Kinetics of Substrate Hydrolysis by Molecular Variants of *Escherichia coli* Alkaline Phosphatase*

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WILL BLOCH† AND MILTON J. SCHLESINGER

From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

SUMMARY

Two isozymes of *Escherichia coli* alkaline phosphatase have been compared in the stopped flow spectrophotometer with respect to the kinetics of hydrolysis of two phosphate esters. Hydrolysis of 2,4-dinitrophenyl phosphate at pH 5.5 is biphasic; release of chromophoric alcohol occurs in a rapid burst followed by a slower steady state. The two isozymes show similar burst rates and amplitudes but their steady state rates differ by a factor of 2. Hydrolysis of p-nitrophenyl phosphate at pH 8.0 shows no significant burst and the homogeneous steady state rate is essentially the same for both isozymes. These observations and the ionic strength dependence of the rates of these reaction steps support the hypothesis, proposed previously in the literature, that the molecular process which determines the steady state rate at pH 8.0 is responsible for the presteady state burst at pH 5.5. The steady state process at pH 5.5 must be a subsequent step in the catalytic mechanism.

A molecular hybrid containing one subunit of wild type alkaline phosphatase and one subunit from an inactive mutationally altered alkaline phosphatase shows one-half the normal burst amplitude at pH 5.5. All transient and steady state rates observed for the hybrid equal those seen for wild type enzyme, once corrected for the halved number of active sites inferred from the burst amplitude. This finding casts doubt on catalytic mechanisms involving alternately functioning subunits that several workers have proposed for *E. coli* alkaline phosphatase.

*Escherichia coli* alkaline phosphatase (EC 3.1.3.1), a nonspecific phosphomonoesterase of molecular weight 86,000 (1), is composed of two identical or nearly identical subunits (2). Starch gel electrophoresis of extracts prepared by sonication or osmotic shock (3) and of purified alkaline phosphatase may reveal several isozymic forms of this enzyme (4). All of these isozymes are products of a single *E. coli* structural gene (5). The three principal phosphatase isozymes (numbered in order of increasing negative charge) have been resolved by ion exchange chromatography (6, 7). Isozyme 1 and isozyme 3 appear each to contain identical subunits, whereas isozyme 2 seems to be a molecular hybrid of the two, containing one subunit of each charge type. Isozymes 1 and 3 are virtually indistinguishable with respect to a variety of properties, including optical rotary dispersion and ultraviolet spectra, stability to heat and extremes of pH, substrate specificity, and steady state kinetic constants (6). A recent study in this laboratory has shown that the isozyme 3 subunit differs from the isozyme 1 subunit in that the latter contains an additional arginine residue at the NH₂ terminus of the polypeptide chain (8).

In this report we present data on the stopped flow kinetics of substrate hydrolysis by the two pure isozymes. This comparison illuminates not only the very modest functional differences between the two isozymes but also contributes to an understanding of the molecular events accompanying the hydrolysis of phosphate esters by alkaline phosphatase.

There is considerable published evidence from rapid kinetic studies (9–15), equilibrium dialysis studies of the binding of inorganic phosphate to enzyme (16–18), and investigations of the covalent binding of phosphate to alkaline phosphatase at acid pH values (15, 19) to indicate that the two subunits of the alkaline phosphatase molecule are nonequivalent or nonindependent in their interaction with substrates, substrate analogues, and inorganic phosphate, a product of substrate hydrolysis. Classically, only one of the two subunits appears to hydrolyze substrate in a rapid burst phase preceding the catalytic steady state at pH 5.5. The two subunits appear to bind phosphate with radically different dissociation constants; and only one phosphoryl group can be attached easily to the active site serine side chain (20) of the alkaline phosphatase molecule, even though the apparent molecular symmetry of the enzyme would lead one to expect the existence of two identical active sites on an enzyme molecule.

This body of information and subtler kinetic anomalies have led two groups of workers (12, 15, 21, 22) to suggest that, in the course of normal enzyme function, powerful subunit interactions keep the enzyme molecule in an asymmetric state. In this state, only one of the two subunits is thought to interact easily with substrate, the other reacting preferentially with product. Furthermore, subunits are conceived to alternate roles in a rigidly maintained reciprocating manner; conformational subunit interactions hold them out of phase with one another in the sequence of reaction steps defining the catalytic mechanism.

One way to test this reciprocating subunit, or “flip-flop” (15), hypothesis is to prepare an enzyme molecule in which only one
subunit can function catalytically. If the proposed model were valid, such a molecule would be expected to have considerably lower catalytic efficiency than a wild type enzyme molecule since the reciprocating interaction of two functioning subunits is proposed to be important for the operation of either subunit. Native dimeric phosphatase molecules can be dissociated reversibly into inactive monomers at pH levels below 5 (1, 23). Molecular hybrids, in which the dimer contains dissimilar subunits, can be made by mixing and then reneutralizing acidified enzyme from two different sources (24, 25). The simplest hybrid to make that might test the flip-flop hypothesis is one between fully active wild type alkaline phosphatase and a catalytically inactive mutant alkaline phosphatase isolated from an isogenic strain of E. coli. A variety of such phosphatase mutant strains have been isolated and partially characterized (14, 24-28).

This paper describes the purification and use of such a molecular hybrid to examine the functional interaction thought to exist between subunits of the alkaline phosphatase molecule. The results described below and in a prior publication (29) suggest that the two active sites on the E. coli alkaline phosphatase molecule may be independent and equivalent, showing none of the subunit interaction previously attributed to them.

EXPERIMENTAL PROCEDURES

Analytical Methods—All materials and methods not described below were discussed in a prior publication (29). These include procedures for assaying enzyme, substrates, and inorganic phosphate, for reacting enzyme and substrate in the stopped-flow spectrophotometer, and for analyzing the stopped-flow data. The following values for extinction coefficients were used: ε\text{mm} \text{cm}^{-1} \text{M}^{-1} 0.77 for alkaline phosphatase (2); ε\text{max} = 16,200 for the hydrolysis of p-nitrophenyl phosphate at pK 8.0 (12); ε\text{min} = 11,600 for the hydrolysis of 2,4-dinitrophenyl phosphate at pK 5.0 (12).

Starch gel electrophoresis was performed in a Turner model 310 apparatus. Two-microliter samples were applied to a thin slab of 10% starch gel (Connaught Medical Research Laboratories) in 4 mM Tris-Cl, pH 8.0, and were electrophoresed at a constant 120 volts for 1 hour at 28°C. The buffer trays contained 40 mM Tris-Cl, pH 8.0. For assay of proteins, samples contained 1 to 10 μg of protein and were visualized with 0.5% buffaloo black NBR (Allied Chemical Co.) in 7.5% acetic acid; for assay of enzymatic activity, samples contained 0.04 to 0.4 μg of protein and were visualized (4) with α-naphthyl acid phosphate and Nucleot fast red dye (Sigma Chemical Co.). Gels were fixed in a solution containing acetic acid, acetone, and water (1:5:5).

For the purification of the mutational altered alkaline phosphatase, the yield of alkaline phosphatase protein was based on its ability to react with antibodies directed against wild type alkaline phosphatase as determined semiquantitatively by Ouchterlony double diffusion (30), using rabbit antiphosphatase serum prepared in this laboratory.

Purification of Wild Type Isozymes 1 and 3—For isozyme 3 (see Fig. 1), wild type alkaline phosphatase was purified from phosphatase-starved cultures of Escherichia coli strain CWS777 by acid extraction, DEAE-cellulose chromatography, and gel filtration on Sephadex G-100 (8, 31). This preparation consists largely of isozyme 3; its ε\text{max}/ε\text{min} spectral ratio was 2.0 to 2.1, and its specific activity varied from 45 to 112 moles of product formed per mole enzyme per s (29). Prior to use for the hybridization studies, the preparation was refractionated by column chromatography on DEAE-cellulose in order to eliminate other isozymes that might complicate the purification of hybrid.

Lyophilized alkaline phosphatase (60 mg) was applied to a column of microgranular DEAE-cellulose (4.0 X 40 cm) (Reeve Angel DE52) and eluted with a 2-liter linear gradient of 0.015 to 0.15 M NaCl in a buffer containing 10 mM Tris-Cl (pH 7.4), 1 mM MgCl2, 1 mM Na2SO4, and 0.02% NaN3. The fractions containing essentially pure isozyme 3 were identified by starch gel electrophoresis, pooled, concentrated 50- to 100-fold by ultrafiltration (Amicon model 8-MC with a PM-30 membrane), and stored at 3°C with no loss of activity over 3 months. Refractionation caused no significant increase in specific activity.

**Fig. 1.** Starch gel electrophoresis of purified molecular variants of Escherichia coli alkaline phosphatases. Five-microgram samples of protein were electrophoresed and stained for protein (see “Experimental Procedures”). All movement was toward the anode. The arrow indicates the site of sample application. From left to right: Channel 1, S-33 isozyme 1; Channel 2, wild type isozyme 3; Channel 3, a molecular hybrid between S-33 isozyme 1 and wild type isozyme 3, after reaction with substrate in the stopped flow; Channel 4, the same molecular hybrid before reaction in the stopped flow; Channel 5, wild type isozyme 1; Channel 6, wild type isozyme 3.

The purification of isozyme 1 of wild type alkaline phosphatase from a bacterial culture containing a high concentration of arginine is described elsewhere (8). Fig. 1 compares the electrophoretic patterns obtained for purified wild type isozymes 1 and 3.

**Purification of Isozyme 1 of S-33 (Mutant) Alkaline Phosphatase—**E. coli strain S-33 (24, 25) was chosen because it produces a catalytically inactive protein that cross-reacts with antibodies raised against wild type alkaline phosphatase and has a lower electrophoretic mobility than the wild type enzyme. Isozyme 1 of the S-33 protein was prepared because it has lower electrophoretic mobility than isozyme 3. Hence, a hybrid between S-33 isozyme 1 and wild type isozyme 3 would have the greatest possible charge difference from the two "parental" proteins; this property facilitated its purification by electrophoresis or ion exchange.

One hundred liters of S-33 cells were grown at 37°C in a Fermentor (New Brunswick) in a pH 7.4 minimal medium containing Tris-Cl and inorganic salts (8), 0.2% lactose, 1.2 X 10^{-4} M phosphate, 0.01% arginine, and 0.05% peptone (ShelbidScientfific Co., commercial source). Three hours after exponential growth of the culture ceased because of phosphate limitation, cells were harvested in a Sharples continuous flow centrifuge. Yield of cells was 125 g wet weight. The cells were washed in 2 liters of 50 mM Tris-Cl (pH 8) at 28°C, resuspended in 1.6 liters of 15% sucrose-0.01 M Tris-Cl (pH 8), chilled to 3°C in an ice bath, and converted to spheroplasts (32); 30 ml of 5 mg per ml of egg white lysozyme (Sigma Chemical Co.) in 50 mM Tris-Cl, pH 8.0, and 7.5 ml of 0.1 M EDTA, pH 8.0, were added in two equal additions 25 min apart. The reaction was stopped by adding 20 ml of 1.0 M MgCl2 and 0.1 ml of 1.0 M ZnSO4 when 80% of the cells had become omsotically sensitive as determined by diluting samples 100-fold with water. Lysis was monitored by the loss of turbidity at 540 nm in a Gilford model 240 spectrophotometer.

The supernatant fraction from centrifugation of the spheroplasts was dialyzed 2 days at 3°C against two 10-liter volumes of 10 mM Tris-Cl, 1 mM MgCl2, 0.1 mM ZnSO4, and 0.02% NaN3 (pH 7.4), and was centrifuged to remove a heavy precipitate that formed in the period. Ultrafiltration (Amicon model TCF10, PM-30 membrane) over 2 days at 25°C reduced the spheroplast extract volume to 80 ml which required another centrifugation to remove a precipitate.

The concentrated extract was chromatographed and rechromatographed on microcrystalline DEAE-cellulose with the same
procedure used for purifying wild type isozyme 3 (above). Peak fractions from each fractionation were concentrated by ultrafiltration (Amicon model 8-MC, PM-30 membrane). The identity of the elution peaks (monitored by $E_{650}$) was determined from gel electrophoresis patterns stained for protein and by tests for immunochemical reactivity with anti-phosphatase serum. A minor impurity was removed from the S-33 protein by gel filtration on a Sephadex G-100 column (2.5 x 80 cm). The final yield of protein, assuming $\text{mol} \cdot \text{cm}^{-1} \cdot \text{g}^{-1}$ of 0.77 (2), was 17 mg. It was estimated to be at least 90% S-33 isozyme 1 on the basis of starch gel electrophoresis (Fig. 1).

**Purification of Hybrid Alkaline Phosphatase**—A mixture of 17 mg of S-33 isozyme 1 and 27 mg of wild type isozyme 3 in 8 ml of 10 mM Tris-Cl (pH 7.4), 1 mM MgCl$_2$, 10 mM ZnSO$_4$, and 0.02% NaN$_3$ was acidified to pH 2.4 with 0.8 ml of 1 N HCl, added with constant stirring. After 70 min of stirring at 25°C, the mixture was dialyzed overnight against 500 ml of the same pH 7.4 buffer, except that 0.1 mM ZnSO$_4$ was used. Starch gel electrophoresis of the parental enzyme stocks and the dialyzed crude hybridization mixture showed that two new bands of protein and phosphatase activity had appeared between the parentals (Fig. 1). Semiquantitative evidence of hybridization also was obtained by measuring the enzyme's sensitivity to heat inactivation (24, 25); wild type enzyme shows no loss of activity after 10 min at 87°C, but the hybrid mixture was 38% inactivated. This particular hybrid has been shown to possess 50% of wild type catalytic activity and to lose approximately 55% of its activity during this heat treatment (20); therefore, the 35% inactivation of our hybrid preparation suggested that 50% of the active phosphatase molecules present in the mixture were hybrid. However, incubation of wild type enzyme at high temperatures promotes the dissociation of subunits that can subsequently reassociate to active enzyme (1). This burst amplitude indicates the concentration of enzyme active sites in solution provided three conditions are met: (a) the reaction is saturated with respect to substrate; (b) the burst rate constant is much greater than the steady state turnover number; and (c) the sequence of unimolecular steps preceding and including that responsible for color formation is thermodynamically weighted strongly in favor of product formation (34).

**RESULTS**

**Comparison of Isozymes 1 and 3**—Steady state assay of enzyme activity at 37°C in 1.0 M Tris pH 8.0, yielded specific activities of 113 and 119 mols of p-nitrophenyl phosphate hydrolyzed per mole of enzyme per s, respectively, for isozymes 1 and 3. These values are comparable to the specific activity found by Malamy and Horecker (33) for crystalline enzyme, equal to 118 mols per mole per s once converted to our assay conditions (29).

Fig. 3 shows typical stopped flow traces for the hydrolysis of 2,4-dinitrophenyl phosphate at pH 5.5 and of p-nitrophenyl phosphate at pH 8.0 by isozymes 1 and 3. The stopped flow monitors the release of the chromophoric reaction products; the dinitrophenolate ion at pH 5.5 and the p-nitrophenolate ion at pH 8.0. At pH 5.5, the hydrolytic reaction proceeds in two steps, a pre-steady state burst followed by a linear steady state phase. When the straight line that can be drawn through the steady state is extrapolated to the time of mixing (3 ms before the observed beginning of substrate hydrolysis, to account for the dead time of the stopped flow apparatus), the absorbance value obtained indicates how many enzyme active sites have hydrolyzed substrate before onset of the steady state. This burst amplitude indicates the concentration of enzyme active sites in solution provided three conditions are met: (a) the reaction is saturated with respect to substrate; (b) the burst rate constant is much greater than the steady state turnover numbers; and (c) the sequence of unimolecular steps preceding and including that responsible for color formation is thermodynamically weighted strongly in favor of product formation (34).
The first two conditions are met in this system (29), and departure from them can be corrected for (34). Evidence is lacking on the third condition, although it generally has been assumed to be satisfied (see "Discussion"). Division of the concentration of active sites by the concentration of enzyme molecules in solution yields a burst amplitude parameter that estimates the number of active sites on an enzyme molecule.

During the burst, the absorbance approaches the steady state rate of increase in a first order manner. A plot versus time of the logarithm of the difference between the observed absorbance and the extrapolated steady line yields a straight line, the slope of which gives the first order rate constant of the burst. When the reaction is saturated with respect to substrate, this rate parameter measures the speed of the slower process (or ensemble of processes) preceding the steady state rate determining step in the catalytic mechanism of alkaline phosphatase. Occasionally, the burst is observed to contain an instant phase of substrate hydrolysis; it is smaller than the usual first order process and is so fast as to be complete within the dead time of the stopped flow apparatus. The molecular significance of this "instantaneous burst," explained in a previous publication (29), may be summarized as follows.

Native alkaline phosphatase contains an approximately stoichiometric amount of tightly bound endogenous phosphate, which appears to occupy the active site. Active sites containing phosphate produce the normal burst transient with a rate constant of 20 to 50 s⁻¹ under conditions of saturation with respect to substrate. However, small amounts of endogenous phosphate may be lost during enzyme purification or dialysis of purified enzyme. Hence, it is convenient to define three burst amplitude parameters: the total, instantaneous, and transient burst amplitudes. The third parameter is that part of the total burst amplitude which is instantaneous, and transient burst amplitudes. The total burst amplitude, defined as the sum of the instantaneous amplitude and the sum of the slower phases, is constant to within the experimental uncertainty of ±0.1 ROH:E (stoichiometric molar ratio of product alcohol, released by substrate hydrolysis in the presteady state burst, to enzyme). Hence, it is convenient to define three burst amplitude parameters: the total, instantaneous, and transient burst amplitudes. The third parameter is that part of the total burst amplitude which is assigned to the first order process that can be followed in the stopped flow. The difference between the two is the amplitude of the instantaneous burst, which, when expressed in units of ROH:E, is equal to the number of phosphate-depleted active sites per enzyme molecule. At pH 8.0, substrate hydrolysis starts at the steady state rate with no transient burst, although careful analysis reveals an instantaneous burst equal in amplitude to that seen with the same sample of enzyme at pH 5.5 (29).

Quantitative analysis of stopped flow traces like those in Fig. 3 yields the kinetic parameters in Table I, from which the following comparisons between isozymes can be made: (a) the burst rate constant at μ = 0.1 is the same for the two isozymes; (b) the burst rate constant at μ = 1.1 is 40% larger for isozyme 1 than for isozyme 3; (c) the steady state rate at pH 5.5 at either ionic strength is 100% larger for isozyme 1 than for isozyme 3. The burst amplitude at pH 3.5 and the steady state rate at pH 8.0 are essentially the same for both isozymes. The marginal but consistent 10 to 15% differences that do appear may be attributable to two trivial causes that can be disposed of at this point.

As is examined in more detail under "Discussion," some sort of partial site inactivation during enzyme purification is the simplest explanation for one striking feature of Table I, the fact that the observed total burst amplitude at pH 5.5 has an average value of 1.5 ROH:E instead of the value of 2.0 that would be expected for an enzyme composed of two identical subunits. If the preparation of isozyme 1 studied here were 10% more inactivated than that of isozyme 3, the observed burst amplitude at pH 5.5 would be proportionately lower, as is the case at μ = 1.1. Furthermore, since the steady state rate is proportional to the number of functioning active sites, a 10% differential in site stoichiometry should lead to the observed 10% difference in steady state rate at pH 8.0, provided the active site turnover number is unchanged. In fact, the active site turnover number at alkaline pH should be the same for the two isozymes, since the burst rate constant at pH 5.5 is the same for both molecular species (Table I) and there is some evidence (14) that the molecular process responsible for the transient burst at pH 5.5 determines the steady state rate at pH 8.0.

In addition, the 15% difference between isozymes in the burst amplitude at μ = 0.1 must derive in part from the mathematical dependence of the burst amplitude on the burst rate constant and the steady state turnover number (34). It is a basic property of systems showing burst kinetics that, as the steady state rate approaches the burst rate, the burst amplitude approaches zero; as the steady state rate becomes much smaller than the burst rate, the burst amplitude approaches the upper bound of the true active site stoichiometry. Given the rate parameters of Table I,

### Table I

**Transient kinetic parameters of isozymes 1 and 3 of Escherichia coli alkaline phosphatase**

| Substrate and buffer | Parameter | Isozyme 1 | Isozyme 3 | Isozyme 1:isozyme 3 |
|----------------------|----------|-----------|-----------|---------------------|
| 2,4-Dinitrophenyl phosphate in pH 5.5 NaOAc | Total burst amplitude (ROH:E) | 2.12 | 1.12 | 1.08 | 1.48 | 1.35 | 1.10 | 0.82 | 0.83 |
| | Total burst rate constant (s⁻¹) | 1.28 | 1.46 | 0.88 | 1.52 | 1.58 | 0.96 | 0.84 | 0.92 |
| | Steady state rate (moles of ROH per mole of enzyme per s) | 16.0 | 30.7 | 0.52 | 18.2 | 34.6 | 0.53 | 0.88 | 0.89 |

**Steady state rate (moles of ROH per 16.0**

Substrate and buffer | Parameter | Isozyme 1 | Isozyme 3 | Isozyme 1:isozyme 3 |
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| 2,4-Dinitrophenyl phosphate in pH 5.5 NaOAc | Total burst amplitude (ROH:E) | 2.12 | 1.12 | 1.08 | 1.48 | 1.35 | 1.10 | 0.82 | 0.83 |
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| | Steady state rate (moles of ROH per mole of enzyme per s) | 16.0 | 30.7 | 0.52 | 18.2 | 34.6 | 0.53 | 0.88 | 0.89 |
it can be shown (34) that the observed burst amplitude for isozyme 1 at \( \mu = 0.1 \) should be only 91% of the true site stoichiometry. The analogous correction factor for isozyme 3 at \( \mu = 0.1 \) is 94%, whereas values of 97% and 98% are needed at \( \mu = 1.1 \) for isozyme 1 and isozyme 3, respectively. When these corrections are made it can be concluded that isozyme 1 possesses 1.4 to 1.5 active sites per enzyme molecule, whereas isozyme 3 has a site stoichiometry of 1.6.

Table I also permits the following comparisons with respect to ionic strength: (a) the transient burst rate at pH 5.5 is 50% to 100% faster at \( \mu = 1.1 \) than at \( \mu = 0.1 \); (b) the steady state rate at pH 5.5 is 30 to 40% slower at \( \mu = 1.1 \) than at \( \mu = 0.1 \); (c) the steady state rate at pH 8.0 is 100% faster at \( \mu = 1.1 \) than at \( \mu = 0.1 \). There are two less significant effects of ionic strength. The total burst amplitude is 4 to 14% larger at \( \mu = 1.1 \) than at \( \mu = 0.1 \), for the trivial mathematical reason discussed above in comparing the burst amplitudes of the two isozymes; the steady state rate is a smaller fraction of the burst rate at \( \mu = 1.1 \) than at \( \mu = 0.1 \), so that the observed burst amplitude approaches the true site stoichiometry more closely at \( \mu = 1.1 \) than at \( \mu = 0.1 \). In addition, the transient burst is a smaller fraction of the total burst at \( \mu = 1.1 \) than at \( \mu = 0.1 \). This effect must result from a greater loss of endogenous phosphate at \( \mu = 1.1 \) as compared to \( \mu = 0.1 \). It suggests that the affinity of enzyme for phosphate diminishes as the ionic strength increases; this phenomenon has no bearing on the issues being raised in this paper.

With the exception of the steady state rate at pH 5.5, the two isozymes appear to be functionally identical. As will be discussed below, the most important conclusions from Table I have to do with differences among the three rate parameters in the response to changing ionic strength and isozyme identity. In general, the burst rate at pH 5.5 behaves like the steady state rate at pH 8.0, whereas the steady state rate at pH 5.5 responds in a qualitatively different way.

Comparison of Hybrid and Wild Type Alkaline Phosphatase—Table II compares the kinetic parameters of hybrid and wild type alkaline phosphatase, obtained from stopped flow reactions at pH 5.5 in a manner similar to that used to study the two wild type isozymes. The sample of wild type isozyme 3 used as a control for these reactions was the peak of unhybridized parental enzyme recovered from the crude hybridization mixture by DEAE-cellulose chromatography (the third major peak in Fig. 2).

Within a margin of 20%, the molecular hybrid that contains one subunit of wild type isozyme 3 and one subunit of catalytically inactive mutant phosphatase protein from *Escherichia coli* strain S-33 shows one-half the wild type burst amplitude at pH 5.5 and one-half the wild type steady state rates at pH 5.5 and 8.0. The parental mutant phosphatase protein (Channel 1, Fig. 1) and the mutant protein recovered chromatographically from the crude hybridization mixture (Peak 1, Fig. 2) possess less than 0.1% of wild type catalytic activity. The transient burst rate constant at pH 5.5 is not affected by hybridization. The total burst amplitude, expressed in units of ROH:E, should equal the numbers of active sites per enzyme molecule. The steady state rate of substrate hydrolysis (expressed per mole of enzyme) should be proportional to the numbers of active sites per enzyme molecule and therefore to the total burst amplitude. Hence, the halving of the burst amplitude and the steady state rates in hybrid as compared to wild type enzyme actually indicates that the wild type subunit of the hybrid molecule is completely unaffected by its association with an inactive mutant subunit.

Hybrid also behaves like wild type isozyme 3 in the following ways (Table II).

1. Preincubation at pH 5.5 before reaction with pH 5.5 substrate in the stopped flow lowers the total burst amplitude approximately 30%. This behavior appears to result from the formation of phosphoenzyme at 30% of the active sites when phosphatase and inorganic phosphate (in this case endogenous) are put at pH 5.5 (29).

2. Dialysis and reaction at high ionic strength creates instantaneous burst at the expense of transient burst. Previously published findings (29) suggest that this phenomenon, also seen in the comparison of wild type isozymes 1 and 3 (Table I), must be due to a weakened binding of endogenous phosphate to phosphatase at high ionic strength.

3. Raising the ionic strength from 0.1 to 1.1 increases the transient burst rate constant at pH 5.5 by 50% and halves the steady state rate at pH 5.5.

None of these treatments affects the 2:1 ratio of wild type to hybrid found for the burst amplitude and steady state rate. One sample of hybrid enzyme has been assayed for endogenous phosphate by the phosphomolybdate procedure (29), giving a value of 0.9 P_i:E (stoichiometric molar ratio of inorganic phosphate to enzyme), one-half that found for wild type enzyme (29).

### Table II

**Kinetics of substrate hydrolysis by hybrid and wild type alkaline phosphatase**

| Enzyme            | Enzyme preincubation pH | Ionic strength | Burst amplitude at pH 5.5 (ROH:E) | Transient burst rate constant at pH 5.5 | Steady state rate |
|-------------------|-------------------------|---------------|----------------------------------|----------------------------------------|------------------|
|                   |                         |               | Total | Transient | Instantaneous | pH 5.5 | pH 8.0* |
| **Hybrid**        | 7.4                     | 1.1           | 0.75  | 0.38     | 0.37         | 33    |
|                   | 7.4                     | 0.1           | 0.71  | 0.66     | 0.05         | 23    |
|                   | 5.5                     | 0.1           | 0.45  | 0.48     | -0.03        | 24    |
|                   | 7.4                     | 1.1           | 1.37  | 0.87     | 0.50         | 32    |
| **Wild type isoenzyme 3** | 7.4           | 0.1           | 1.36  | 1.22     | 0.14         | 21    |
|                   | 5.5                     | 0.1           | 1.06  | 0.88     | 0.18         | 20    |

* pH 8.0 steady state data were all collected at 37° in 1.0 M Tris, regardless of enzyme preincubation buffer.
**DISCUSSION**

**Comparison of Wild Type Isozymes 1 and 3**

Differences between Isozymes—The data of Table I indicate only one clear functional difference between the two isozymes. The steady state rate of dinitrophenyl phosphate hydrolysis at pH 5.5 by isozyme 1 is twice that by isozyme 3. Isozymes 1 and 3 also appear to differ significantly in the sensitivity of their rate parameters to the ionic strength at pH 5.5. A shift in the ionic strength from 0.1 to 1.1 raises the burst rate constant of isozyme 1 100% but raises that of isozyme 3 only 50%; the steady state rate of isozyme 1 drops 45%, as opposed to almost 45% for isozyme 3, upon shift from $\mu = 0.1$ to $\mu = 1.1$. Although beyond the probable margins of experimental uncertainty, these differences are too small to conclude from them that alkaline phosphatase function is seriously affected by the distribution of enzyme between the two isozymes. The usual environment of cultured *E. coli* has a pH closer to 8.0 than 5.5. At pH 8, in vitro, no significant functional difference between the two isozymes has been observed (6, 7) (Table I).

Mechanism of Substrate Hydrolysis by Alkaline Phosphatase—The principal significance of the data presented above lies in their contribution to our understanding of the catalytic mechanism of alkaline phosphatase. Previous stopped flow and temperature jump studies (9-14, 21, 22, 29) suggested that the enzyme hydrolyzes phosphate esters by the mechanism shown in Scheme 1, where $S$ = substrate, $P_i$ = inorganic phosphate, ROH = the product alcohol which generates the signal monitored by the stopped flow spectrophotometer, $E$ and $E'$ = two isomeric forms of alkaline phosphatase, $E-P_i$ = a noncovalent enzyme-phosphate complex; $E'-P_i$ and $E-P_i$ are covalent phosphorly enzyme intermediates. At pH 5.5, Step 1 or Step 3 appears to determine the transient burst rate, and Step 7 determines the steady state rate. At pH 8.0, Step 1 or Step 3 is the steady state rate-determining (9-12). Of the two enzyme isomers, $E$ binds phosphate well and substrate poorly, whereas $E'$ binds substrate well and phosphate poorly or not at all (12, 21, 22); the $E \rightleftharpoons E'$ equilibrium thermodynamically favors $E'$ (29). Most active sites of the native enzyme contain endogenous phosphate and are in the $E$ isomeric state. They must undergo Step 1 or Step 3 before substrate hydrolysis can occur and therefore show normal burst kinetics at pH 5.5. Active sites lacking phosphate are in the $E'$ state and do not need to undergo Steps 1 or 3 before phosphate-ester cleavage; they hydrolyze a stoichiometric amount of substrate very rapidly in Step 5 before onset of the steady state.

A very important feature of this mechanism is the proposal that the pre-steady state rate-determining process at pH 5.5 is the steady state rate-determining process at pH 8.0 (9, 12, 14). The strongest single piece of evidence for this idea comes from a comparison of the stopped flow kinetics of substrate hydrolysis by wild type alkaline phosphatase, largely isozyme 3, and phosphatase purified from *E. coli* strain S-19, carrying a point mutation in the phosphatase gene. The latter enzyme hydrolyzes phosphate ester at 10% of the wild type steady state rate at pH 8.0 and 10% of the wild type burst rate at pH 5.5; however, the steady state rate at pH 5.5 is unaffected by the single amino acid substitution in the phosphatase primary structure that is caused by this mutation (14).

The data in this paper (Table I) support the conclusions of the work with S-19 mutant phosphatase, although in somewhat less detail. The steady state process at pH 5.5 appears to differ from that at pH 8.0 in two respects: (a) the difference in primary structure between isozyme 1 and isozyme 3 (8) leads to a doubling of the steady state rate at pH 5.5 without affecting the rate at pH 8.0; and (b) a rise in ionic strength from 0.1 to 1.1 diminishes the steady state rate at pH 5.5 by 40%, but doubles the steady state rate at pH 8.0. Our data support the notion that the transient burst process at pH 5.5 is identical to the steady state process at pH 8.0: an increase in the ionic strength from 0.1 to 1.1 doubles the steady state rate at pH 8.0 for both isozymes and doubles the first order rate constant of the transient burst for isozyme 1 at pH 5.5. In contrast, it increases the burst rate constant for isozyme 3 at pH 5.5 by only 50%. However, it is not necessary for a molecular process to show exactly the same ionic strength dependence at two different pH values. Hence, this quantitative difference between the two isozymes in no way disproves the hypothesis that in both isozymes the same molecular process limits the rate of the pH 5.5 burst transient and the pH 8.0 steady state.

These effects have a straightforward molecular interpretation within the framework of Scheme 1. A rise in ionic strength from 0.1 to 1.1 doubles the rate of isomerization of enzyme-substrate complex (Step 3) and almost halves the rate of hydrolysis of phosphoryl enzyme (Step 6 or 7, the two processes have not been resolved yet). The loss of an arginine residue from the NH$_2$ terminus of alkaline phosphatase, the process which appears to result in the formation of isozyme 3 from isozyme 1 (8), halves the rate of hydrolysis of the covalent phosphoryl enzyme catalytic intermediate. It should be interesting to interpret this clear correlation of a structural change with a functional alteration when the complete three dimensional structure of phosphatase has been determined to atomic resolution by x-ray crystallography.

**Comparison of Hybrid and Wild Type Alkaline Phosphatase**

Identity and Purity of Hybrid Enzyme—There are four criteria for concluding that the hybrid enzyme was the expected molecular species, containing one subunit from wild type isozyme 3 and one subunit from mutant (*E. coli* strain S-33) isozyme 1.

1. It eluted from DEAE-cellulose at an ionic strength identical to the salt concentrations needed to elute the parental S-33 isozyme 1 and wild type isozyme 3 (Fig. 2).

2. Its electrophoretic mobility was exactly intermediate between those of the two parental molecules (Fig. 1); isozymes 1 and 3 of *E. coli* alkaline phosphatase have been shown each to consist of two identical subunits (5) so that a molecular hybrid should be a single species of intermediate charge. Fig. 1 shows that the electrophoretic pattern of purified hybrid is essentially unchanged by reaction with substrate in the stopped flow. Therefore, hybrid does not dissociate and reassociate to form the parental wild type and mutant protein molecules during reaction in the stopped flow. The kinetic properties described in Table II can be assigned safely to the hybrid molecule and not to wild type molecules that might be made from hybrid. The electrophoretic band that runs slightly ahead of the principal hybrid...
band in Fig. 1 and which appears to be slightly enhanced after reaction in the stopped flow is also attributed to a hybrid species. If a sample of such enzyme is heat-treated (see below) before electrophoresis and if the starch gel is stained for enzyme activity rather than protein, both the front-running and the main hybrid bands no longer appear, although the band for a similarly treated sample of wild-type isozyme 3 is unaffected.

3. The specific activity of hybrid is half that of wild-type enzyme, as has been reported before (25). In that study, hybrid was not purified, but the absence of wild-type molecules was insured by hybridizing wild-type enzyme with a considerable excess of mutant protein; phosphatase subunits appear to associate in an approximately binomial manner during hybridization.

4. The hybrid enzyme was largely inactivated by incubation for a brief period at 87°, an operation which does not affect wild-type enzyme. Starch gel electrophoresis indicated that any residual activity was not due to wild-type isozyme 3, which was completely resolved from hybrid during chromatography.

Flip-Flop Model for Subunit Interaction—It has been proposed that enzymes possessing 2-fold molecular symmetry may gain considerable catalytic efficiency through rigid integration of the catalytic steps at opposed active sites in a reciprocating manner (35). That is, strict conformation coupling of molecular events on different subunits might force an intrinsically exergonic process in the catalytic cycle at one active site to occur simultaneously with an energetically less favored step at the partner active site, thereby lowering the activation energy of the latter. The net result of such a phenomenon should be a lessening of the spread of activation barrier heights among the chemical processes in the catalytic mechanism, increasing the steady-state rate by raising the turnover number per active site.

Variants of this reciprocating subunit, or flip-flop, mechanism have been applied to E. coli alkaline phosphatase by several authors (12, 15, 21, 22) in attempts to explain several kinds of kinetic and thermodynamic anomalies, having to do in general with the reported half-site burst stoichiometry and phosphate binding anticooperativity. The need for such explanations is rendered less clear by the indications we presented in a preceding paper (29) that at least some of the evidence for anticooperative subunit interactions may be artifactual. As is discussed below, the two active sites on a completely functional alkaline phosphatase molecule may be identical and noninteracting. Nevertheless, the failure to find a significant difference in active site catalytic efficiency between hybrid and wild-type enzyme poses a separate serious challenge to the flip-flop mechanism, quite independently of other evidence for or against subunit interaction.

From a teleological viewpoint one would expect reciprocating subunit interaction to evolve biologically only if it significantly enhanced catalytic efficiency over that attainable in the absence of subunit interaction. Therefore, an enzyme molecule modified to prevent one subunit from completing the catalytic cycle would be predicted to turn over substrate at a much lower rate than each subunit in a native molecule. The normal mode of subunit cooperation would be interrupted.

Application of a purely chemical principle, that of microscopic reversibility, leads to an identical prediction. If the normal catalytic mechanism of E. coli alkaline phosphatase includes the features of the flip-flop hypothesis, subunit reciprocation must result in a lower series of activation energy barriers separating reactants from products than does any alternative catalytic pathway, such as one in which there is no subunit interaction.

Hence, elimination of reciprocating subunit interaction in a molecular hybrid like the one constructed here should raise the rate-determining energy barriers experienced by a functioning subunit.

The hybrid molecule constructed and characterized in the work described here should test the flip-flop model adequately. If subunit reciprocation existed in alkaline phosphatase, the subunit turnover number (the steady-state rate divided by the number of active sites per enzyme molecule) should be significantly lower for hybrid than for wild-type enzyme. Since the molecular process responsible for steady-state rate limitation at pH 8.0 is the same as that causing the pre-steady-state burst transient at pH 5.5 (9, 12, 14), one might also expect to see a lower burst rate constant for hybrid than for wild-type phosphatase.

In fact, neither expectation is met. The first order rate constant for the burst at pH 5.5 is unchanged by hybridization, as is the subunit turnover number at pH 8 and 37°, calculated to be near 90 moles of ROH per mole of active sites per s if the total burst amplitude at pH 5.5 following enzyme preincubation at pH 7.4 is taken as a measure of the number of active sites per enzyme molecule (29). Therefore, there should be no biologically adaptive advantage to guide natural selection toward a flip-flop mechanism. In the chemical context of microscopic reversibility, elimination of the possibility of subunit reciprocation does not lower the catalytic efficiency of a functional subunit. Hence, subunit reciprocation could not be facilitating catalysis by the wild-type enzyme dimer. Every detail of the catalytic function of hybrid that has been examined so far suggests that it is composed simply of an active subunit of wild-type isozyme 3 linked to a completely nonfunctional mutant subunit. The latter has a single role: stabilization of the conformation of the functional subunit. All conditions found so far to dissociate phosphatase dimers into monomers (in which, of course, there could be no subunit interaction) also destroy catalytic activity (1, 23). Even the properties of hybrid with respect to endogenous phosphate fit into this interpretation. The hybrid contains half as much endogenous phosphate as wild-type enzyme. The effect of preincubating hybrid at pH 5.5 before reaction with substrate at pH 5.5 in the stopped flow (Table II) is what would be expected if the phosphate were covalently bound at the single functioning active site to the same degree that phosphoenzyme is formed with native alkaline phosphatase at pH 5.5 (29).

We must qualify our rejection of the flip-flop mechanism for phosphatase in one respect. We have not yet performed the equilibrium dialysis needed to conclude that the mutant subunits in hybrid do not bind substrate and phosphate without catalyzing substrate hydrolysis. It is conceivable but unlikely that reversible binding of substrate or product to the mutant subunit in a hybrid molecule occurs and triggers flip-flop interactions that allow the wild-type subunit to show the normal burst and steady-state rate constants. This possibility is rendered remote by the fact that hybrid enzyme contains half the native wild-type amount of endogenous, tightly bound, phosphate; but it will require the purification of a considerable amount of hybrid for definitive testing.

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Subunit Interactions in Alkaline Phosphatase

True Active Site Stoichiometry—The central question raised in this and the preceding paper (29) concerns the number of active sites on the alkaline phosphatase molecule and their degree of...
independence. The previously accepted value of the pre-steady state burst amplitude for native enzyme at pH 5.5 in the stopped flow was 1.0 mole of alcohol produced per mole of enzyme, from which it was inferred that some sort of enzyme-subunit interaction rendered the two potentially identical active sites on the phosphatase molecule functionally nonequivalent (9-15). In contrast, we observe total burst amplitudes of 1.3 to 1.0 (Tables I and II; see also Ref. 29), which raise the possibility that both active sites are actually identical and independent but that 25% of the sites have been prevented from functioning for some reason. We already have discussed reasons for considering our burst amplitudes to be a better indication of active site concentrations than those of earlier workers, as well as a possible explanation for the discrepancy which exists in the literature. Now it is necessary to consider reasons why a burst amplitude of only 1.5 might be seen if the true site stoichiometry is 2.0.

The observed nonintegral stoichiometry would result if the published $E_{30}$, 0.1₄ for enzyme of 0.72 (33, 36) to 0.77 (2) were 50% too high (for a true stoichiometry of one site per molecule) or 25% too low (for two active sites per molecule). However, it is hard to imagine how the operations involved in determining, a protein extinction coefficient could generate such a large error. Furthermore, this explanation would not account for the disagreement which now exists in the literature since all workers have used extinction coefficients in this range.

One simple explanation for the site stoichiometry of 1.5 remains; in our preparations of alkaline phosphatase, 25% of the total of two sites per molecule have been inactivated irreversibly by some unknown process during bacterial growth or enzyme purification. This suggestion is rendered unattractive by the unusual stability of the phosphatase molecule to extremes of temperature (24, 25) and pH (1, 31). However, the discovery that native enzyme is almost saturated with endogenous phosphate (29) presents the possibility that the tightly bound phosphate is responsible for this stability. Active sites depleted of phosphate might be more susceptible to irreversible inactivation. Since bacteria grown for phosphatase production commonly are starved for phosphate for at least several hours before harvesting, and since phosphatase is secreted into the bacterial periplasm (37, 38), where the phosphate concentration must be much lower than that within the cell, it is possible that a significant fraction of the final crop of enzyme has experienced phosphate depletion and partial irreversible inactivation.

The burst amplitudes observed for hybrid alkaline phosphatase and for the wild type control enzyme that is recovered from the hybridization procedure (Table II) suggest that 25% of the active sites in the original wild type enzyme were inactive. Hybrid does not show a burst amplitude of 1.0, as one would expect if hybridization selected one potentially fully functional wild type subunit to pair with an inactive mutant subunit. Rather, the hybrid burst amplitude is 0.7 to 0.8, just half that of native wild type enzyme. The native isozyme 3 recovered from hybridization has a burst amplitude of 1.4; acidification and renaturation do not eliminate the hypothesized 25% of inactivated subunits from the population, nor do these operations revive the dead active sites. If it is true that the observed nonintegral site stoichiometry is an artifact of the enzyme purification procedure, it should be possible to revise the scheme in a way to prevent the hypothesized inactivation. We currently are investigating ways to do this.

It is necessary to consider a less trivial explanation for the nonintegral burst amplitude. Particular attention should be paid to the possibility that the nonintegral site stoichiometry arises from the existence of a finite amount of enzyme-substrate complex during the catalytic steady state. If the equilibrium constant of Step 5 in Scheme 1 (above) does not favor the formation of phosphoenzyme completely, all of the enzyme active sites would not reach the $E'$-P₁ and $E$-P₁ states before the onset of the steady state. Hence, the amount of chromophoric alcohol released before onset of the steady state might be less than the number of functioning active sites.

However, there exists one experimental observation (29) which would be hard to reconcile with the explanation for the nonintegral site stoichiometry that was just suggested. Alkaline phosphatase which has been purged completely of endogenous inorganic phosphate shows no burst transient at pH 5.5, but has an instantaneous burst of amplitude of 1.5 ROH:$E$. Transient burst is restored at the expense of instantaneous burst in proportion to the amount of phosphate added back to purged enzyme. Only 1.5 P₁:$E$ are required to restore the transient burst completely. This finding, which is independent of the fact that the total burst amplitude is 1.5 ROH:$E$, bears no obvious relationship to the value of the equilibrium constant for Step 5 in Scheme 1. Furthermore, it argues against other more complicated explanations of the submaximal burst stoichiometry such as might arise from molecular models retaining some degree of subunit interaction. On the other hand, it is easily explained by the hypothesis that 25% of the possible active sites cannot react with either phosphate or substrate because they have been permanently inactivated.

In order to reinforce the conclusions reached here, it may prove useful to purify other phosphatase molecular hybrids, especially ones in which both subunits, instead of just one, show catalytic function. However, it is unclear that more complicated explanations of the submaximal burst stoichiometry would be hard to reconcile with the explanation for the nonintegral site stoichiometry that was just suggested. Alkaline phosphatase a mechanism as general as the flip-flop model (15). The preceding paper (29) raises an even more general mechanistic question, that of whether alkaline phosphatase shows any interdependence of active sites, and discusses the discrepancy which exist between our results and those of previous investigators using different experimental techniques.

In light of the present ambiguity regarding the true site stoichiometry for E. coli alkaline phosphatase, it is most important now to re-examine the question of whether any functional subunit interaction occurs in this enzyme rather than to continue the search for mechanisms to explain an interaction that may not exist.

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Will Bloch and Milton J. Schlesinger

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