PHOSPHORYLATION OF ALKALINE PHOSPHATASE (E. coli) WITH *o*- AND *p*-NITROPHENYL PHOSPHATE AT pH <6[1]

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The phosphorylation of alkaline phosphatase (E. coli) by inorganic phosphate and glucose-6-phosphate has been studied by several workers (Engstrom, 1961, 1962; Schwartz and Lipmann, 1961; Levinthal *et al.*, 1962; Schwartz, 1963; Aldridge *et al.*, 1964; Pigretti and Milstein, 1965). These experiments, however, did not permit direct observation of the reaction between enzyme and phosphorylating agent nor did they provide an accurate determination of the number of active sites on the enzyme nor a clear understanding of enzyme behavior in acid media.

This paper reports the preliminary results of experiments in which phosphosylation of alkaline phosphatase by *o*- and *p*-nitrophenyl phosphate[3] at pH 3.9-5.7 was observed directly by spectrophotometric methods. At enzyme concentrations of 10^{-6} to 10^{-5} M detectable amounts of nitrophenol are produced along with phosphorylated enzyme during a very rapid reaction which precedes the steady-state process. This technique provides an excellent means of determining the number of active sites on the enzyme and of studying quantitatively the interaction between inorganic phosphate and enzyme.

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[3]Abbreviations: *o*-NPP - Disodium *o*-nitrophenyl phosphate,
p-NPP - Disodium *p*-nitrophenyl phosphate.

The data suggest but do not establish the following conclusions:

(1) Alkaline phosphatase (E. coli) contains two principal active sites per dimeric unit (M.W. 86,000).

(2) The number of active sites determined experimentally is independent of pH (3.9-5.7), but it is somewhat dependent on substrate (*o*-NPP or *p*-NPP) and/or substrate concentration. (The number of sites found was 1.7 ± 0.1 at [*o*-NPP] = 5 X 10^{-5}- 2 X 10^{-4} M, [*p*-NPP] = 2 X 10^{-5} M and 2.7 ± 0.3 at [*o*-NPP] = 2 X 10^{-3} M.)

(3) During preincubation with enzyme at pH 4.65 (25° C), inorganic phosphate blocks one of the two active sites to phosphorylation by substrate but does not affect enzymic activity.

(4) Phosphorylated enzyme is more stable than native enzyme in acidic media.

Experimental

Materials -- Alkaline phosphatase (E. coli) was obtained chromatographically pure from Worthington Biochemicals Corporation as a slurry with 0.65 saturated ammonium sulfate solution. The enzyme was separated from the slurry by centrifugation and dissolved in N-ethylmorpholine-HCl buffer (0.05 M, pH 8.05, μ = 0.1 M [NaCl]) to obtain stock solutions which remained stable for several months. Disodium *p*-nitrophenyl phosphate was purchased from Sigma Chemical Company. Disodium *o*-nitrophenyl phosphate was prepared by the method of Bessey and Love (1962). All other reagents were "Reagent Grade" materials and they were used as supplied by the manufacturer. The distilled water was obtained from a central supply and was shown to be free of extraneous effects (adventitious metal ions, etc.).

Assay Procedure -- Aliquots of stock solutions of enzyme (10^{-6}-10^{-5} M, pH 8.05), substrate (10^{-5}-10^{-3} M), and in some experiments sodium phosphate (10^{-6}-10^{-2}M) were added to cuvettes (1 cm. path) which contained sufficient

buffer[4] to make 2.0 ml of reaction mixture. When enzyme was preincubated at pH 4.65 with or without inorganic phosphate, reaction was initiated by adding substrate to complete the reaction mixture. In all other experiments enzyme was added last.

The formation of *o*- or *p*-nitrophenol was followed with a Cary 15 spectrophotometer. (λ = 355 mμ for *o*-nitrophenol and 330 mμ for *p*-nitrophenol.) Recording of absorbancy vs. time curves was started within 5-6 seconds after initiation of reaction. Extrapolation of the absorbancy vs. time curves (Figure 1) to zero time gave absorbancy values from which the concentration of nitrophenol released in an "instantaneous burst" was calculated. The concentration of nitrophenol in the burst is assumed to be equal to the concen-

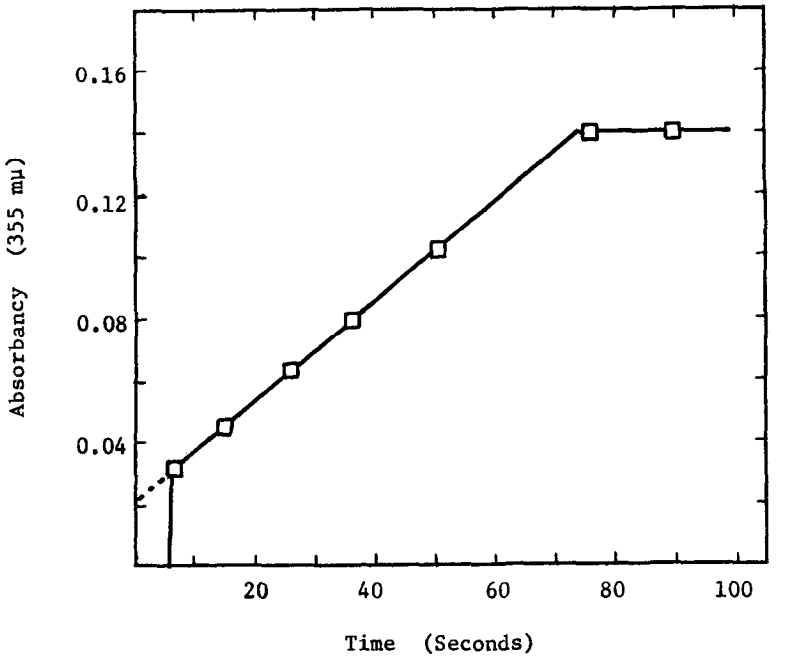

Fig. 1. Alkaline phosphatase catalyzed hydrolysis of *o*-nitrophenyl phosphate at pH 4.65. [Enzyme] = 4.55 X 10^{-6} M, [*o*-NPP] = 5.25 X 10^{-5} M, temperature = 25.04 ± 0.05° C.

[4]The buffers were all 0.05 M and were adjusted to an ionic strength of 0.1 M with sodium chloride. Their pH ranges are given below: Potassium Biphthalate - Hydrochloric Acid, pH 3.7-3.9; Acetic Acid - Sodium Acetate, pH 4.1-5.0; Potassium Biphthalate - Sodium Hydroxide, pH 5.2-5.7.

tration of active sites on the enzyme (Hartley and Kilby, 1954). Enzymic activity[5] was determined from the slopes of the absorbancy vs. time curves which remained linear to 100% reaction in nearly all cases. The reaction appears to be zero order in substrate at pH <6.

Results and Discussion

A study of the effect of substrate concentration, pH, and inorganic phosphate on phosphorylation of alkaline phosphatase (E. coli) by *o*-NPP and *p*-NPP is summarized and discussed in this section. Both concentration of active sites (magnitude of nitrophenol burst) and enzymic activity appear to depend on substrate concentration (Figure 2).[6] The data obtained with *p*-NPP can be interpreted on the basis of the usual enzyme-substrate saturation phenomenon. The same case can be made for some of the *o*-NPP data ([*o*-NPP] $<10^{-3}$ M). However, at high *o*-NPP concentrations (2×10^{-3} M) the burst data require nearly three active sites (2.7 ± 0.3) and enzymic activity is clearly higher than that obtained at lower substrate concentrations. Unfortunately, corroborative studies with high concentrations of *p*-NPP cannot be carried out due to its strong absorption at 330 mμ ($\varepsilon \simeq 4 \times 10^3 M^{-1}cm^{-1}$, pH 4.65).

Although the continued increase in burst with increasing substrate concentration is puzzling, it is consistent with the biphasic relationship between V and V/S (where V is the reaction velocity determined from the slopes of absorbancy vs. time curves and S is the substrate concentration) found in this work (pH <6) and reported earlier by Heppel *et al.* (1962) (pH >7).

The burst appears to be independent of pH over the range 3.9 to 5.7 and enzymic activity increases smoothly with pH as expected (Figure 3). The low

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[5]Enzymic activity is defined as the Slope of Absorbancy vs. Time Curve for NPP Hydrolysis divided by Enzyme Concentration (M^{-1} sec.$^{-1}$).

[6]A good linear relationship between enzyme concentration and burst or enzymic activity was obtained with *o*-NPP (1×10^{-3} M) and *p*-NPP (1×10^{-4} M).

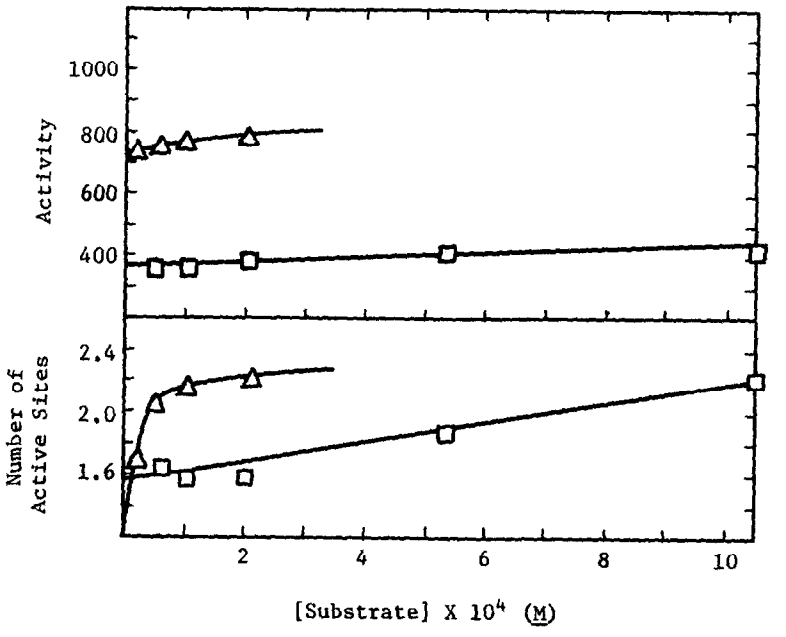

Fig. 2. Effect of substrate concentration on number of active sites ([Nitrophenol] in Burst/[Enzyme]) and enzymic activity (Slope of Absorbancy vs. Time Curve/[Enzyme]). [Enzyme] = 2-10 X 10^{-6} M, pH = 4.68 ± 0.05, Temperature = 25.04 ± 0.05° C, Substrate: □-*o*-NPP, △-*p*-NPP.

value (1.6) for the number of active sites at pH 5.7 is probably due to a poor value for the difference extinction coefficient (ε [*o*-NPP] -ε [*o*-nitrophenol]). It is interesting that the pH dependence of *o*-NPP hydrolysis seems to differ somewhat from that of *p*-NPP.

The nature of the interaction between phosphate ion and alkaline phosphatase was studied by preincubating the enzyme at pH 4.65 for various lengths of time in the presence and absence of phosphate ion. The results are illustrated in Figures 4 and 5. In the absence of inorganic phosphate, the decrease in burst and loss of enzymic activity with increasing preincubation time parallel one another (Figure 4). This means that the concentration of enzymic active sites is decreasing with time, a consequence which can be attributed to destruction of active sites by acid (Schwartz, 1963; Pigrette and Milstein, 1965; Schlesinger, 1965).

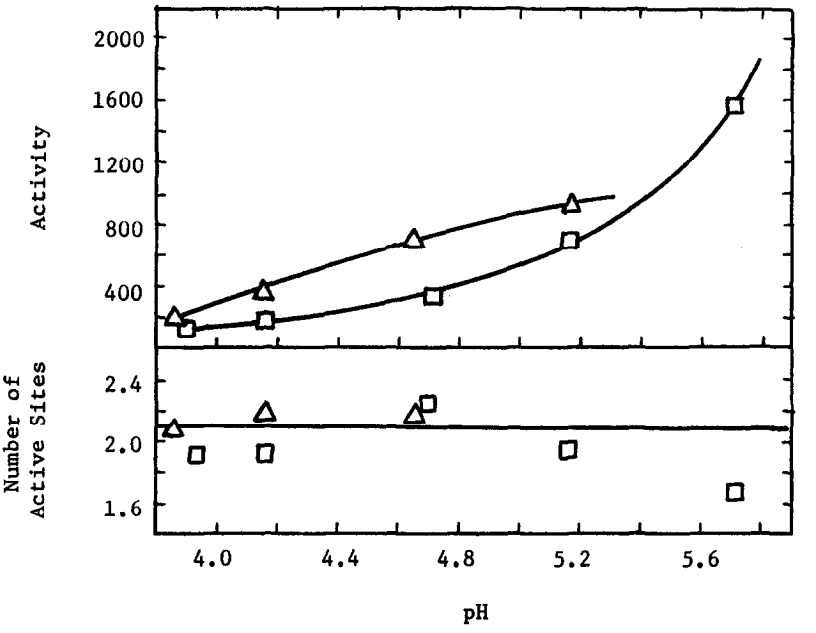

Fig. 3. pH Dependence of Enzymic Activity (Slope of Absorbancy vs. Time Curve/[Enzyme]) and Number of Active Sites ([Nitrophenol] in Burst/[Enzyme]). [Enzyme] = 5 X 10^{-6} M, Temperature = 25.04 ± 0.05° C, Substrate: □-*o*-NPP-1.05 X 10^{-3} M, △-*p*-NPP-2.02 X 10^{-4} M.

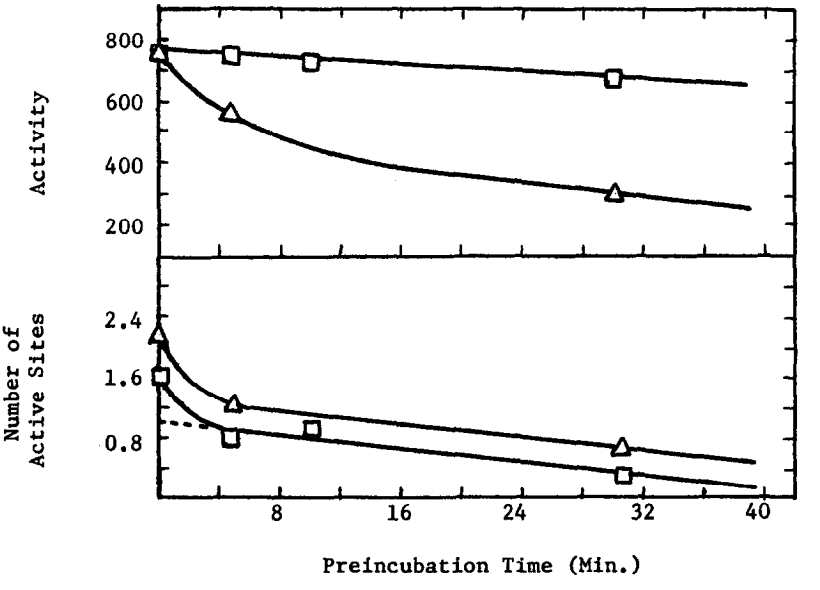

Fig. 4. Effect of Preincubation of Enzyme at pH 4.65 in the Presence and Absence of Phosphate Ion. [Enzyme] = 5 X 10^{-6}M, Temperature = 25.04 ± 0.05° C, Substrate: *p*-NPP; 1.05 X 10^{-4} M. □[HPO_4 =] = 100 [Enzyme] = 5 X 10^{-4} M; △ [HPO_4 =] = 0.

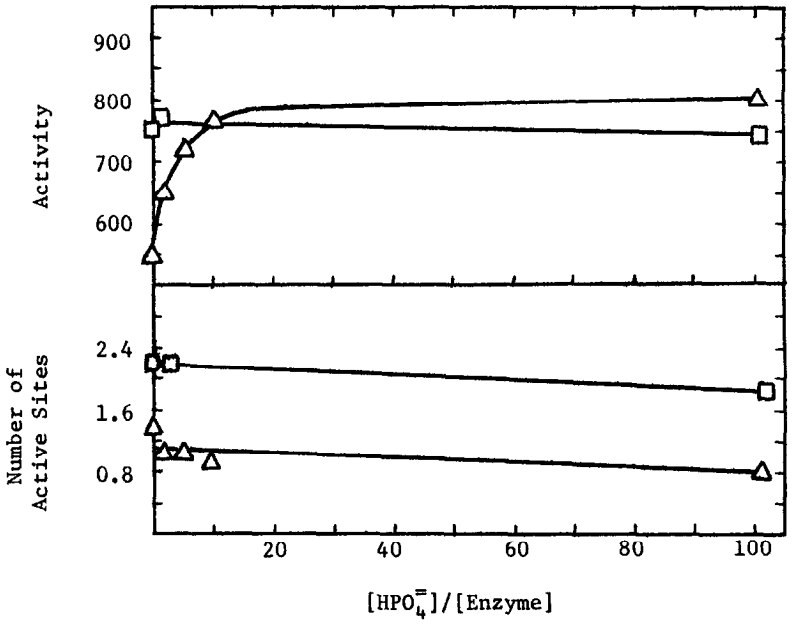

Fig. 5. Effect of Preincubation of Enzyme at pH 4.65 in the Presence and Absence of Phosphate Ion. [Enzyme] = 5 X 10^{-6} M, Temperature = 25.04 ± 0.05° C, Substrate: *p*-NPP, 1.05 X 10^{-4} M. □ No Preincubation; △ Preincubation Time = 5 min.

Preincubation of enzyme at pH 4.65 in the presence of a large excess of inorganic phosphate ([HPO_4 =]/[Enzyme] = 100) produces a striking difference between the burst and activity behavior. The burst falls off rapidly to approximately one-half the reference value of 2 sites (?) and then decreases in somewhat the same fashion as in the experiments containing no phosphate. Enzymic activity, however, exhibits no sudden drop but decreases slowly and linearly with preincubation time. The presence of inorganic phosphate evidently protects enzyme against acid denaturation as cited earlier by Schwartz (1963), but at the same time it causes a decrease in the number of active sites available for instantaneous phosphorylation. These results are consistent with the observations of Schwartz and Lipmann (1961) and Levinthal *et al.*, (1962); namely, that only one phosphate ion was found attached to alkaline phosphatase in experiments with P^{32} labelled inorganic phosphate

but the enzyme was shown to have two sites for binding phosphate in equilibrium dialysis studies.

The effect of inorganic phosphate was illustrated in another way. The dependence of burst and activity on [HPO_4 =]/[Enzyme] ratio was displayed for one preincubation time (5 minutes) (Figure 5). The activity increased sharply with increasing [HPO_4 =]/[Enzyme] ratio until it reached a plateau at [HPO_4 =]/[Enzyme] = 10. When enzyme was preincubated with inorganic phosphate the burst was approximately one-half that obtained when enzyme was added directly to reaction mixtures which contained the same amount of inorganic phosphate. An increase in inorganic phosphate concentration relative to that of enzyme produced a small but noticeable decrease in burst. These results indicate that phosphorylation of enzyme by phosphate ion is slow compared to phosphorylation by phosphate ester. However, it is noteworthy that combination of phosphate ion with enzyme must be fast compared to the acid denaturation process because inorganic phosphate protects enzyme against acid denaturation (Figure 4).

Although the experiments described above suggest that alkaline phosphatase contains at least two active sites of which one is rapidly phosphorylated in acid media, they fail to clarify several aspects of enzymic behavior in acid media. Not the least troublesome is the fact that inorganic phosphate, a potent inhibitor in alkaline solution (Torriani, 1960; Garen and Levinthal, 1960), does not inhibit the enzyme at pH <6. This result cannot be accounted for by making dephosphorylation of the enzyme the rate-limiting step in the hydrolysis process. If this were the case, *o*-NPP and *p*-NPP should hydrolyze at the same rate. The data (Figure 2) indicate that *p*-NPP is hydrolyzed approximately twice as fast as *o*-NPP at pH 4.65.

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