The Phosphate Content of *Escherichia coli* Alkaline Phosphatase and Its Effect on Stopped Flow Kinetic Studies*

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**SUMMARY**

Alkaline phosphatase purified from *Escherichia coli* contains up to 2 molecules of tightly bound inorganic phosphate per enzyme molecule. Removal of this endogenous ligand alters the stopped flow traces obtained for the hydrolysis of substrates at pH 5.5 and at pH 8.0. The pre-steady state burst transient of product release normally seen at pH 5.5 (τ = 20 s⁻¹) is transformed into an instantaneous burst of equal amplitude; an equivalent instant phase is created at pH 8.0, where no burst normally is seen. Addition of 1.5 eq of phosphate to phosphate-free enzyme restores the stopped flow trace completely to the normal burst transient. The maximum burst amplitude seen at all stoichiometries of enzyme preincubated with phosphate at pH 7.4 before reaction with substrate at pH 5.5 is 1.4 to 1.6 moles of product released per mole of enzyme and not 1.0 as previously reported. This amplitude is decreased if enzyme is preincubated with endogenous or added phosphate at pH values below 6.5.

These results suggest that: (a) during the steady state turnover of substrate, the enzyme shuttles between two conformations, one of which binds substrate preferentially and catalyzes substrate hydrolysis and one of which binds phosphate preferentially and has no catalytic activity; the former conformation is thermodynamically preferred in the absence of phosphate; and (b) the active site on the alkaline phosphatase molecule may be equivalent and independent, at least in the pre-steady state.

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Alkaline phosphatase from *Escherichia coli* (EC 3.1.3.1), a nonspecific phosphomonoesterase of molecular weight 86,000 (1), is composed of 2 identical or nearly identical subunits (2). The normal alkaline phosphatase molecule contains 2 to 4 tightly bound zinc ions that are essential for catalytic activity (3-6). Enzyme is inactivated by exposure to 0.1 M nitritotriacetic acid, a chelating agent with high affinity for zinc ion; replacement of the removed metal restores activity rapidly (5). Alkaline phosphatase is reported to bind inorganic phosphate, a product of substrate hydrolysis, in an anticooperative manner: 1 mole of phosphate per mole of enzyme appears to be bound with a dissociation constant of approximately 10⁻⁶ M, another 1 or more moles being held much more loosely (5, 7, 8). This functional asymmetry in a molecule composed of identical subunits implies the existence of subunit interactions which operate to inhibit phosphate binding to one subunit's active site when the other's active site is occupied.

Stopped flow study of the kinetics of substrate hydrolysis and stopped flow and temperature-jump study of the kinetics of binding of a substrate analogue (9-12) suggest that *E. coli* alkaline phosphatase may operate by the mechanism in Scheme I.

\[
\begin{align*}
E' + \text{ROP} & \rightleftharpoons E':\text{ROP} \rightleftharpoons E':\text{P} + \text{ROH} \\
E + \text{ROP} & \rightleftharpoons E:\text{ROP} \\
E + \text{P} & \rightleftharpoons E':\text{P} \\
E' + \text{ROP} & \rightleftharpoons E':\text{ROP} \\
E' + \text{P} & \rightleftharpoons E':\text{P}
\end{align*}
\]

Steps 1, 3, and 6 represent enzyme isomerizations, perhaps conformational changes. The existence of Steps 1 and 3 has been inferred directly from transient (9, 10) and relaxation (11, 12) studies in which they appear as relatively slow (τ⁻¹ = 10 to 100 s⁻¹) binding-linked unimolecular processes. These steps are rate-limiting in the steady state hydrolysis of substrate at pH levels above 7 and appear as a pre-steady state burst transient at aci pH levels. \(E' - \text{P}\) and \(E - \text{P}\) represent covalent enzyme-product catalytic intermediates. At pH levels <6, a covalent phospho-enzyme is thermodynamically more stable than the noncovalent enzyme-phosphate complex (13, 14). Hydrolysis of phospho-enzyme is believed to be rate-limiting in the catalytic mechanism at acid pH levels (15-17). Step 6 has never been observed. It or an analogous isomerization of the enzyme-phosphate noncovalent complex, \(E' - \text{P}\), must exist if Steps 1 and 3 are on the steady state catalytic pathway and are not merely signs of a pre-steady state hysteresis from inactive enzyme to a catalytically active species (18).

In the stopped flow kinetics of substrate hydrolysis, usually monitored by the release of a chromophoric or fluorescent alcohol in Step 5 (Scheme I), the amplitude of the burst transient at acid pH levels has indicated that 1 molecule of substrate is hydrolyzed per enzyme molecule before the steady state is reached (9, 15-17). As the dimeric enzyme should contain two identical active sites, this half-site burst has been taken as further evidence for subunit interaction coupled to the reaction of enzyme with...
substrate or product. It suggests that only 1 of the 2 subunits in a molecule can be in the rate-limiting molecular form at a time.

In addition to the usual burst transient with a first order rate constant of 10 to 100 s⁻¹, some workers have observed at both acid and alkaline pH levels an instantaneous burst, representing some process that precedes the rate-limiting step and is complete within the 3-ms dead time of the stopped flow (9, 12, 19). Although the appearance of this "instant phase" at the expense of the usual burst transient has been correlated with the diminution of alkaline phosphatase molecules to form a catalytically active tetramer of mass 172,000 daltons (12), instant phase also has been reported without mechanistic interpretation under nontetramerizing conditions (9).

In what follows, we report that native alkaline phosphatase, which previous workers have assumed to be phosphate-free (state E in Scheme I), in fact contains a site-stoichiometric quantity of inorganic phosphate and therefore should be considered to be in state E-P of Scheme I. Details of the effect this endogenous phosphate has on the stopped flow kinetics of substrate hydrolysis lead to surprising mechanistic conclusions. They suggest a general model for the origin of instantaneous burst but also indicate that the true upper bound on the amplitude of the normal burst transient is 1.5 moles of product released per mole of enzyme rather than 1.0. Indeed, a complete interpretation of the data reported here calls to question most of the existing evidence for subunit interactions in the function of E. coli alkaline phosphatase.

**EXPERIMENTAL PROCEDURES**

**Methods**

**Assay for Enzyme and Substrates—**Enzyme E₉₀ was measured in a Gilford 240 spectrophotometer with a 1-cm light path and 1.7-mm slit width; ε₉₀₅₄₀ of 0.77 was assumed for alkaline phosphatase (2). Enzyme activity was assayed in 1.0 M Tris-HCl, pH 8.0, at 37°C by measuring the hydrolysis of 0.02% (0.76 mM) p-nitrophenyl phosphate at 410 nm in the 1-cm thermostatted cuvette of a Beckman DU spectrophotometer equipped with a Gilford 2000 recorder. Spectrophotometric assay of substrate solutions used in the stopped flow involved adding 10 µg of alkaline phosphatase to approximately 3 ml of 0.1 M p-nitrophenyl phosphate in pH 8.0 buffer (Δε₅₄₀₅₄₀ = 16,200) or 2.4-dinitrophenyl phosphate in pH 5.5 buffer (Δε₅₄₀₅₄₀ = 11,600) (9).

**Assay for Phosphate—**Inorganic phosphate was assayed by a modification of the procedure of Ames and Dubin (20). Zero to 17 umoles of phosphate (0 to 7 umoles of alkaline phosphatase) in 0.6 to 1.2 ml of 0.010 M Tris-Cl, pH 7.4, were evaporated to dryness at 120°C in acid-washed Pyrex tubes (15 × 125 mm); 0.6 ml of constant-boiling HCl was added to each tube, and the tubes were sealed under vacuum. After hydrolysis for 18 to 24 hours at 100°C, the samples were dried in a vacuum desiccator over NaOH pellets. A freshly prepared solution of 1.4% ascorbic acid and 0.36% ammonium molybdate-4H₂O in 0.86 M H₂SO₄ (0.5 ml) was added to each tube and incubated at 37°C for between 1 and 2 hours before reading the E₉₀ in a Gilford 240 spectrophotometer. Phosphate standards were run with each series of assays. E₉₀ was linear with phosphate between 0 and 150 umoles (E₉₀ = 0.036) and 150 umoles (E₉₀ = 0.545). Estimated accuracy was ±0.1 umole.

**Preparation of Phosphate-free (Purged) and Phosphate-supplemented Enzyme—**Purged alkaline phosphatase was obtained by dissolving 20 mg of lyophilized wild type alkaline phosphatase in 2 ml of 10 mM Tris-Cl, pH 7.4, 0.1 M NaCl, 10 mM nitrotoluic acid, and 10 mM Tris-Cl, pH 7.4, 0.1 M NaCl, 10 mM Na₂SO₄, and dialyzing overnight against 250 ml of 10 mM Tris-Cl, pH 7.4, 0.1 M NaCl. Glassware was thoroughly rinsed free of detergents to maintain phosphate-free solutions. Enzymatic assay before renaturation with zinc showed a lag phase of 10 min before reaching a maximum initial rate that was less than 5% of that obtained for untreated enzyme. Although steady state assays of purged enzyme fell off more rapidly from initial linearity than assays of untreated phosphatase, the initial rates were identical: 110 to 120 moles of product formed per mole of enzyme per s. Supplemental enzyme was obtained by adding limited amounts of P₁ or p-nitrophenyl phosphate to purged enzyme.

**Measurement of Stopped Flow Kinetics—**Experiments were run at 20°C in a Durrum Gibson stopped flow spectrophotometer with a light path of 2 cm and a dead time of 2 to 4 ms. The photomultiplier time constant was 0.5 ms, and 100% transmittance, obtained by mixing substrate with the enzyme buffer, was kept at an 800-ns photomultiplier signal by controlling the photomultiplier-tube dynode voltage. A monochromator slit width of 1 mm was used for study of dinitrophenyl phosphate hydrolysis at 390 nm (blue filter in place). An 0.4-mm slit was used for observing p-nitrophenyl phosphate hydrolysis at 410 nm.

The stopped flow was calibrated as a spectrophotometer at the two wave lengths with solutions of the two chromophoric reaction products, 2,4-Dinitrophenolate anion at pH 5.5 and p-nitrophenolate anion at pH 8.0. Linearity was obtained to within 2% of absorbance, and agreement with the Gilford spectrophotometer was within 3%. The transmittance of a 1:1 mixture of substrate solution and enzyme buffer was checked before each set of reactions with a given syringe load to minimize the effect of lamp and photomultiplier drift.

Reaction curves were traced from the storage-oscilloscope screen onto a matte-finish 0.010-inch thick vinyl sheet. The light signal at 5- to 500-ms intervals was measured from the tracings with underlay graph paper and transformed algebraically into transmittance and absorbance. The steady state base-lines for semilogarithmic plots versus time of the pre-steady state burst of dinitrophenyl phosphate hydrolysis were obtained by graphic back extrapolation to zero time of a straight line through the absorbances measured between 250 and 500 ms after flow stoppage. Zero time in the log plots was placed 3 ms before flow stoppage to take account of the instrumental dead time. Log plots were linear for at least two half-times. The burst transient first order rate constant (r⁻¹) = −2.3 × Δ log₁₀ ΔE (steady state minus transient)/Δ time.

Stock enzyme concentrations before 1:2 dilution with substrate in the stopped flow were 0.3 to 1.0 mg per ml (3.5 to 11.6 µM). Enzyme solutions were stored covered at 25°C before and after stopped flow reaction. Substrate stocks, stored at 3°C until 10 min before reaction, were 0.90 ± 0.05 × 10⁻⁴ M for dinitrophenyl phosphate and 1.05 ± 0.05 × 10⁻⁴ M for p-nitrophenyl phosphate. 2,4-Dinitrophenyl phosphate was dissolved in 0.1 M NaOH, pH 7.0. p-Nitrophenyl phosphate was dissolved in 0.1 M Tris-Cl, pH 8.0. Unless otherwise stated, the enzyme was dissolved in 10 mM Tris-Cl, 0.10 M NaCl, pH 7.4. The final pH of a 1:1 mixture of enzyme buffer and 0.1 M NaOAc, pH 5.5, was 3.3. Enzyme was completely stable in this buffer for at least 1 day at 25°C and for months at...
The stopped flow reaction conditions and the operations of purging, phosphate assay, and stopped flow data analysis are described under "Experimental Procedures."

Native enzyme refers to preparations of lyophilized, purified alkaline phosphatase dissolved in 10 mM Tris-Cl, 0.1 M NaCl, pH 7.4, and dialyzed against a 50-fold excess of the same buffer for at least 4 hours at 4°C. Reclaimed enzyme was purged enzyme that had been reacted with excess 2,4-dinitrophenyl phosphate in the stopped flow, concentrated by Diaflo ultrafiltration, and dialyzed against at least two 100-fold excess volumes of 10 mM Tris-Cl, 0.1 M NaCl, pH 7.4, at 4°C. Supplemented enzyme was prepared by adding small amounts of p-nitrophenyl phosphate to two of the three preparations of purged enzyme several minutes before reaction with 2,4-dinitrophenyl phosphate in the stopped flow.

Total, transient, and instantaneous burst amplitudes are defined in the text. Calculated phosphate contents were obtained by adding the amount of phosphate assayed in purged enzyme to an amount equivalent to the p-nitrophenol released at pH 7.4 before reaction in the stopped flow (ε₂₈₀ = 1.24 × 10⁴ M⁻¹ cm⁻¹ at pH 7.4). Total burst amplitudes for supplemented samples were corrected for the expected absorbance at the reaction pH of the p-nitrophenol assayed at pH 7.4 (ε₂₈₀ = 1.18 × 10⁴ M⁻¹ cm⁻¹ at pH 5.5).

Table I. Phosphate Content of Wild Type Alkaline Phosphatase

| Enzyme          | Phosphate content | Burst amplitude at pH 5.5 | Transient burst rate constant at pH 5.5 | Steady state rate of pH 5.5 |
|-----------------|-------------------|---------------------------|----------------------------------------|---------------------------|
|                 | Assayed           | Calculated                | Total                                   | Transient                 | Instantaneous             |                                 |
| P₁:E            |                   |                           |                                         |                           |                           |                                 |
| Native          | 1.04              | 1.47                      | 1.39                                    | 0.09                      | 22                        | 0.62                          |
| Native          | 2.05              | 1.45                      | 1.09                                    | 0.37                      | 18                        | 0.41                          |
| Reclaimed       | 1.47              | 1.44                      | 1.44                                    | 0.00                      | 21                        | 0.56                          |
| Reclaimed       | 2.21              | 1.50                      | 1.54                                    | 0.04                      | 18                        | 0.61                          |
| Purged          | 0.46              | 1.43                      | 0.38                                    | 1.05                      | 23                        | 0.65                          |
| Purged          | 0.15              | 1.40                      | 0.24                                    | 1.16                      | 16                        | 0.52                          |
| Supplemented    | 0.86              | 1.43                      | 1.06                                    | 0.37                      | 20                        | 0.54                          |
| Supplemented    | 1.76              | 1.50                      | 1.46                                    | 0.04                      | 19                        | 0.54                          |
| Purged          | 0.16              | 1.45                      | 0.10                                    | 1.39                      | 16                        | 0.48                          |
| Supplemented    | 0.61              | 1.52                      | 0.62                                    | 0.90                      | 23                        | 0.52                          |
| Supplemented    | 1.33              | 1.51                      | 1.13                                    | 0.38                      | 19                        | 0.52                          |
| Supplemented    | 1.90              | 1.61                      | 1.40                                    | 0.45                      | 20                        | 0.52                          |

3°C. There is no evidence that the pH-jump involved in mixing this buffer with the more concentrated substrate buffer in the stopped flow affected the kinetic behavior of alkaline phosphatase except in one easily interpreted way described under "Results."

Materials

Nitrilotriacetic acid from Pierce Chemical Co. was used without further purification. Tris, egg-white lysozyme, and disodium p-nitrophenyl phosphate were obtained from Sigma Chemical Co. The di-lutidinium salt of 2,4-dinitrophenyl phosphate was the generous gift of Dr. S. E. Halford. Its extinction at 390 nm, 0.16 for a 10⁻⁴ M solution at pH 5.5, suggested that approximately 10% had hydrolyzed during storage undesiccated in the crystalline state at 25°C. All inorganic reagents were of the highest purity available. Alkaline phosphatase was purified from phosphate-starved cultures of E. coli strain CW3747 by acid extraction (21). Freshly Millipore-filtered solutions had an ε₂₈₀/E₂₈₀ of 2.0 to 2.1. Enzyme activity was between 4700 and 6000 uoles of product formed per mg of enzyme per hour (112 and 143 moles per mole per s) in the assay system described above. These values compare with 143 moles per mole per s obtained by Reynolds for enzyme saturated with zinc (5) and 118 moles per mole per s calculated for our assay conditions from specific activity data obtained by Malamy and Horecker at a different temperature, and assuming a different protein extinction coefficient (22).

RESULTS

Phosphate Content of Wild Type Alkaline Phosphatase—Analysis of purified native wild type alkaline phosphatase for phosphate (Table I) revealed the presence of 1.6 to 2.1 P₁:E.³ Purginc the enzyme of this endogenous phosphate by nitrilotriacetic acid treatment and renaturation with zinc left 0.1 to 0.5 P₁:E. Enzyme reclaimed from purging and reaction with excess substrate in the stopped flow contained 1.5 to 2.3 P₁:E, despite exhaustive dialysis. Hence, wild type native alkaline phosphatase binds approximately 2 molecules of phosphate per enzyme molecule too tightly to be removed during purification or subsequent dialysis. Nitrilotriacetic acid removes zinc ion from the enzyme's active site by chelation and also liberates the tightly bound phosphate which unlike zinc is not essential for catalytic activity.

Effect of Enzyme Phosphate Content on Transient Kinetics of Substrate Hydrolysis—Fig. 1 shows representative stopped flow traces for the hydrolysis of 2,4-dinitrophenyl phosphate at pH 5.5 and of p-nitrophenyl phosphate at pH 8.0 using alkaline phosphatase purified of phosphate and purged enzyme supplemented with 2.0 P₁:E. At both pH levels, purged enzyme showed an instantaneous burst of 1.3 to 1.5 ROH:E before onset of the steady state. In contrast, purged enzyme supplemented with P₁ showed a negligible instantaneous burst at either pH, but had a slower burst phase at pH 5.5, with an amplitude of 1.5 ROH:E and a first order rate constant of 17 to 23 s⁻¹. At

³ The abbreviations used are: P₁:E, stoichiometric molar ratio of inorganic phosphate to enzyme; ROH:E, stoichiometric molar ratio of product alcohol, released by substrate hydrolysis in the pre-steady state burst, to enzyme.
Table II

Comparative kinetics of substrate hydrolysis at pH 5.5 and pH 8.0 by purged alkaline phosphatase before and after supplementation with 2 moles of phosphate per mole of enzyme.

Total, transient, and instantaneous burst amplitudes are defined in the text. Phosphate content of supplemented enzyme = phosphate content of purged enzyme + known amount added of a standard Pι solution. Data are from Fig. 1.

| pH | Enzyme       | Phosphate content | Burst amplitude | Transient burst rate constant | Steady state rate |
|----|--------------|-------------------|-----------------|-------------------------------|------------------|
|    |              | Pι:E              | ROH:E           | s⁻¹ mole product/mole enzyme/s |                  |
| 5.5| Purged       | 0.16              | 1.53            | 0.19                          | 1.34             | 18    | 0.50 |
|    | Supplemented | 2.2               | 1.52            | 1.52                          | 0.00             | 19    | 0.53 |
| 8.0| Purged       | 0.16              | 1.47            | 0.00                          | 1.47             | 18    |      |
|    | Supplemented | 2.2               | 0.05            | 0.00                          | 0.05             | 18    |      |

pH 8.0, supplemented enzyme released p-nitrophenol prior to onset of the steady state.

Examination on an expanded transmittance scale of the reaction trace for purged enzyme at pH 5.5 revealed a small burst transient with a rate constant of 18 s⁻¹ in addition to the much larger instantaneous phase. The observation that the burst could be partitioned between two kinetic steps has led to the adoption of the following definitions. The total burst amplitude is calculated from the absorbance measured by back extrapolation to zero time of the steady state absorbances observed from 0.5 to 5 s after mixing enzyme with substrate, or 0 to 0.1 s at pH 8.0, where the steady state rate is much faster than at pH 5.5. The transient burst amplitude is measured from the semi-logarithmic plot used to obtain the burst rate constant. It is the difference between the absorbances measured by back-extrapolation to zero time of both the steady state and the linear log plot of the burst transient. The instantaneous burst amplitude is the difference between the total and transient burst amplitudes.

Table II compares the transient kinetic parameters of purged and phosphate-supplemented purged alkaline phosphatase from the experiments shown in Fig. 1. The amount of phosphate bound to enzyme appears to affect the amount of instantaneous burst at pH 8.0 and the distribution of burst between transient and instantaneous phases at pH 5.5, but not the burst or steady state rates. Furthermore, the instantaneous burst has the same amplitude within experimental error (±0.1 ROH:E) for the two different substrates, 2,4-dinitrophenyl phosphate at pH 5.5 and p-nitrophenyl phosphate at pH 8.0. The steady state rate should be proportional to the number of functioning active sites on an enzyme molecule. Both its value and that of the total burst amplitude at pH 5.5 are relatively invariant over a wide range of conditions (Tables I and II), thus indicating that total burst amplitude at pH 5.5 is a more reliable measure of the number of active sites than is the phosphate-dependent transient burst amplitude.

The kinetic behavior of phosphate-supplemented, purged enzyme closely resembles that of native wild type alkaline phosphatase (Table I). However, native enzyme occasionally shows a significant amount of instantaneous burst, although the total burst is invariant within experimental error and phosphate assay reveals no obvious deficiency in endogenous phosphate (Table I). To resolve this ambiguity and to confirm the hypothesis that the distribution of the kinetic burst at pH 5.5 between transient and instant phases is controlled by the amount of phosphate bound to the enzyme before reaction with substrate, purged enzyme was supplemented with small amounts of p-nitrophenyl phosphate in pH 7.4 buffer several minutes before stopped flow reaction with 2,4-dinitrophenyl phosphate at pH 5.5. The enzyme hydrolyzed the p-nitrophenyl phosphate immediately, as judged by the development of yellow color, releasing quantitatively the p-nitrophenol and inorganic phosphate in the phosphate ester. The amount of phosphate could be measured accurately from the E₂₄₅ of the p-nitrophenol in the stock enzyme solution. This spectrophotometric procedure is simpler and less error-prone than the digestion and phosphomolybdic acid colorimetric method used for total phosphate assays. The p-nitrophenol released does not interfere seriously with the measurement of total burst amplitude at pH 5.5, because its pKₐ of 7.04 (9) renders it un-ionized and almost colorless at 390 nm at pH 5.5.

The results of p-nitrophenyl phosphate supplementation, summarized in Table I and Fig. 2, lead to two conclusions. (a) The transient burst amplitude at pH 5.5 is equivalent to the phosphate content of alkaline phosphatase up to 1.5 Pι:E (calculated from p-nitrophenol release when there is a choice), and is constant for stoichiometries of 1.5 to 2.0 Pι:E; Table III extends this conclusion to higher phosphate concentrations. (b) At pH 5.5, the total burst amplitude, transient burst rate, and steady state rate are phosphate-independent and insensitive to the purging process.

Nitrilotriacetic acid treatment is not the only way to purge native enzyme of endogenous phosphate. Prolonged dialysis with four or five changes of buffer has created, but not reproducibly, instantaneous burst as high as 1.0 ROH:E (data not shown). Dialysis at an ionic strength of 1.1 is more efficient in this regard than at an ionic strength of 0.1. The instantaneous burst generated by dialysis, like that of purged enzyme, is transformed into transient burst upon addition of stoichiometric amounts of phosphate.

The effect of stoichiometric levels of phosphate on the burst of dinitrophenol release at pH 5.5 is discontinuous with the effect of higher concentrations of phosphate on the burst. Table III
shows that greater than $10^{-4}$ m phosphate is needed before the burst rate constant is affected, and even $10^{-3}$ m phosphate does not diminish the burst amplitude. $10^{-2}$ m phosphate decelerates the burst phase so much that it is no longer easily separable from the steady state. Initial 2,4-dinitrophenyl phosphate concentrations of $5 \times 10^{-6}$ to $5 \times 10^{-4}$ all produce burst amplitudes at pH 5.5 of 1.4 to 1.5 ROH:E and burst rate constants of 18 to 22 s$^{-1}$ (data not shown) when the stoichiometric phosphate concentrations in the stopped flow cell were half those tabulated here. The final enzyme concentration was 2.4 to 2.6 $\mu$m. Total, transient, and instantaneous burst amplitudes are defined in the text.

**Effect of Preincubating Enzyme at Different pH Levels on Burst Amplitude at pH 5.5**—There is evidence that the rate-limiting step in substrate hydrolysis at pH 5.5 may be the dephosphorylation of a covalent phospho-enzyme catalytic intermediate (15-17). Since phospho-enzyme is more stable than the noncovalent phosphate-enzyme complex at pH levels below 6 (13, 14), this hypothesis can be tested by preincubating native enzyme, which contains a stoichiometric amount of endogenous phosphate (Table I), at acid pH levels before reaction with 2,4-dinitrophenyl phosphate at pH 5.5 in the stopped flow. The phospho-enzyme formed during preincubation should start hydrolyzing substrate at the steady state rate without the usual pre-steady state burst, provided dephosphorylation really is rate-limiting at pH 5.5. The results of such an experiment are shown in Table IV and plotted in Fig. 3 along with the pH dependence of phospho-enzyme stability found by other workers (14). Preincubation of native enzyme in acid does lower the burst amplitude without affecting the burst rate constant, although lower pH levels are required than would be predicted from the published data on phospho-enzyme stability. One possible explanation for the decline, that preincubation in acid inactivates the enzyme (14, 23), can be discounted with the steady state rate data in Table I.

**TABLE IV**

| Enzyme sample | Enzyme preincubation pH | Final reaction pH | Burst amplitude | Transient burst rate constant | Steady state rate |
|---------------|-------------------------|------------------|-----------------|-----------------------------|-----------------|
| Native......... | 4.5                     | 5.5              | 0.32            | 0.36            | 0.01            | 25              | 0.26            |
| Native......... | 5.0                     | 5.5              | 0.61            | 0.56            | 0.12            | 24              | 0.37            |
| Native......... | 5.5                     | 5.5              | 1.01            | 1.09            | 0.06            | 25              | 0.47            |
| Native......... | 6.1                     | 5.5              | 1.32            | 1.38            | 0.06            | 24              | 0.48            |
| Native......... | 6.5                     | 5.5              | 1.48            | 1.46            | 0.01            | 23              | 0.46            |
| Purged......... | 5.5                     | 5.5              | 1.49            | 1.49            | 0.02            | 23              | 0.47            |
| Purged......... | 7.4                     | 5.5              | 1.53            | 1.53            | 0.17            | 18              | 0.50            |

a Preincubation buffer was 0.1 M NaOHc, pH 5.5.

b Preincubation buffer was 0.01 M Tris-Cl, 0.10 M NaCl, pH 7.4.
If the effect of preincubating native enzyme in acid on the burst amplitude is due to the preformation of the steady state rate-limiting phospho-enzyme species, the instantaneous burst of purged enzyme should be unaffected by the preincubation pH. Table IV shows that such is the case at the one low preincubation pH tested.

**DISCUSSION**

**Mechanistic Significance of Endogenous Phosphate**

The importance of endogenous phosphate to our understanding of the mechanism of action of alkaline phosphatase depends on one central question. Does endogenous phosphate bind to the enzyme in exactly the same way as phosphate added to enzyme or produced during substrate hydrolysis, or does it occupy a special site, perhaps allosteric in nature, with a higher affinity for phosphate than the catalytic site has? We believe that the first alternative is correct for the following reasons.

1. Comparison of Tables I and II shows that the effect of removing endogenous phosphate (purging) on the transient kinetics of substrate hydrolysis by alkaline phosphatase can be reversed either by adding back a stoichiometric amount of inorganic phosphate or by allowing purged enzyme to hydrolyze a stoichiometric amount of a phosphate ester immediately before reaction in the stopped flow with a chromogenic substrate. Therefore the “endogenous site” is readily accessible to phosphate generated in the catalytic site as well as to added inorganic phosphate.

2. As will be argued in detail below, the effect of endogenous phosphate removal on substrate hydrolysis by alkaline phosphatase (Fig. 1) is qualitatively consistent with a molecular mechanism for substrate turnover (Scheme I) that was developed from experiments performed before the discovery of endogenous phosphate. No special or allosteric properties need be ascribed to endogenous phosphate.

3. The effect on substrate hydrolysis of preincubating native enzyme (containing endogenous phosphate) at acid pH levels (Fig. 3) is consistent with the hypothesis that endogenous phosphate reacts at the catalytic site to form a covalent phospho-enzyme whose rate of hydrolysis is steady state rate-determining at pH 5.5. Long before the discovery of endogenous phosphate, kinetic, thermodynamic, and structural studies concluded that such a compound was formed during substrate hydrolysis (15-17) and as a consequence of adding inorganic phosphate to native phosphatase at pH levels below 6 (13, 14). In this way too, endogenous phosphate behaves in the manner expected for added phosphate.

4. Purged alkaline phosphatase (Fig. 1) and tetrameric enzyme (12) show the same transient kinetics of substrate hydrolysis. Stoichiometric replacement of removed endogenous phosphate has the same effect on purged enzyme that excess phosphate has on tetramer, namely, the restoration of the kinetic behavior seen with dimeric native enzyme.

5. Published reports on the equilibrium dialysis of native alkaline phosphatase with $^{32}$PO$_4$ disagree radically in their estimates of how tightly phosphate binds to enzyme, although they agree that binding is anticooperative (7, 8). As will be explained below, both the inconsistency and the apparent anticooperativity would be expected if nonradioactive endogenous phosphate were present and competed with added labeled phosphate for a single kind of binding site.

If the assumption is accepted that there is only one kind of phosphate-binding site on alkaline phosphatase, the discovery of endogenous phosphate on native enzyme seriously affects the interpretation of any mechanistic study involving enzyme (and endogenous phosphate) concentrations in the vicinity of or greater than the dissociation constant for phosphate. At such concentrations, endogenous phosphate should compete with added ligand for the enzyme active site. Studies in this category include examinations of the transient kinetics of substrate hydrolysis (9, 12, 16, 17, 24), of the transient and relaxation kinetics of substrate-analogue binding (10, 11), and of the thermodynamics of binding of inorganic phosphate (5, 7, 8). For this reason, the following discussion and the Appendices include detailed considerations of the effects of endogenous phosphate on the relevant rate equations and on the apparent binding isotherm for isotopically labeled inorganic phosphate.

In addition, existing ideas about subunit interaction in *E. coli* alkaline phosphatase are gravely affected by our observations that wild type enzyme has significantly more than 1.0 functioning active site per enzyme molecule, at least in the pre-steady state. Data presented here show that 1.5 to 2.2 sites per native enzyme molecule contain tightly bound endogenous phosphate (Table I) and that 1.5 sites must contain phosphate before reaction with substrate in the stopped flow in order to annihilate completely the instantaneous burst (Fig. 2). It is important to consider in detail the ways such a nonintegral site stoichiometry might arise experimentally and the possible reasons why our results differ from those already in the literature.

**Catalytic Mechanism of Alkaline Phosphatase**

**Pre-steady State Process Observed at Acid pH—Phosphate-free enzyme is in a molecular state which hydrolyzes a stoichiometric amount of substrate instantaneously before onset of the catalytic steady state and without the usual pre-steady state burst rate constant of 10 to 100 s$^{-1}$. Such a state, with identical kinetic behavior, has been trapped by reversibly transforming enzyme from a dimer of identical subunits into a tetramer (12).**

The significance of the tetramerization reaction is discussed below. This behavior is what would be expected for the species, $E'$, in Scheme I, whether reaction occurs at acid or alkaline pH. At acid pH, Step 7 in Scheme I appears to be steady state rate-determining (15-17); Step 1 appears to be pre-steady state rate-determining ($\tau^{-1} = 10$ to $100$ s$^{-1}$), Steps 2 and 5 being too fast to observe in the stopped flow (9, 17). Hence, if enzyme were in the $E'$ state of Scheme I, one active site equivalent of ROH would be released during Step 5 before onset of the steady state. At alkaline pH, Step 1 or Step 3 appears to be steady state rate-limiting (9, 17), so that if enzyme initially were in the $E'$ state, one active site equivalent of ROH would be released in the very fast pre-steady state rush of enzyme from $E'$ all the way to $E$ or $E$-ROP. The fact that this process appears instantaneous in a stopped flow with a dead time of 3 ms indicates that if Scheme I is valid, Steps 5, 6, 7, and 8 must have first order rate constants in the forward direction that exceed $10^3$ s$^{-1}$.

Hence, purging appears to stabilize an enzyme species, $E'$, whose existence previously has been inferred from the substrate concentration dependence of the burst rate constant at acid pH (9) and from the substrate-analogue concentration dependence of a unimolecular process with a similar rate constant, seen in stopped flow and temperature-jump studies of analogue binding at alkaline pH (10, 11). The existence of a rate-determining $E \rightarrow E'$ transition before substrate binding also has been inferred from the properties of an instantaneous burst found at pH 9 (18). The completely new information contained in our kinetic results is that, if Scheme I truly describes the catalytic
mechanism of alkaline phosphatase, the equilibrium constant of the \(E \rightarrow E'\) isomerization in the absence of all ligands must be strongly in favor of \(E'\), the enzyme form with higher affinity for substrate and lower affinity for phosphate (9, 11, 12, 10). Otherwise, the transient burst amplitude would not extrapolate to zero for purged enzyme that is completely phosphate-free (Fig. 2). Previous workers had concluded that the \(E\) state was thermodynamically preferred (9, 11, 19) because the unsuspected presence of endogenous phosphate on their native enzyme displaced the isomerization equilibrium in favor of \(E\), as would be predicted from application of Le Chatelier's principle to Scheme I. A necessary consequence of the conclusion that \(E'\) is strongly preferred over \(E\) thermodynamically is that the \(E \rightarrow E'\) equilibrium should be very difficult to study kinetically, in either transient or relaxation systems, in the absence of phosphate.

However, the discovery of endogenous phosphate on native alkaline phosphatase demands a reassessment of the kinetic inferences that led to the formulation of Scheme I, for two reasons. (a) The rate equations used to analyze the transient and relaxation kinetics in terms of binding-linked isomerizations might be inapplicable when phosphate is present. (b) The dissociation of tightly bound endogenous phosphate, if sufficiently slow, might itself appear to be a unimolecular kinetic process linked to substrate or substrate-analogue binding. The second possibility is particularly serious. All of the phenomena which previously have been attributed to a mechanistically important enzyme isomerization might turn out to be artifactual, based instead on the unsuspected presence of endogenous phosphate on native enzyme.

“Appendix A” details the reasons, based on pre-steady state rate equations derived in “Appendix B,” why both objections are groundless. The rate equations derivable from Scheme I under certain simplifying conditions are not altered with respect to the form of their dependence on the substrate or substrate-analogue concentrations. They lead to the same mechanistic conclusions proposed before endogenous phosphate was known to exist. However, the apparent microscopic rate constants obtained from appropriate plots of transient or relaxation rate data should be sensitive to endogenous phosphate. Hence, quantitative conclusions from rapid kinetic studies should be made carefully. In addition, the kinetic model in which the binding-linked unimolecular process is nothing more than a slow dissociation of endogenous phosphate from native enzyme leads to a rate equation predicting an acceleration of the transient burst as the phosphate concentration is increased. It is well established (9, 16; cf. Table III) that the reverse trend is followed. Therefore this particular alternative to Scheme I may be discounted. The best explanation for the pre-steady state rate-determining process at acid pH is that it represents an enzyme isomerization linked to substrate binding. This process appears to be steady state rate-limiting at alkaline pH (25).

Steady State Rate-limiting Process at Acid pH—The original kinetic inference that the hydrolysis of phospho-enzyme was steady state rate-limiting at acid pH was based on three observations (15-17). Such an hypothesis predicts the appearance of a pre-steady state burst of \(p\)-nitrophenol release at acid pH and explains why the steady state rate of phosphate ester hydrolysis at acid pH is independent of the structure of the alcohol leaving group. In addition, the logarithm of the steady state rate at pH levels below 6.0 is a linear function of pH, as might be expected if nucleophilic attack of an hydroxyl ion on the phospho-enzyme phosphate ester leads to formation of the transition state reflected in the steady state rate of substrate hydrolysis.

Given that phospho-enzyme is more stable than the noncovalent \(E-P\) complex at acid pH (13, 14), the hypothesis that phospho-enzyme hydrolysis is rate-limiting at acid pH predicts that incubation of enzyme with inorganic phosphate at acid pH before reaction with substrate in the stopped flow should diminish the amplitude of the pre-steady state burst. Such preincubated enzyme should already be in the rate-limiting state at time of mixing in the stopped flow and should not need to undergo any sort of pre-steady state process to reach that state. However, it has been reported previously that incubation of the enzyme in the presence of phosphate at pH 5.5 does not diminish the burst amplitude from that seen when the enzyme is incubated at pH 7.4 before reaction with substrate at pH 5.5 (9). In order to preserve the hypothesis of rate-limiting enzyme dephosphorylation, Halford (9) had to propose that enzyme subunits alternate roles during substrate hydrolysis so that rate-limiting dephosphorylation of one active site on an enzyme molecule does not interfere with substrate binding and reaction at the other site on the same molecule.

The acid preincubation results of this paper (Fig. 3 and Table IV) contradict the previous findings. Incubation of native phosphatase (or native phosphatase supplemented with \(10^{-4}\) m phosphate) at acid pH before stopped flow reaction with 2,4-dinitrophenyl phosphate at pH 5.5 lowers the amplitude of the burst phase without affecting the steady state rate once the latter is corrected to constant final pH (15). The absence of any effect on the steady state rate indicates that acid preincubation does not lessen the number of active sites, but only affects their initial distribution between molecular states on either side of the steady state rate-determining process. The preincubation effect does not occur with purified enzyme, whose burst amplitude is independent of preincubation pH (Table IV), and thus is most probably the result of formation of phospho-enzyme during preincubation. The pH dependence for the loss of burst amplitude has a lower half-maximal pH than does the published pH dependence of the \(E-P \rightarrow E-P\) equilibrium (14). This discrepancy may well be due to the difference in ionic strength between that study (ionic strength = 1.1) and the experiments reported here and may help to explain the contradiction in the literature (9) concerning the kinetic effect of preincubation of enzyme with phosphate in acid.

In summary, our results are in perfect accord with the hypothesis that dephosphorylation of phospho-enzyme is steady state rate-limiting at acid pH, without recourse to hypothesis of any sort of subunit interaction.

Position of \(E \rightarrow E'\) Isomerization in Steady State Turnover of Substrate—The question must be asked whether the enzyme isomerization observed in the pre-steady state at pH 5.5 also exists in the steady state or whether it represents a one-shot hysteresis (18) of enzyme from an inactive to an active conformation. It has been argued that the transient burst process at pH 5.5 is the same as the steady state rate-limiting process at pH 8.0, because of the similarity in rate constants (15-17) (see also Table II) and because the two processes are altered similarly in a mutant form of alkaline phosphatase (25). We have additional data to support that assignment on the basis of results from studies of the kinetics of substrate hydrolysis by two isozymes of wild type enzyme.

The principle of mechanistic continuity with respect to pH would place the transient burst process on the steady state pathway at pH 5.5 because it is there at pH 8.0. The results

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of this paper support that argument in two ways. Mechanistic continuity with respect to pH is implied by the fact that an operation (purging) which bypasses this process during the first turnover of substrate at pH 8.0 also does so at pH 5.5, giving an instantaneous burst whose amplitude is pH-independent (Table II). That purging bypasses the process rather than accelerating it is indicated by its failure to accelerate the steady state rate at pH 8.0. In addition, the effect of preincubation pH on the burst amplitude at pH 5.5 (Fig. 3) implies that the $E \rightleftharpoons E'$ isomerization communicates with the rate-limiting dephosphorylation of $E \rightarrow P$ at both ends and therefore is a link in the steady state catalytic cycle. If preincubation of enzyme with phosphate at acid pH did not diminish the burst amplitude, one might well argue that the burst process represents a one-shot activation of enzyme from a dormant conformation that cannot equilibrate directly with inorganic phosphate or substrate (9).

**Relationship of Purging to Alkaline Phosphatase Tetramer Formation—** At high ($10^{-4}$ M) zinc ion concentrations and pH levels above 8, alkaline phosphatase exists as a tetramer, molecular weight 172,000, instead of a dimer (12, 26). Although it originally was reported that tetramer bound more than 2 Pi : E (29), it later became clear that phosphate binding is antagonistic to tetramer formation (12). Furthermore, the transient kinetics of substrate hydrolysis by tetramer is exactly like that of purged wild type phosphatase (Fig. 1). Addition of phosphate to tetramer restores the kinetic behavior of native dimeric phosphatase, just as stoichiometric amounts of phosphate return purged enzyme to a state showing “normal” kinetics (Fig. 1). This behavior is most simply explained if the tetrameric state is accessible only to phosphate-free enzyme ($E'$ in Scheme I) and is stabilized by the binding at high pH of zinc to relatively low affinity sites. Then zinc and phosphate binding would exist at opposite ends of a series of coupled equilibria, linked by the $E \rightleftharpoons E'$ isomerization and the dimerization of $E'$ to form tetramer. Zinc would bind to the tetrameric form of $E'$; phosphate would bind to dimeric $E$. Zinc binding should indirectly displace phosphate, and vice versa.

However, it is conceivable that tetramer is synonymous with rather than a special case of the $E'$ state, and that the function of $10^{-4}$ M zinc at pH levels above 8 is to complex endogenous phosphate more effectively than enzyme does. This hypothesis is rendered unattractive by the fact that $\text{Zn}^{2+}$ binds the $\text{HPO}_4^{-2}$ ion with a dissociation constant of $4.0 \times 10^{-4}$ M at 25° and 0.1 ionic strength (27). Such a low affinity should prevent zinc from competing effectively with enzyme whose dissociation constant for phosphate is on the order of $10^{-6}$ M (13, 14, 28, 29). Nevertheless, it remains to be shown that purged enzyme at pH 7.4 in the absence of added zinc is not tetrameric.

**Subunit Interaction in Alkaline Phosphatase**

**Anticooperativity in Binding of Phosphate to Alkaline Phosphatase—** From equilibrium dialysis of alkaline phosphatase with $^{32}$P-labeled phosphate it has been reported that one site per enzyme molecule binds phosphate with a dissociation constant of $6 \times 10^{-7}$ M to $2 \times 10^{-5}$ M, but that additional phosphate is bound more loosely, as though there were another site with a dissociation constant of $1 \times 10^{-6}$ M to $4 \times 10^{-5}$ M (5, 7, 8). Since the 2 subunits of alkaline phosphatase have the same primary structure (2), these results have been considered strong evidence for the existence of subunit interactions that hinder phosphate binding at the active site of 1 subunit of an enzyme molecule when its partner on the other subunit already is occupied.

The discovery of endogenous phosphate on native alkaline phosphatase calls into question both the quantitative and the qualitative conclusions of previous equilibrium dialysis studies. Although some of the considerable disagreement in the literature about the exact values of the dissociation constants can be attributed to differences in temperature, pH, and ionic strength among the studies, differences in the endogenous phosphate content and enzyme concentration certainly should lead to variation in the effectiveness with which labeled phosphate competes with endogenous phosphate for enzyme binding sites. This variability should affect the measured, or apparent, dissociation constant for labeled phosphate; larger values should be obtained as more concentrated enzyme (and therefore endogenous phosphate) is used. In fact, the authors of one of the binding studies reported an inexplicable effect of protein concentration on the equilibrium dialysis results and suggested that it was responsible for the disagreement in the literature about the exact affinity of enzyme for phosphate (8).

More significantly, the presence of endogenous unlabeled phosphate in proportion to the enzyme concentration may explain completely the apparent anticooperativity in the binding of added labeled phosphate.

$$K_{\text{apparent}} = \frac{([E \cdot P] + [E'] \cdot [P])}{(E \cdot [P])} = 1 + \frac{[P]}{K_{\text{diss}}} \cdot \frac{([E \cdot P])}{(E \cdot [P])} = K_{\text{diss}} + [P]$$

Equation 1 shows the apparent dissociation constant for the binding to enzyme ($E$) of labeled phosphate ($P$) in competition with unlabeled phosphate ($P$) (both with the same true dissociation constant, of course). When the enzyme and endogenous phosphate concentrations are on the order of or greater than the true dissociation constant ($K_{\text{diss}}$), as always has been the case (5, 7, 8), most of the unlabeled phosphate is enzyme-bound at low concentrations of labeled phosphate, but most is free in solution at high $P$ concentrations. Hence, the apparent dissociation constant increases with progressive saturation of the enzyme with titrant. The significance of the perturbation on the titration curve depends on the relative values of the true dissociation constant, probably equal to the inhibition constant of around $10^{-6}$ M measured by steady state kinetics (13, 14, 28, 29), and the stoichiometric concentration of endogenous phosphate, probably between 1 and 2 times the enzyme concentration. If the enzyme concentration is $10^{-5}$ to $10^{-4}$ M, the apparent dissociation constant should increase 10- to 100-fold with the progressive saturation of enzyme with $P$. In the Scatchard plots commonly used to display the results of equilibrium dialysis, the slope is equal to $-1/K_{\text{apparent}}$. Equation 1 predicts that the magnitude of this slope should diminish as $\text{PO}_4$ displaces endogenous phosphate from the enzyme. This is the effect that has been observed with increasing saturation of alkaline phosphatase with $\text{PO}_4$ (7, 8). Therefore, it is no longer certain that alkaline phosphatase binds phosphate anticooperatively; anticooperativity may be an artifact generated by the unsuspected presence of endogenous phosphate on native alkaline phosphatase.

**Number of Functioning Active Sites on Enzyme Molecule—** Historically it has been found that only 1 mole of substrate is hydrolyzed per mole of enzyme in the pre-steady state, and
inferred that only 1 of the 2 subunits on a phosphatase molecule is engaged in the steady state turnover of substrate (9, 12, 15, 17, 25). This half-site reactivity was what might be expected for an enzyme which appears to bind phosphate anti-cooperatively (7, 8). The observations here that 1.5 sites per molecule experience the pre-steady state process at pH 5.5 and that 1.5 to 2 sites per molecule bind phosphate too tightly to be removed by dialysis (Table I) challenge that view. The value of 1.5 has been reached by two other paths as well, the instantaneous burst amplitude for purified enzyme at pH 8.0 (Table II) and the amount of phosphate that must be added to purified enzyme to transform the instantaneous burst completely into transient burst (Fig. 2). Furthermore, a total binding stoichiometry of 1.5 has been observed, without explanation, in equilibrium dialyses with 32P-labeled phosphate (8).

These findings lead to two questions. Why is a nonintegral stoichiometry of 1.5 rather than the expected 2.0 sites observed? Why do our results differ from those already published (9, 12, 15–17, 25)? We shall discuss the first issue in a subsequent report that will describe the catalytic behavior of a hybrid type and a mutationally altered form of this enzyme (2).

The most likely reason for the difference between the present (Table I) and previously published values of the burst amplitude is that previous workers were not aware that phosphate depletion of native alkaline phosphatase leads to submaximal transient burst amplitudes. In one case a small and variable instantaneous burst was reported but was dismissed as an artifact of enzyme purification (9). In our hands too, the transient and instantaneous burst amplitudes of native enzyme are variable, but their sum, the total burst amplitude, is reproducible. Furthermore, addition of substoichiometric amounts of phosphate to native enzyme always raises the transient burst amplitude to the same value found for the total burst amplitude: 1.5 ROH: enzyme.

One kind of phosphate-binding study probably is not affected by the discovery of endogenous phosphate. This is investigation of the amount of labeled phosphate covalently bound to alkaline phosphatase at low pH (8, 14, 30), in which the enzyme, and therefore the endogenous phosphate, concentration often is less than 10–7 M and in which the saturation level of phosphorylation is the principal variable being determined. Such studies have shown that alkaline phosphatase is saturated with 1 Pi:E bound covalently at phosphate concentrations below 5 × 10–6 M, although a second site appears to be labeled at phosphate concentrations up to 10–5 M (30). They suggest that anti-cooperativity and subunit interaction may exist at least at the level of covalent phosphorylation of enzyme.

The theoretical foundation for such experiments is somewhat dubious because they involve “fixing” covalent phosphoenzyme by rapid irreversible acid denaturation that transforms phosphatase into a stable phospho-protein. It is assumed that denaturation is complete and faster than any linked processes such as phosphorylation or dephosphorylation. The experiments on which Fig. 3 and Table IV of this paper are based suggest a better approach to measuring the amount of covalently bound phosphate on alkaline phosphatase, namely, the determination of the extent of quenching of the burst amplitude by preincubation of enzyme and phosphate at acid pH before reaction with substrate at pH 5.5 in the stopped flow. Although interpretation of such results requires acceptance of a model for the mechanism of substrate hydrolysis, it is not compromised by unproven assumptions about the kinetics of acid denaturation. The limited data in Fig. 3 give no indication of site heterogeneity with respect to phosphorylation.

In conclusion, it should be noted that many thermodynamic and kinetic studies of alkaline phosphatase must be repeated with purged enzyme to eliminate the perturbing effects of tightly bound endogenous phosphate. As discussed above, some studies on purged enzyme will be unprofitable because phosphate must be present to sustain the phenomenon of interest (e.g. the E → E' isomerization equilibrium). In such cases phosphate-containing enzyme must be used, and the phosphate content must be determined. Until more work is done, and especially until it is understood why native alkaline phosphatase appears to have 1.5 active sites per enzyme molecule instead of 1.0 or 2.0, the status of alkaline phosphatase as the archetype of enzymes showing “negative homotropic subunit interaction” (7, 31) and “half-of-the-sites reactivity” (32) must remain in question.

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Appendix A

Predictions of Several Kinetic Models with Respect to Effect of Phosphate on Burst Rate

Reaction Schemes—It is hard to predict the effect of phosphate on the transient burst rate from Scheme I because two unimolecular processes would be operating simultaneously. However, Scheme I may be considered to be a composite of two simpler mechanisms, shown in Schemes II and III, which are seen to be the limiting cases of Scheme I at low and high substrate (or substrate analogue) concentrations, respectively.

\[
\begin{align*}
\text{ROP} + \text{E.P} & \xrightleftharpoons[k_3]{k_5} \text{P} + \text{E + ROP} + k_{1} \xrightarrow{k_{3}} \text{P} + \text{E'} \\
\text{ROP} + \text{E.P} & \xrightarrow{k_3} \text{P} + \text{E'.ROP} + k_{5} \xrightarrow{k_{3}} \text{P} + \text{ROH} \\
\text{ROP} + \text{E.P} & \xrightarrow[k_3]{k_5} \text{P} + \text{E'.ROP} + k_{5} \xrightarrow{k_{3}} \text{P} + \text{ROH} \\
\end{align*}
\]

At concentrations below \(K_d = k_{4}/k_{3}\), the dissociation constant for the \(E\cdot\text{ROP}\) complex, reaction would tend to proceed via the pre-steady state rate-limiting isomerization of \(E\) to \(E'\), the enzyme form which binds substrate tightly (Scheme II). At concentrations above \(K_d\), enzyme would tend to bind substrate in a rapid bimolecular reaction before undergoing a pre-steady state rate-limiting isomerization of \(E\cdot\text{ROP}\) to \(E'\cdot\text{ROP}\) (Scheme III). It should be clear that even if Scheme I were operating, kinetic data might appear to fit Schemes II or III, depending on reaction conditions.

The possibility that dissociation of tightly bound endogenous phosphate from native \(E\cdot\text{P}\) has been rate-limiting in previous kinetic studies requires the consideration of the simpler mechanism, containing no binding-linked isomerizations, shown in Scheme IV.
that it is rate-determining is being tested separately in Scheme IV. The association of phosphate from \(E\cdot P\) is very fast, since the proposal is that the temperature-jump studies where they could be observed directly are much faster than the associated unimolecular process in temperature-jump studies where they could be observed directly (11). Implicit in Schemes II and III is the notion that dissociation of phosphate from \(E\cdot P\) is very fast, since the proposal that it is rate-determining is being tested separately in Scheme IV.

Most important is the assumption that all processes except the postulated pre-steady state rate-determining step in the respective reaction schemes are so rapid as to be at virtual equilibrium in the time interval used to study the pre-steady state rate-determining process. This assumption is empirically based. Step 5 has never been found to be of kinetic significance, for if it were, the rate of some observable process should vary with the structure of the ROH leaving group of the phosphate ester. Such an effect never has been observed (15, 17). The bimolecular binding reactions (Steps 2 and 4) have been observed to be much faster than the associated unimolecular process in temperature-jump studies where they could be observed directly (11). Implicit in Schemes II and III is the notion that dissociation of phosphate from \(E\cdot P\) is very fast, since the proposal that it is rate-determining is being tested separately in Scheme IV.

It is also assumed, that under the experimental conditions used here (pH 5.5, 20°C), Step 7 is sufficiently slower than the pre-steady state rate-determining process that the transient burst may be considered an approach to equilibrium of all reaction steps preceding Step 7, which is steady state rate-limiting (9, 15, 17). In certain kinds of experiments, such as studies of substrate-analogue binding to alkaline phosphatase at alkaline pH (10, 11), this assumption is true rigorously because analogue is not altered chemically by the enzyme (k₂ and k₆ = 0). Kinetic interpretation of the reversible binding of substrate analogue is simplified further by the fact that Step 5 does not occur either in this case; \(k₅ = k₅/₆₅\) must be infinitely large. This situation is in sharp contrast to that occurring with phosphate ester hydrolysis at acid pH levels, where one must assume, from the fact that the burst amplitude is so large (so close to the maximum permissible site stoichiometry), that \(k₅\) is much smaller than 1. It should be noted that Step 5 is really two reactions; a phosphoryl group transfer and a dissociation of alcohol. It is tolerable to treat it as a single reaction that can be described by a pseudo-equilibrium constant, \(K₅ = [E''\cdot P] / [E - P]\), because of the evidence that \(K₅\) is either negligibly small or infinitely large, depending on the reaction studied.

The rate equations given below are considered to apply to temperature-jump studies of substrate-analogue binding to enzyme. Rigorously derived rate expressions for the relaxation kinetics of coupled binding-isomerization systems (11) include concentration terms for free (unliganded) enzyme, something lacking in our rate equations. We consider the omission to be insignificant in this experimental system, because there should be negligible free enzyme in any reaction mixture containing endogenous phosphate. The enzyme concentrations needed for temperature-jump study (and therefore the endogenous phosphate concentration) exceed the enzyme dissociation constant for phosphate so much that any active site that does not contain substrate-analogue should contain phosphate.

Finally, it should be noted that \(k₁ >> k₋₁\) (Scheme II) and \(k₃ >> k₋₃\) (Scheme III). These relationships, justified by the apparent imbalance of the \(E \leftrightarrow E'\) equilibrium in favor of \(E'\), do not contribute to the derivation of the rate equations but may simplify their interpretation.

**Rate Equations**—Appendix B derives completely Equation 2, the rate equation for the isomerization step in Scheme II. The derivations of Equation 3 (Scheme III) and Equation 4 (Scheme IV) are strictly parallel to that of Equation 2, differing primarily in the assignment of normal variable.

\[
\tau⁻¹ = k₁/[1 + (P)/K₅] + k₋₁/[1 + (ROP)[1 + K₁]/K₂K₃] (2)
\]
\[
\tau⁻¹ = k₋₂K₄/[1 + K₂] + k₃/[1 + K₄][1 + (P)/K₄][ROP] (3)
\]
\[
\tau⁻¹ = k₄ + k₋₄K₄/[1 + (ROP)[1 + K₄]/K₂K₃] (4)
\]

In these equations, \(\tau⁻¹\) is the first order rate constant for the approach to the steady state rate of substrate hydrolysis at acid pH or the first order rate constant for the approach to equilibrium in the binding of substrate analogue to enzyme at alkaline pH; \(K₅ = k₅/k₆\); \(K₂ = k₂/k₆\); \(K₃ = k₃/k₆\); \(K₄ = k₄/k₆\). The rate expressions indicate that as long as the free phosphate and free substrate-analogue concentrations are approximately constant in the course of the "equilibration" being observed, the burst should appear to be a first order approach to the steady state. In temperature-jump reactions, where the approach to a true equilibrium is a small fraction of the stoichiometric reaction, it is assured that (ROP) and (P) will be relatively invariant during the observed relaxation.

**Interpretation**—When compared to the existing rapid kinetic literature on \(E. coli\) alkaline phosphatase, Equations 2 and 4 yield several interesting observations.

1. The effect of phosphate on the burst rate constant allows an unambiguous test of Scheme IV. Equations 2 and 4 both show a substrate concentration-dependence for the burst rate constant like that seen for low concentrations of substrate (9). However, only Equation 2 corresponds qualitatively to the observed inhibition of the burst rate constant by high concentrations of phosphate (Table III). Equation 4 predicts a rate enhancement by phosphate. Hence, the effect of phosphate on the burst eliminates Scheme IV from consideration. Previous workers' conclusion that the transient burst at pH 5.5 derives from a binding-linked isomerization (9, 10, 11) remains valid.

2. Phosphate product-inhibition data should determine the conditions under which Scheme II or Scheme III applies. Previously published kinetic studies support the notion that alkaline phosphatase obeys Scheme II and Equation 2 at low substrate concentrations (9) and obeys Scheme III and Equation 3 at high substrate-analogue concentrations (10). In the first case the burst rate constant decreases with increasing substrate concentration. In the second, the rate constant of a binding-linked unimolecular process increases to a plateau with increasing substrate analogue. As discussed above, Scheme I would resolve this apparent paradox, predicting such simplified kinetic behavior at the limits of low and high substrate concentration. Equations 2 and 3 suggest how detailed phosphate product-inhibition data might reinforce this conclusion. At high substrate or analogue concentrations (Equation 3), phosphate should be a competitive inhibitor, as actually has been observed (16). At low substrate or analogue concentrations, phosphate should be a noncompetitive inhibitor (Equation 2). Data are lacking to test this latter point.

3. Apparent microscopic rate constants and dissociation constants, fitted to burst rate data without consideration of the...
effect of phosphate binding or of the effect of Step 5 will depend on the kind of experiment in which the data were obtained, for two reasons. Comparison of the kinetics of substrate hydrolysis with the kinetics of substrate analogue binding is complicated by the fact that $K_s < 1$ in the first case and $K_s \gg 1$ in the second. This difference affects the apparent affinity for ligand under conditions where Equation 2 is obeyed and the apparent value of $k_{-4}$ under conditions favoring Equation 3. In addition, experiments conducted with high concentrations of enzyme (and endogenous phosphate) are hard to compare with studies at much lower enzyme levels, because of the effect of the phosphate concentration on the apparent value of $k_1$ in Equation 2 and on the apparent affinity for substrate or substrate analogue in Equation 3. This incompatibility is most significant in comparing relaxation-kinetic experiments performed with $10^{-4}$ M enzyme (11) to stopped flow experiments, in which the enzyme concentration never exceeded $10^{-6}$ M (9, 10, 15–17). It may explain a noted discrepancy between isomerization rate constants observed in the temperature-jump apparatus and in the stopped flow (11). It should be noted from Equation 2 that the presence of endogenous phosphate would depress the apparent value of $k_1$ ($k_1/(1 + (P)/K_s)$). This effect might make $k_1$ appear to be smaller than $k_{-1}$, as has been concluded in previous kinetic studies (9, 11). Recall that a principal conclusion from Fig. 2 of this paper (see “Discussion”) is that $k_1 \gg k_{-1}$.

APPENDIX B

Pre-steady State Rate Equation Assuming That Rate-determining Process ($r^{-1} \sim 10$ to $100$ s$^{-1}$) Is Enzyme Isomerization between Dissociation of Phosphate from, and Binding of Substrate to, Enzyme

1. Assume that in Scheme II (“Appendix A”), Step 1 is pre-steady state rate-determining and Step 7 is steady state rate determining.

2. Assume pre-equilibrium (Steps 8, 2, and 5 much faster than Step 1): $E = (E)(P)/K_s$; $(E' \cdot ROP) = ((E')(ROP)/K_s$; $(E' - P) = (E'(ROP)/K_s$. The equilibrium constants are defined under “Appendix A.”

3. Conserve enzyme species: $E_0 = (E)(P) + (E') + (E' \cdot ROP) + (E' - P)$.

4. Condense the conservation expression, using the pre-equilibrium assumption: $E_0 = (E)(1 + (P)/K_s) + (E')(1 + (ROP)/1 + K_s/K_sK_s)$. Define $\alpha$ and $\beta$:

$$\alpha = 1/(1 + (ROP)(1 + K_s)/K_sK_s)$$
$$\beta = 1 + (P)/K_s$$

Then $E_0 = \beta(E) + (E')/\alpha$.

5. Choose the normal variable. Since Step 1 is completely rate-determining on the time scale of the burst transient, but the burst transient is much faster than the steady state, the burst transient measures the equilibration of all of the enzyme species to the left of Step 1 in Scheme II with all of the species to the right of Step 1. Choose as normal concentration variable, either

$$\Sigma = (E)(P) + (E) = \beta(E)$$
$$\Sigma' = (E') + (E' \cdot ROP) + (E' - P), = (E')/\alpha = E_0 - \Sigma$$

As will be seen below, this variable must be normalized to its final (equilibrium or steady state) value.

6. Derive the rate equation:

$$\dot{\Sigma} = -k_1(E) + k_{-1}(E') = -k_1(E) + k_{-1}(E') = -k_{-1}(E) + k_1(E') = -k_{-1}(E) + k_1(E'),$$

where $r^{-1} = k_{-1} + k_1/\beta$.

7. In the steady state (subscript $= s$), Step 1 has come to equilibrium: $k_{-1}(E') = k_1(E)/\Sigma = -k_{-1}(E)/\Sigma = -k_1(E)/\Sigma$. Therefore

$$E_0 = k_{-1}(E) + k_1(E') = k_{-1}(E) + k_1(E') = (E)(1 + K_s/K_sK_s) + k_1(1 + (P)/K_s).$$

8. Substitute into the rate equation: $\dot{\Sigma} = \dot{\Sigma} - \dot{\Sigma} = [\Sigma - \Sigma] = \tau^{-1}[\Sigma - \Sigma]$. Therefore $\Sigma$ will approach $\Sigma$ in a first order manner, provided $\alpha$ and $\beta$ are effectively constant during the burst. The first order rate constant, $r^{-1}$, $= k_{-1}/(1 + (ROP)(1 + K_s/K_sK_s) + k_1(1 + (P)/K_s).$

9. Chromogenic ROIL will appear at the same rate that $E' - P$ does. Because of pre-equilibrium,

$$(E' - P) = \Sigma/(1 + K_s + K_sK_s/(ROP))$$
$$= E_0/[1 + K_s + K_sK_s/(ROP)]$$
$$= \Sigma/[1 + K_s + K_sK_s/(ROP)]$$
$$[E'(P) - (E' - P)] = [\Sigma - \Sigma]/[1 + K_s + K_sK_s/(ROP)]$$
$$= \tau^{-1}[\Sigma - \Sigma].$$

10. Primary difference is in the choice of normal concentration variable. For Scheme III, use either $[(E)(P) + (E)(ROP)]$ or $[(E')(ROP) + (E' - P)]$. For Scheme IV, use either $(E')(ROP)$ or $[(E)(ROP) + (E']$.

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