The reaction catalyzed by this enzyme is similar to that catalyzed by glutamine synthetase. However, purified glutamine synthetase does not catalyze the formation of γ-glutamylcysteine nor is the present enzyme active with ammonia. The slight but definite activity observed with hydroxylamine is consistent with the formation of an activated glutamate intermediate. It is pertinent to note that ^{18}O studies have shown that there is a transfer of oxygen from glutamate to inorganic phosphate in the course of γ-glutamylcysteine synthesis (Strumeyer and Bloch, 1960). There is now substantial evidence that enzyme-bound γ-glutamyl phosphate is an intermediate in the reaction catalyzed by glutamine synthetase and it seems probable that a similar intermediate is involved in the synthesis of γ-glutamylcysteine. The availability of large amounts of homogeneous γ-glutamylcysteine synthetase from rat kidney now makes possible a variety of studies on the mechanism of the reaction and on the structure of the enzyme.

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Conformational Isomers of Alkaline Phosphatase in the Mechanism of Hydrolysis*

Ted W. Reid† and Irwin B. Wilson‡

ABSTRACT: The level of phosphoryl-enzyme formed when alkaline phosphatase (*Escherichia coli*) and [^{32}P]phosphate esters are rapidly mixed at 25° and pH 8.0 was measured as a function of time from 10 to 200 msec using a rapid mixing and sampling device. There is an initial rapid phosphorylation of more than 30% of the enzyme. The phosphoryl-enzyme level then falls to about 10% in about 50 msec which is somewhat longer than the turnover time. The earliest measurements were made at 10 msec. At this time the level of phosphorylation is on its way down. When P_i was added to the substrate no change was observed, but when P_i was added to the enzyme the initial high level of phosphorylation did not appear; instead there was a slow rise to the steady-state level of 10%. The time required was about 45 msec. Similarly in stopped-flow experiments using *p*-nitrophenyl phosphate as a substrate an extremely rapid burst of *p*-nitrophenol requiring less than 3 msec, the dead time of our instrument, was observed. About 50% of the enzyme was phosphorylated. When P_i was added to the enzyme no burst was found. Addition of P_i to the substrate was without effect. Similar results were obtained with the inhibitor *p*-chloroanilidophosphonate. We interpret these results as indicating that there are two equally stable conformations of the enzyme, E_α and E_β. Only E_β can react with substrates and only E_α with P_i. The slow step in the hydrolysis of substrates is $E_\alpha \rightarrow E_\beta$. The steady-state level of only 10% phosphorylation indicates that dephosphorylation is not the rate-limiting step. Yet a burst is obtained. A complete scheme is presented which explains these observations and also explains why a phosphate acceptor such as Tris increases the rate of utilization of substrate even though dephosphorylation is not rate limiting.

Early studies with alkaline phosphatase from *Escherichia coli* showed that the enzyme catalyzes the hydrolysis of a wide range of phosphate anhydrides and esters at the same rate (Garen and Levinthal, 1960; Heppel *et al.*, 1962). This fact, plus the finding that Tris (and similar compounds) by acting as a phosphate acceptor could accelerate the rate of

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substrate hydrolysis with little change in the rate of P_i release, led to the proposal that the rate-determining step was dephosphorylation of a phosphoryl-enzyme intermediate (Wilson *et al.*, 1964).

In disagreement with the above proposal Aldridge *et al.* (1964) found that for enzyme which was phosphorylated at pH 5.5 with P_i (Engstrom and Agren, 1958; Schwartz and Lipmann, 1961) and was rapidly mixed with buffer at pH 8.4, the rate of dephosphorylation was twice as fast as the turnover of the enzyme at pH 8.0. Also, transient-state kinetic studies by Fernley and Walker (1966, 1969) showed a rapid release (burst) of alcohol followed by a steady-state release of alcohol only at pH <7. Thus, these data would seem to indicate that at pH $\geq$7, the rate determining step is phosphorylation.

In order to account for the fact that almost all substrates are hydrolyzed at the same rate at high pH, even though dephosphorylation is not the sole rate-determining step, Trentham and Gutfreund (1968) proposed that the mechanism involves a first-order conformation change of the enzyme–substrate complex. This step is assumed to be slow compared with the subsequent transfer of phosphate from substrate, S, to enzyme, the final step being the liberation of phosphate from a phosphoryl-enzyme intermediate

$$E + S \rightleftharpoons E\cdot S \overset{\text{slow}}{\rightleftharpoons} E^*\cdot S \longrightarrow \text{phosphorylation, etc.}$$

In order to test this scheme, the kinetics of the combination of the reversible competitive inhibitor, 2-hydroxy-5-nitrobenzylphosphonate, with the enzyme were studied by the stopped-flow and temperature-jump techniques (Halford *et al.*, 1969). A relatively slow change in optical absorption was found. This was interpreted as indicating that a first complex between inhibitor and enzyme occurs rapidly but leaves the absorption unchanged. This rapid and silent complexation step is followed by a slow conformational change in the complex which is reported by a change in optical absorption. The rate of this conformational change is postulated as the rate of hydrolysis of substrates. This is a reasonable interpretation of the changes in absorption, but this new hydrolytic scheme has some shortcomings. It does not automatically explain why all substrates are hydrolyzed at about the same rate. Trentham and Gutfreund assume that the conformational change of the E·S complex occurs at a fixed rate independent of the nature of S. This assumption, while better, is not too different from assuming that the change E·S → E–P + ROH is independent of S. Secondly the increased rate of production of ROH in the presence of an acceptor such as enthanolamine is not explained nor is the observation that there is little or no change in the rate of formation of phosphate. Indeed, this mechanism predicts that the rate of formation of phosphate would be decreased by an acceptor. The present study was undertaken in order to explore these questions.

Experimental Section

Chemicals. [^{32}P]P_i was obtained from New England Nuclear, [^{32}P]*p*-nitrophenyl phosphate from International Chemical and Nuclear, [^{32}P]sodium pyrophosphate monolabeled, from New England Nuclear. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (1964). *p*-Chloroanilidophosphonic acid was obtained from Pfaltz and Bauer and phenylarsonic acid from Aldrich Chemical.

Enzyme. The enzyme was obtained from *Escherichia coli* by the osmotic shock technique of Neu and Heppel (1964, 1965) and purified by chromatography on DEAE-cellulose using a sodium chloride gradient (Simpson *et al.*, 1968). The final preparations were found to be 90–105% pure, as compared to the crystalline enzyme of Malamy and Horecker (1964).

Enzyme Assay. The enzyme concentration in each experiment was determined by assaying the activity with *p*-nitrophenyl phosphate in 1 M Tris at 27°, and comparing this value with the one obtained for crystalline enzyme by Malamy and Horecker (1964). The molecular weight was assumed to be 89,000 (Simpson *et al.*, 1968).

K_i for p-Chloroanilidophosphonic Acid. Initial rates of hydrolysis of *p*-nitrophenyl phosphate (2×10^{-5} M) were measured in the presence of seven concentrations of *p*-chloroanilidophosphonic acid (1.18×10^{-4}–1.65×10^{-5} M) in 0.01 M Tris, pH 8.0, 25°. The total volume of the reaction mixture was 3 ml and the quantity of enzyme was 0.279 μg. Plots of v^{-1} *vs.* *p*-chloroanilidophosphonic acid concentration yielded straight lines intercepting at the ordinate, V_0^{-1}. The slope under these conditions is K_m(of *p*-nitrophenyl phosphate)/V_0K_i(S).

K_i for Phenylarsonic Acid. Initial rates of hydrolysis of *p*-nitrophenyl phosphate (8.9×10^{-6} M) were measured in the presence of six concentrations of phenylarsonic acid (4.4×10^{-3}–8.9×10^{-4} M). The determinations were carried out in the same manner as those for *p*-chloroanilidophosphonic acid.

K_m Determination for PP_i. Initial rates of hydrolysis of *p*-nitrophenyl phosphate (2×10^{-4} M) were measured in the presence of ten concentrations of pyrophosphate (1.6×10^{-5}–1.6×10^{-4} M). These experiments were carried out in the same manner as those of *p*-chloroanilidophosphonic acid.

K_m Determination for ATP. Initial rates of hydrolysis of *p*-nitrophenyl phosphate (2×10^{-4} M) were measured in the presence of nine concentrations of ATP (2.5×10^{-5}–2.5×10^{-4} M). These experiments were carried out in the same manner as those for *p*-chloroanilidophosphonic acid.

Stopped-Flow Studies. These experiments were carried out using a Durrum–Gibson stopped-flow spectrophotometer. The enzyme concentration after mixing was 4.25×10^{-6} M. The buffer was 0.01 M Tris (pH 8.0). The concentrations after mixing were: *p*-nitrophenyl phosphate, 5×10^{-5} M; phosphate, 1×10^{-5} M; phenylarsonic acid, 1×10^{-3} M; *p*-chloroanilidophosphonic acid, 4×10^{-5} M. The temperature for all experiments was 25°.

Rapid Sampling Apparatus. The two reactants in solution were contained in two syringes. The plungers were driven by a synchronous motor and the contents of the syringes were forced into an eight jet mixing chamber at the head of a stainless steel capillary wire. Wires of varying lengths were substituted to change the reaction time. The reaction mixture exited from the wire into a quenching solution to stop the reaction "instantaneously." The wires, within which the reaction occurred, were calibrated by filling them with a solution of *p*-nitrophenol (0.5 mM) in 0.01 M Tris (pH 8.0) while they were in the instrument. They were then flushed out with 0.01 M Tris (pH 8.0), and the absorbance of the solution was determined after dilution to 2.0 ml. In this way the volume of the mixing chamber (~5 μl) was included in the volume of the wire. The accuracy of the instrument was checked by following the hydrolysis of 2,4-dinitrophenyl acetate (0.62 mM after mixing) with sodium hydroxide (0.25 M after mixing) at 25°. The reaction was quenched in 2 M

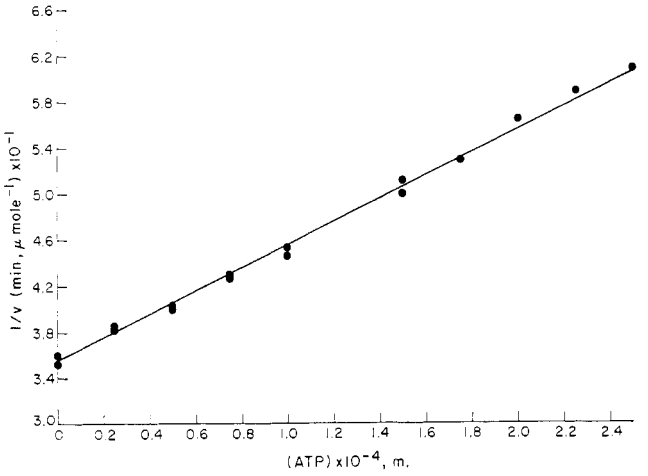

FIGURE 1: Rate of hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase in the presence of ATP. Conditions: 2×10^{-4} M *p*-nitrophenyl phosphate, 0.093 μg/ml of enzyme, $(0.25–2.5) \times 10^{-4}$ M ATP–0.01 M Tris, pH. 8.0, 25°.

TABLE I: Kinetic Parameters.

Substrate or Inhibitor	K_m or K_i ($\times 10^6$)[a]
PP_i	0.87
ATP	1.6
p-Nitrophenylphosphate[b]	1.0
p-Chloroanilidophosphonate	4.3
Phenyl arsonate	500
P_i[b]	0.6

[a] 0.01 M Tris, pH 8.0, 25°. [b] Wilson and Dayan (1965).

acetate buffer (pH 4.5). The absorbance was then read at 400 nm. The rate constant so determined was compared to the value determined using the intermediate speed sampling apparatus of Froede *et al.* (1970). In the latter experiments a lower NaOH concentration was used in order to slow the reaction.

Dephosphorylation Experiments. The initial enzyme concentration was 0.06 mg/ml in 0.01 M Tris (pH 7.0). This solution was allowed to incubate for various periods of time (2 hr–1 month). Enough phosphate was added just prior to doing the experiment to make the solution 10^{-6} M in phosphate and enough [^{32}P]phosphate was added to the solution to yield 10^6 cpm/ml. In order to determine the rate of dephosphorylation, the above solution of phosphoryl-enzyme was mixed in the rapid mixing and sampling apparatus with an equal volume of 0.2 M Tris (pH 8.0). After specified times the enzyme solution (1 ml) was quenched by rapid mixing with 1 ml of 12 N perchloric acid and 0.1 M phosphate. Within 10 sec after quenching 0.5 ml of 3-mg/ml bovine serum albumin was added, followed by 8 ml of water which precipitated the protein. The solution was centrifuged and the precipitate was resuspended in 5% trichloroacetic acid and recentrifuged. The pellet was dissolved in 0.8 ml of 1.6 N NaOH by heating at 80° for 30 min. The solution was washed into a counting vial with 2 ml of Bio-Solve and 10 ml of scintillation cocktail. The cocktail consisted of 7 g of 2,5-diphenyloxazole and 0.42 g of 1,4-bis[2-(4-methyl-5-phenyloxazoylyl)]benzene in 1 l. of toluene. Similar experiments were carried out using enzyme obtained from Worthington Biochemical Corp. In these experiments the enzyme was labeled by reaction with [^{32}P]phosphate at pH 5.5, 6.0, and 7.0, and the reaction conditions for dephosphorylation were: 1 M Tris, 1 M NaCl; 0.01 M Tris, 1 M NaCl; 0.01 M Tris, 0.01 M NaCl; and 1 M Tris, 0.01 M NaCl.

Phosphorylation Experiments. Using the rapid mixing and sampling apparatus, 0.5 ml of enzyme solution was mixed with 0.5 ml of ^{32}P-labeled substrate solution, and after a given reaction time, this solution was quenched in 1 ml of 12 N perchloric acid and 0.1 M phosphate. The labeled and denatured protein was then worked up in the same manner as described above for the dephosphorylation experiments. In these experiments the initial enzyme concentration was usually around 1.8×10^{-6} M in 0.01 M Tris (pH 8.0). The substrate solutions were generally about 4×10^{-5} M plus enough ^{32}P-labeled compound to yield 4×10^8 cpm/ml (10° cpm/μmole) in 0.01 M Tris (pH 8.0). In some experiments the enzyme solution was 10^{-5} M in phosphate. In other experiments different Tris and NaCl concentrations were used (these are specified in the text).

Quenching. With simple high-speed jets 98% mixing is obtained in approximately 3 msec. In our worst case we need only a few per cent mixing which would drop the pH to 1. Since our quenching solution is 12 N perchloric acid, probably less than 0.1 msec is required. Since this time is at least an order of magnitude less than the half-time for dephosphorylation of the enzyme at its fastest rate, we conclude that only negligible dephosphorylation can occur as the enzyme, still undenatured, passes through various pH conditions during the quenching reaction. Similarly only negligible phosphorylation can occur during quenching as evidenced by the fact that both addition of enzyme to a quench solution containing the labeled substrate and addition of labeled substrate to a quench solution containing enzyme produced negligible labeling. These conditions were used to determine the background. Again very little labeling occurred when the enzyme was allowed to react with the substrate for very long periods of time (10 sec–1 hr) before quenching.

Results

K_m and K_i Determinations. Values for the various K_m's and K_i's are shown in Table I. These values are accurate in relationship to one another but are only numerically approximate because they are all based upon the approximate K_m value for *p*-nitrophenyl phosphate (Wilson and Dayan, 1965). The results of a typical K_m determination are shown in Figure 1.

Hydrolysis of 2,4-Dinitrophenyl Acetate with Sodium Hydroxide. Typical results from the use of the rapid mixing and sampling apparatus for studying the hydrolysis of 2,4-dinitrophenyl acetate with NaOH are shown in Figure 2. The value obtained for the second order rate constant was 56.8 sec^{-1} M^{-1} which is in good agreement with the value of 58.2 sec^{-1} M^{-1} obtained using the intermediate-speed sampling apparatus of Froede *et al.* (1970).

Labeling of Alkaline Phosphatase with [^{32}P]Labeled Substrates. The time course of labeling alkaline phosphatase with [γ^{32}P]ATP or [^{32}P]*p*-nitrophenyl phosphate is shown in Figure 3. The counts per minute represent the acid precipitable count at a given time. The value at zero time was obtained

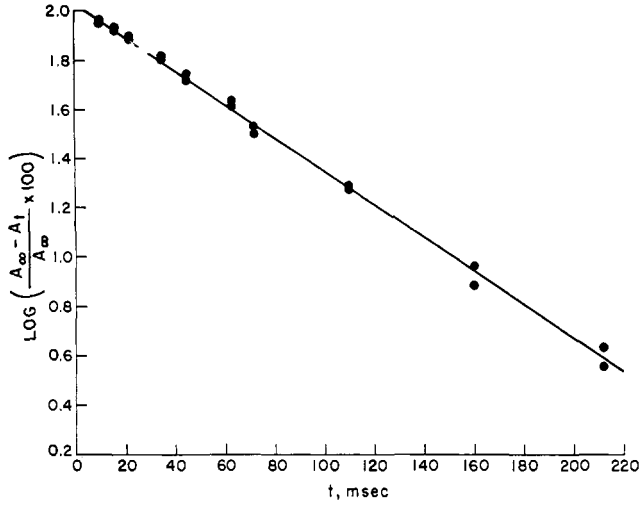

FIGURE 2: Hydrolysis of 2,4-dinitrophenyl acetate by sodium hydroxide. Conditions: 0.62 mM dinitrophenyl acetate–0.25 M NaOH. Absorbance was at 400 nm in 2 M acetate (pH 4.5).

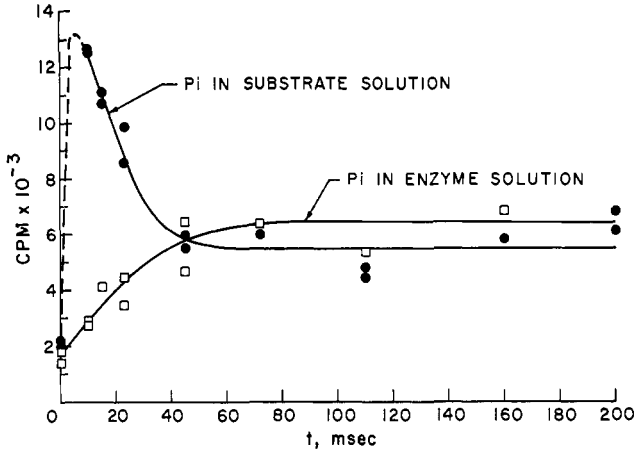

FIGURE 4: ^{32}P labeling of alkaline phosphatase with [^{32}P]pyrophosphate in the presence of inorganic phosphate. Conditions: 2×10^{-5} M [^{32}P]PP_i (8.9×10^7 cpm/μmole), 0.92×10^{-6} M enzyme, and 0.01 M Tris, pH 8.0, 25°, enzyme incubated with 10^{-5} M P_i (□); 2×10^{-5} M [^{32}P]PP_i (8.7×10^7 cpm/μmole), 0.89×10^{-6} M enzyme, and 0.01 M Tris, pH 8.0, 25°, 10^{-5} M P_i added with the substrate (●).

by the addition of enzyme to the quench solution followed by addition of the labeled substrates or the reverse order of addition, since both yielded the same values. Figure 3 shows that rapid mixing of either [γ-^{32}P]ATP or [^{32}P]*p*-nitrophenyl phosphate with enzyme followed by rapid quenching of the reaction mixture produced an initial labeling of approximately 30–40% of the enzyme within 10 msec. The amount of label then decreases within 50–60% msec to a steady-state level of approximately 10%. As seen in Figure 4, similar results were obtained with [^{32}P]pyrophosphate. However, if the enzyme is preincubated with inorganic phosphate before it is mixed with [^{32}P]pyrophosphate, no rapid initial labeling is observed. Also, Figure 5 shows that if the reaction with [^{32}P]pyrophosphate is carried out in 1 M NaCl or in 1 M NaCl plus Tris, no rapid initial labeling is observed and the amount of steady-state labeling is smaller. Table II shows the amounts of steady-state labeling under various conditions.

Stopped-Flow Studies. The results of the stopped-flow studies are shown in Figure 6. For this figure, zero absorbance was taken as the absorbance of the substrate solution mixed with buffer. The results show that when the enzyme is preincubated with *p*-chloroanilidophosphonate and then mixed with *p*-nitrophenyl phosphate in the stopped-flow apparatus, no initial burst of *p*-nitrophenol is observed. However, if *p*-chloroanilidophosphonate is added to the *p*-nitrophenyl phosphate solution an initial burst is observed which corresponds to approximately 46% of the enzyme present. Similar results were obtained using inorganic phosphate in place of *p*-chloroanilidophosphonate.

Since the dead time of the instrument is approximately 3 msec, it was not possible to determine how fast the burst reaction occurs. Within the experimental error of the instrument, the burst was complete by the time the reaction regis-

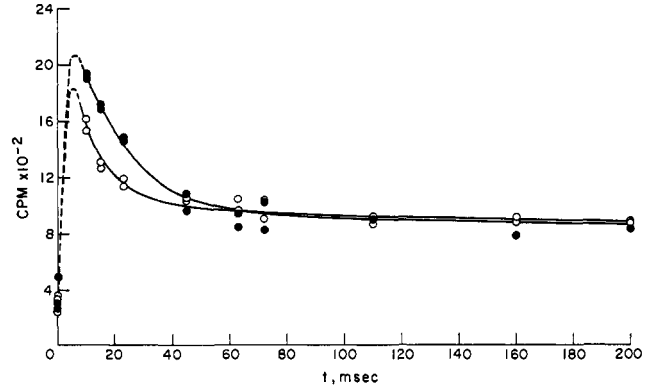

FIGURE 3: ^{32}P labeling of alkaline phosphatase with [γ-^{32}P]ATP and [^{32}P]*p*-nitrophenyl phosphate. Conditions: 6.5×10^{-5} M [^{32}P]*p*-nitrophenyl phosphate (2.4×10^7 cpm/μmole), 5.7×10^{-7} M enzyme, 0.01 M Tris, pH 8.0, 25° (●); 1.1×10^{-5} M [γ-^{32}P]ATP (4.5×10^7 cpm/μmole), 2.3×10^{-7} M enzyme, and 0.01 M Tris, pH 8.0 25° (○).

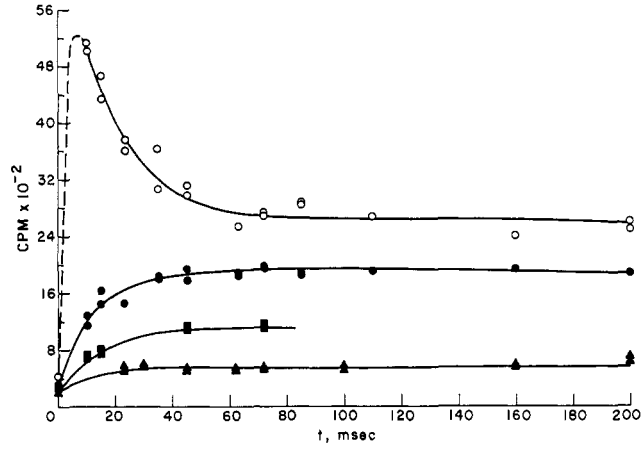

FIGURE 5: ^{32}P labeling of alkaline phosphatase with [^{32}P]pyrophosphate in the presence of various concentrations of Tris and sodium chloride. Conditions: 0.61×10^{-5} M [^{32}P]PP_i (1.3×10^8 cpm/μmole), 3.1×10^{-7} M enzyme, 0.1 M Tris, pH 8.0, 25° (○); 0.61×10^{-5} M [^{32}P]PP_i (1.5×10^8 cpm/μmole), 2.3×10^{-7} M enzyme, 1.0 M NaCl, 0.01 M Tris, pH 8.0, 25° (●); 0.62×10^{-5} M [^{32}P]PP_i (1.4×10^8 cpm/μmole), 2.7×10^{-7} M enzyme, 1.0 M NaCl, 0.5 M Tris, pH 8.0 25° (■); 0.61×10^{-5}M [^{32}P] PP_i ($1.9 \times 10_8$ cpm/μmole), 2.3×10^{-7} M enzyme, 1.0 M NaCl, 1.0 M Tris, pH 8.0, 25° (▲).

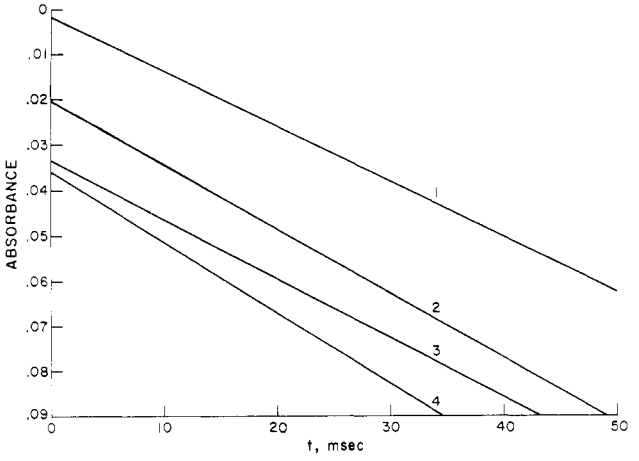

FIGURE 6: Stopped-flow results of the reaction of *p*-nitrophenyl phosphate with alkaline phosphatase in the presence of various inhibitors. Conditions: 5×10^{-5} M *p*-nitrophenyl phosphate, 4.25×10^{-6} M enzyme, and 0.01 M Tris, pH 8.0, 25°; (1) enzyme incubated with 8×10^{-5} M *p*-chloroanilidophosphonic acid; (2) enzyme incubated with 2×10^{-3} M phenylarsonic acid; (3) nothing added to the enzyme, 8×10^{-5} M *p*-chloroanilidophosphonic acid added with substrate; (4) no inhibitor present.

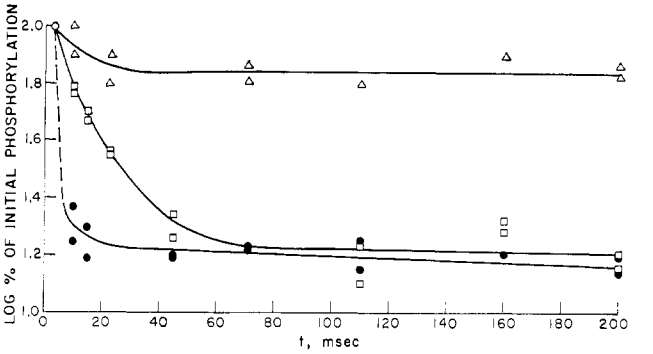

FIGURE 7: First-order plot of the dephosphorylation of the phosphoryl-enzyme. Conditions: 0.01 M Tris (pH 8.0)–10^{-6} M P_i, 25°, using enzyme (0.06 mg/ml) which was incubated at pH 7.0 in 0.01 M Tris for 2 hr (●), 24 hr (□), and 1 month (△).

tered on the oscilloscope. Thus, "$t_{1/2}$" for the burst appears to be less than 1 msec.

When phenylarsonate was incubated with the enzyme, a 27% burst was obtained. Thus, it appears to have a different effect than either *p*-chloroanilidophosphonate or inorganic phosphate.

As seen in Figure 6, if no inhibitor is present a slightly larger burst and a slightly faster steady-state rate are obtained than is the case where the inhibitor was added with the substrate. If a kinetic inhibition constant for *p*-chloroanilidophosphonate is calculated from the difference in the rates shown in Figure 6, a value of 4.2×10^{-6} M is obtained. This is in agreement with the value of 4.3×10^{-6} M obtained by other means. The burst data are summarized in Table III.

Dephosphorylation Rate Studies. The rate of dephosphorylation for enzyme which is phosphorylated at low pH and is then rapidly brought to a higher pH, in order to cause dephosphorylation, can be seen in Figure 7. The point at 3 msec is the zero time value, moved over to correct for the mixing time of the instrument. It is seen that enzyme which was kept at pH 8.0 and then incubated at pH 7.0 for 2 hr in the presence of phosphate prior to dephosphorylation showed an extremely rapid rate of dephosphorylation ($t_{1/2} \sim 2$ msec). Enzyme which was incubated overnight (24 hr) at pH 7.0 with no phosphate present and was then used for dephosphorylation studies showed a slower rate of dephosphorylation ($t_{1/2} \sim 11$ msec). Enzyme which was incucated 3 months at pH 7.0 (no phosphate present) showed a much slower rate of dephosphorylation ($t_{1/2} > 200$ msec). Thus, the results show fast, intermediate, and slow rates of dephosphorylation, depending on the incubation time of the enzyme at pH 7.0. The enzyme was completely active in a standard assay at pH 8.0.

Dephosphorylation studies were also carried out with Worthington enzyme, which is obtained as an ammonium sulfate suspension. Incubation times ranging from 2 hr to 3 days yielded intermediate rates of dephosphorylation. A typical example is shown in Figure 8.

In addition to the effect of incubation time on the dephosphorylation rate, the results show a marked deviation from first-order kinetics. The curves shown in Figures 7 and 8 appear to be biphasic in nature. Over 50 determinations carried out under different conditions all showed this biphasic nature. Various conditions used were: ionic strength varying from 0.1 to 2.0; Tris concentrations varying from 0.1 to 1.0 M; starting pH values of 5.5, 6.0, and 7.0; final pH values of 7.4, 8.0 and 9.0; phosphate concentrations varying from 10^{-4} M to 10^{-9} M; different enzyme purification methods such as with and without heating and with and without ammonium

TABLE II: Per Cent Steady-State Labeling of Alkaline Phosphatase.

Substrate	Conditions	% Steady-State Labeling
ATP	0.01 M Tris, pH 8.0	8.5 ± 1
p-Nitrophenyl phosphate	0.01 M Tris, pH 8.0	10.8 ± 1
PP_i	0.01 M Tris, pH 8.0	10.3 ± 1
	0.10 M Tris, pH 8.0	10.0 ± 1
	0.01 M Tris, pH 8.0, 1 M NaCl	8.0 ± 1
	0.50 M Tris, pH 8.0, 1 M NaCl	4.0 ± 1
	1.00 M Tris, pH 8.0, 1 M NaCl	1.5 ± 1

TABLE III: Stopped-Flow Burst under Various Conditions.

Absorbance Burst Size[a]	%
Enzyme preincubated with P_i	1
Enzyme preincubated with *p*-chloroanilidophosphonate	1
Enzyme preincubated with phenylarsonate	27
No preincubation	
(substrate + *p*-chloroanilidophosphonate)	46
(substrate only)	49

[a] The per cent burst size is moles of *p*-nitrophenyl released in burst, divided by moles of enzyme, times 100.

sulfate precipitation; and different isozymes obtained from DEAE-cellulose chromatography.

Discussion

Although we have seen that the rate of dephosphorylation of the phosphoryl-enzyme at pH 8.0 depends on the past history of the enzyme solution, it can be very fast and is very fast in the usual catalytic process. Dephosphorylation therefore is not the rate controlling step and we have to have some other explanation for the lack of specificity of the enzyme and its enhanced activity in the presence of phosphate acceptors.

Trentham and Gutfreund have suggested that there is a rate determining conformational change in the enzyme–substrate complex that proceeds at the same rate regardless of the substrate. Let us change this proposal in the following way. Assume that there are two enzyme conformational E_α and E_β in slow equilibrium and that E_β binds substrate better than E_α. Then

$$E_\alpha \overset{\text{slow}}{\rightleftharpoons} E_\beta$$
$$E_\beta + S \rightleftharpoons E_\beta \cdot S \text{ etc.} \qquad (1)$$

Thus the addition of substrate will shift the equilibrium toward the β conformation which now will consist of E_β and $E_\beta \cdot S$. Thus in this formulation the slow conformational change is automatically independent of S. This interpretation is thermodynamically equivalent to the interpretation of Trentham and Gutfreund but it is kinetically different and leads to different kinetic predictions. Also in this scheme there is no silent complex.

In order to simplify the discussion of this mechanism we will treat the interactions of substances with the enzyme as all and none. Thus rather than say that S combines more readily with E_β than with E_α we will say S combines with E_β and not at all with E_α. But we emphasize that this is only to simplify the discussion. Now we complete the scheme for hydrolysis of a substrate.

$$\begin{array}{ccccc} E_\beta + S & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & E_\beta \cdot S & \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} & E_\beta\text{–P} + ROH \\ k_5 \downarrow \uparrow k_6 & & & & k_3 \downarrow \uparrow k_{-3} \\ E_\alpha + P_i & \overset{k_5}{\underset{k_{-5}}{\rightleftharpoons}} & E_\alpha \cdot P_i & \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} & E_\alpha\text{–P} + H_2O \end{array} \qquad (2)$$

It will be expedient to consider initial hydrolysis in which there is no added P_i or ROH so that single arrows, *i.e.*, unidirectional steps, can be used in reactions 2, 4, and 5. As a further simplification we assume that $k_4 \ll k_5$. However, it is necessary to complicate the scheme a little by adding an acceptor, ROH, such as Tris or ethanolamine. Thus reaction 2 becomes

$$\begin{array}{ccccc} E_\beta + S & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & E_\beta \cdot S & \xrightarrow{k_2} & E_\beta\text{–P} + ROH \\ k_6 \uparrow \downarrow k_{-6} & & & & k_{-3} \uparrow \downarrow k_3 \quad \searrow^{k_7,\ +\ R'OH} \\ E_\alpha + P_i & \xleftarrow[+H_2O]{k_4} & E_\alpha\text{–P} & & E_\beta + R'OP \end{array} \qquad (3)$$

In these schemes S reacts only with E_β. The phosphoryl-enzyme E_β–P must be able to react with any leaving group of a substrate (microscopic reversibility) but cannot react with water. The second phosphoryl-enzyme, E_α–P, reacts only with water. The enzyme form E_α must be able to react with P_i (microscopic reversibility) although it does not react with S. Similarly, E_β cannot react with P_i.

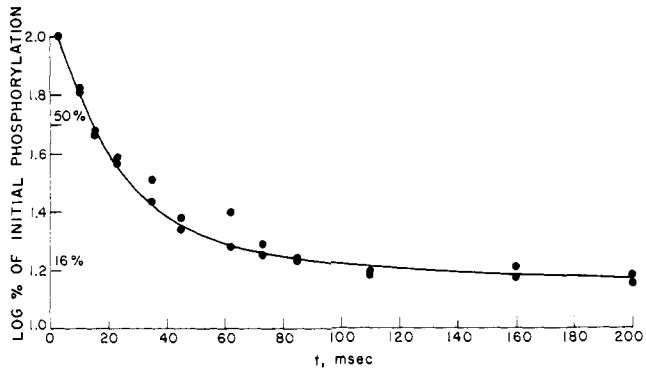

FIGURE 8: First-order plot of the dephosphorylation of the phosphoryl-enzyme. Conditions: 1.0 M Tris (pH 8.0), 25°, 10^{-6} M P_i, 1 M NaCl, 25°, using Worthington enzyme suspended in ammonium sulfate until incubated with P_i.

The increase in the rate of liberation of *p*-nitrophenol, ROH, in the presence of ethanolamine, R'OH, is readily explained by this scheme even though dephosphorylation is not the rate limiting step. The rate is increased because ethanolamine reacts with E_β–P to form E_β so that the slow step $E_\alpha \rightarrow E_\beta$ is passed by.

It would be possible for E_β–P to re-form so rapidly that the formation of P_i would hardly be affected. In the simpler phosphoryl-enzyme theory it would be necessary for dephosphorylation to be very much the rate controlling step. However, this explanation of why the rate of formation of P_i is not much affected by the introduction of an acceptor may not be correct as we shall see.

The rate equation for reaction 3 has the Michaelis–Menten form

$$v = \frac{k(E^0)}{1 + \dfrac{K_m}{(S)}}$$

with

$$K_m = \frac{\left(\dfrac{k_{-1}+k_2}{k_1}\right)\left(1 + \dfrac{k_7}{k_4}\dfrac{k_4+k_{-3}}{k_3}\right)\left(1 + \dfrac{k_{-6}}{k_6}\right)}{\dfrac{k_2}{k_6} + \left(1 + \dfrac{k_7}{k_4}\dfrac{k_4+k_{-3}}{k_3}\right) + \dfrac{k_2}{k_3}\left(1 + \dfrac{k_4+k_{-3}}{k_3}\right)} \qquad (4)$$

$$k_{P_i} = \frac{k_2}{\dfrac{k_2}{k_6} + \left(1 + \dfrac{k_7}{k_4}\dfrac{k_4+k_{-3}}{k_3}\right) + \dfrac{k_2}{k_4}\left(1 + \dfrac{k_4+k_{-3}}{k_3}\right)} \qquad (5)$$

$$k_{ROH} = \frac{k_2\left(1 + \dfrac{k_7}{k_4}\dfrac{k_4+k_{-3}}{k_3}\right)}{\dfrac{k_2}{k_6} + \left(1 + \dfrac{k_7}{k_4}\dfrac{k_4+k_{-3}}{k_3}\right) + \dfrac{k_2}{k_4}\left(1 + \dfrac{k_4+k_{-3}}{k_3}\right)} \qquad (6)$$

The ratio of products ROH: P_i is given by

$$1 + \frac{k_7}{k_4}\frac{k_4+k_{-3}}{k_3} \qquad (7)$$

In these equations we have lumped the concentration of water into k_4 and the concentration of ROH into k_7; thus k_7 is dependent upon R'OH and is found to be directly proportional to R'OH below a certain concentration. In the absence of acceptor, $k_7 = 0$.

The original purpose for introducing the conformational change was to explain the lack of specificity of this enzyme in V_{max} values, without recourse to dephosphorylation as the rate controlling sequence. This requires us to assume that k_6 is considerably smaller than k_2 and also rather smaller than $k_4/(1 + ((k_4 + k_{-3})/k_3))$. This later condition requires that k_6 is considerably smaller than both k_4 and k_3 and that k_{-3} cannot be so much larger than k_3 that $k_4(k_3/k_{-3})$ is not larger than k_6.

However this scheme leaves it somewhat uncertain whether the experimental measurements of dephosphorylation in which the loss of label was quite rapid indicate that the dephosphorylation sequence is really fast. This uncertainty arises because the labeled enzyme consists of two forms and we do not know whether the amount of E_β–P in equilibrium with E_α–P when the enzyme is labeled with [^{32}P]P_i is substantial or not. If the labeled enzyme should be mostly E_α–P, our measurements would indicate only that k_4 is large and it is possible that k_3 may not be large. This question is clarified later and it is shown that dephosphorylation is fast.

Our labeling experiments, using ^{32}P substrates (Figure 3), were at first quite puzzling since we expected to see the extent of labeling rise toward the steady-state value. What we observe is a fall toward the steady-state value. We soon realized, however, that these observations could be explained by reaction 2. If at equilibrium before the addition of substrate there is about 50% E_β we would get a 50% "labeling burst" provided k_2 were considerably larger than at least one of k_3 and k_4. Then as the reaction proceeded the amount of labeled protein would fall to the steady-state value, which in this case is about 10% and is about the same for the three substrates that were used.

Using reaction 2 we predicted that P_i should pull the equilibrium toward E_α and therefore if substrate were now introduced there should be no "labeling burst" and the amount of labeled protein should rise toward the steady-state level. This prediction proved correct (Figure 4).

In the presence of high salt no labeling burst is evident (Figure 4). Evidently high salt shifts the equilibrium toward E_α. The steady-state level of labeling falls somewhat to 8%. High salt increases the rate of hydrolysis of substrates by about 50% at pH 8.0. Evidently both the steady-state overall effective rate constants for phosphorylation and for dephosphorylation are increased about 50%. (The steady-state labeling is given by $(E_\beta\text{–P}) + (E_\alpha\text{–P})$, $S \gg K_m$.)

The ratio of products *p*-nitrophenol:P_i increases linearly with the concentration of Tris. At 0.5 M the ratio is about 1.5 (eq 7). It is also found that the rate of release of *p*-nitrophenol increases linearly with Tris up to about 0.5 M at which concentration the rate has been increased by 50%. Thus from eq 6 we can judge that k_2/k_6 must be considerably greater than 1, a conclusion that we have already reached. From eq 5 we would expect that the rate of formation of P_i would not be much changed by Tris (or ethanolamine, etc.) which is in agreement with experiment. However, these same equations predict that the steady-state labeling would not be much affected by 0.5 M Tris but there is a substantial drop from 8% to 4%. This suggests that the effect of 0.5 M Tris is not restricted to Tris serving as a kinetic acceptor but that 0.5 M Tris is a substantial altered medium. This is not too surprising and we have already seen that 1 M NaCl substantially increases the overall rate of hydrolysis of substrate and also decreases the steady-state phosphorylation level. It does in fact appear from Figures 4 and 5 that 1 M NaCl and 1 M NaCl + 1 M Tris do increase the rate of phosphorylation. In low salt about 40 msec is required to reach the steady-state level of phosphorylation but in 1 M NaCl only about 30 msec are required and in 1 M NaCl + 1 M Tris less than 20 msec are required.

The "labeling burst" must have its counterpart in an "ROH burst." We therefore investigated the burst of *p*-nitrophenol, using stopped flow, when large amounts of enzyme are mixed with *p*-nitrophenyl phosphate. We found an "instantaneous" increase in optical absorbance at 400 nm (Figure 6) corresponding to 49% of the enzyme concentration. This value is consistent with our "labeling burst" of about 35% (Figures 3, 4 and 5) when one considers that in the latter experiments our first point at 10 msec catches the labeled protein on its way down toward the steady-state level. Evidently about half the enzyme is initially in the β form, indicating that k_6 and k_{-6} are about equal.

As in the case of the "labeling burst," the "absorbance burst" is eliminated if P_i is added to the enzyme solution at a concentration well above its K_m value but too small to affect the rate of hydrolysis of the substrate which was present in even higher concentration relative to its K_m value. As expected, the addition of P_i to the substrate solution had no effect.

While the scheme demands that P_i shift the equilibrium toward E_α, there is no requirement for inhibitors such as *p*-chloranilidophosphonate or phenylarsonate. Both compounds are bound better by E_α, Table III, but while *p*-chloranilidophosphonate is bound 50 times better, phenylarsonate is bound only three times better. When *p*-chloranilidophosphonate was added to the substrate there was only a slight effect on the burst. It would be interesting to find an inhibitor that is better bound by E_β.

The steady-state level of phosphorylation at $S \gg K_m$ derived from the scheme is

$$\frac{(E_\alpha\text{–P}) + (E_\beta\text{–P})}{(E^0)} = \frac{1}{1 + \left(\frac{1}{k_6} + \frac{1}{k_2}\right)k_4\left(1 + \frac{k_4 + k_{-3}}{k_3}\right)}$$

The steady-state effective rate constant for phosphorylation, $E_\alpha \rightarrow E_\beta\text{–P}$, is

$$\left(\frac{1}{k_6} + \frac{1}{k_2}\right)^{-1}$$

and the effective steady-state rate constant for dephosphorylation, $E_\beta\text{–P} \rightarrow E_\alpha$, is

$$k_4\left(1 + \frac{k_4 + k_{-3}}{k_3}\right)^{-1}$$

Since the steady-state label is 10%, it is apparent that the steady-state effective rate constant for dephosphorylation is 9 times greater than the steady-state effective rate constant for phosphorylation. This result confirms the assumption that dephosphorylation is not rate controlling.

The turnover time for this enzyme is about 38 msec which

now can be divided up with 34 msec for $E_\alpha \rightarrow E_\beta$–P, almost all of which is consumed in the step $E_\alpha \rightarrow E_\beta$, and 4 msec for E_β–P $\rightarrow E_\alpha$. The phosphorylation of E_β with *p*-nitrophenyl phosphate is very fast and requires less than 3 msec, the "dead time" of our stopped-flow instrument.

It is generally accepted that dephosphorylation is rate controlling below pH 7, based upon "absorbance burst" kinetics in which a 100% burst was obtained. In view of the present findings and the new scheme this assignment is not necessarily correct because it is possible that at low pH the E_α, E_β equilibrium is shifted completely toward E_β. Thus, it is interesting to note that this enzyme represents a case where a burst does not indicate a covalent intermediate, although the phosphoryl-enzyme intermediate for this enzyme is well documented (Barrett *et al.*, 1969; Levine *et al.*, 1969; Reid *et al.*, 1969).

The dephosphorylation studies (Figures 7 and 8) indicate that the enzyme undergoes some kind of change when incubated at pH 7.0 for a relatively long period of time. When the time at pH 7.0 is relatively short, dephosphorylation at pH 8.0 is rapid; $t_{1/2}$ is about 2 msec which agrees with the steady-state labeling experiments. The curves are biphasic indicating that some 15% of the label comes off very slowly, far too slowly to be a phosphoryl-enzyme intermediate.

In conclusion, this work has indicated that there are two forms of the enzyme, E_α and E_β, at equilibrium in equal concentrations at pH 8.0, 25°. The form E_β reacts better with substrates and E_α reacts better with phosphate. The slowest step in the hydrolysis of phosphate esters is the conversion of E_α to E_β. Dephosphorylation is about ten times faster.

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