared to the first term. Therefore, the value of k_3 that we calculate is nearly independent of actual value of k_4 (or of k_2). Thus, it is possible to assess the role of k_3 in determining k_{cat} by comparing the two without regard to the values of k_2 and k_4. It is apparent that k_3 is the major term in determining k_{cat} at pH 7 and 7.5 and decreases in importance as the pH increases, becoming of small importance (10%) at pH 10 (Table I).

We calculated the minimum value of k_4 by assuming that k_2 was very large with respect to k_{cat}. In fact, there is evidence that k_2 is large with respect to k_{cat}. Since k_{cat} must be smaller than k_2, k_3, and k_4, it follows that if k_2 is large with respect to k_3 or k_4 it will be large with respect to k_{cat}. Now it has been repeatedly observed that k_{cat} is approximately the same for a large number of esters, some with good and some with poor leaving groups. Although it is possible that k_2 is the same for all esters, it appears much more reasonable to explain the constancy of k_{cat} by assuming that k_2 is so large that it scarcely influences the value of k_{cat}, eq 3. This will occur when k_2 is substantially larger than k_3 or k_4. Similarly, we observe that the degree of phosphorylation is the same for several substrates (pH 7.5). Again, this can be explained in similar fashion with eq 5. Finally, the occurrence of an instant burst in stopped-flow kinetics with *p*-nitrophenyl phosphate indicates that k_2 must be considerably larger than k_3.

Therefore, the values listed in Table I as minimum values of k_4 are not far from the actual values. For example, the calculated values for k_4 based upon the assumption that $k_2 = 10k_{cat}$ for *p*-nitrophenyl phosphate are 65, 31, 30, 28, and 25 for the entries in the table for pH 7 to 10.

There may be some danger in assuming that k_2 is much larger than k_{cat} at pH 9 and 10 because the data on which this conclusion was based were obtained at pH 8.0 and lower. However, no assumptions are made in evaluating k_3.

It is interesting to note that k_3, the rate constant for dephosphorylation, is strongly pH dependent and increases rapidly with increasing pH, whereas k_4 is nearly constant (Figure 7).

Our values of k_4 can be compared with those obtained by NMR studies using inorganic phosphate and estimating the rate of dissociation by line widths. In one study, k_4 was evaluated as 24 s^{-1} at pH 7 and <25 s^{-1} at pH 8.0 (Hull et al., 1976). In another study at pH 8 based upon the line width of P with E·P_i in equilibrium with P_i, k_4 was determined to be 60 ± 20 s^{-1} (Chlebowski et al., 1977). Our minimum values of 32 and 25 s^{-1} at pH 7 and 8 are in reasonably good agreement with the NMR studies. However, there is some leeway, and it may be possible to slip an extra rate-influencing step, such as a conformational change, into the scheme.

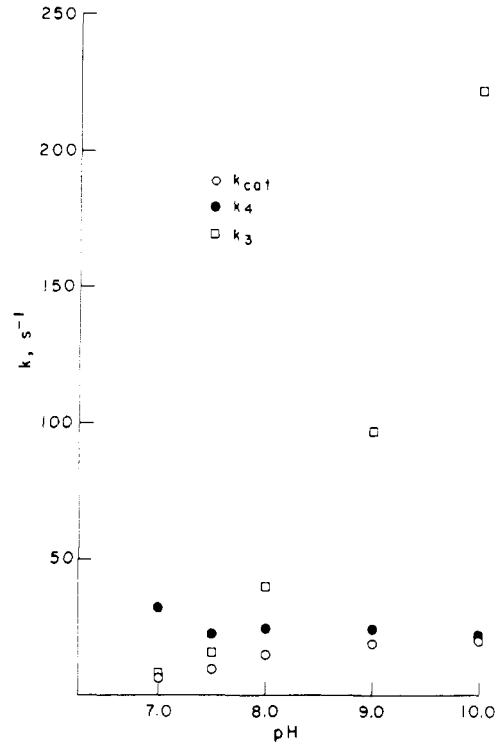

FIGURE 7: The pH dependence of the kinetic parameters that were derived assuming the applicability of the mechanism in eq 1.

The pH variation of k_{cat} and $([E-P]/2[E]_0)_{max}$ roughly resembles ionization curves with a pK_a of about 7.7, although the variation is too slow. Yet it is interesting that the resemblance is there, because we know that k_{cat} derives from two rate constants that, with respect to pH, behave very differently from k_{cat}. The rate constant k_4 is approximately independent of pH, whereas the other rate constant, k_3, keeps increasing with pH. Yet the two combine to give an apparent ionization curve (Figure 7).

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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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