Chemostat Culture of *Escherichia coli* K-12 Limited by the Activity of Alkaline Phosphatase

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The growth-limiting reaction of a chemostat culture of *Escherichia coli* K-12 was the hydrolysis of $\beta$-glycerophosphate by alkaline phosphatase. The culture was buffered at pH 5.2 where alkaline phosphatase was unable to supply phosphate to the cells at a rate sufficient to sustain the maximum rate of growth. Alkaline phosphatase activity in this system is discussed in terms of the so-called Flip-Flop mechanism.

The alkaline phosphatase of *Escherichia coli* (EC 3.1.3.1) is a nonspecific phosphomonoesterase (16) localized in the periplasmic space (6). The enzyme is a dimer comprised of identical subunits (12). Its molecular weight is about 86,000 (2). Activity requirements include dimerization (in the periplasmic space) (14) and divalent cations (usually Zn$^{2+}$) (15).

Alkaline phosphatase is coded by the structural gene $phoA$ (12). Its synthesis is controlled by two regulatory genes $phoR$ and $phoS$ (4); a mutation in either regulatory gene overcomes a repression produced by the presence of orthophosphate. These three genes have been mapped by both time of entry (4) and recombination techniques (1, 3, 17).

Alkaline phosphatase demonstrates classical Michaelis-Menten kinetics with negative (or anti) cooperativity for substrate binding between distinct active sites on identical monomers. Lazdunski et al. (5) have proposed a functional interrelationship between the two active sites which is mediated by a structural modification called the Flip-Flop mechanisms. The mechanism entails alternating functions for each of the two active sites of the phosphatase.

We describe in this paper a chemostat culture of *E. coli* K-12 where all chemostat parameters are functions of the Zn$^{2+}$ alkaline phosphatase activity. This was accomplished by defining the culture medium such that the growth-limiting reaction in the chemostat was hydrolysis of $\beta$-glycerophosphate by alkaline phosphatase. We present further an interpretation of alkaline phosphatase activity (and growth) in this system in terms of the Flip-Flop mechanism.

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**MATERIALS AND METHODS**

The chemostat (7, 10) is a continuous culture apparatus used to maintain microbial populations at constant growth rates under constant cultural conditions over long periods of time.

**Organism.** *E. coli* K-12 was provided by the *E. coli* Genetic Stock Center, Yale University. Strain K-12 was used in all experiments except one control experiment which required a phosphatase-negative mutant, P1, that was isolated in our lab from K-12.

**Media.** The amounts of the various ingredients indicated are those required for preparation of 1 liter of medium. Agar (2%) was added for solid medium. Inorganic salts are as follows: NaCl, 4.68 g; KCl, 1.49 g; NH$_4$Cl, 1.07 g; Na$_2$SO$_4$, 426 mg; MgCl$_2$, 20 mg; CaCl$_2$, 29 mg; FeCl$_3$, 0.54 mg; ZnCl$_2$, 0.55 mg. The carbon source was glucose (8.0 g). Tris-maleate buffer contained (0.05 M in each): tris(hydroxymethyl)-aminomethane (Tris), 6.05 g; maleic acid, 5.80 g; the pH was adjusted with either 1 N NaOH or 1 N HCl. The phosphate source for the equilibrium chemostat was: filter-sterilized $\beta$-glycerophosphate (disodium salt, pentahydrate) (6 mg). The phosphate source for $\mu_{max}$ estimation was either $\beta$-glycerophosphate (120 mg) or KH$_2$PO$_4$ (100 mg).

**Chemostat culture conditions.** All cultures were maintained at 37°C in a 200-ml chemostat.

$\mu_{max}$ estimation. An operating chemostat was employed to measure the parameter under the experimental conditions obtaining in the chemostat. In an operating chemostat a population of cells, if its density is sufficiently below the equilibrium density, will reproduce at its maximum rate ($\mu_{max}$) because all essential nutrilites are in excess and will increase in density according to the equation

$$N_t = N_0 e^{\mu_{max} t - D t}$$

where $N_t$ is the density of the population at time $t$, $N_0$ is the density initially, and $(\mu_{max} - D)$ is the difference between the maximum growth rate and the rate at which the culture is being diluted. With a high dilution rate, several days may be required before density increased to the point where the concentration
of an essential nutrient becomes limiting. Estimates of \( \mu_{\text{max}} - D \) were taken from the regression of \( \ln(N)/t \) time according to the equation \( \ln(N) = \alpha + \beta t + e \), where \( \beta = \mu_{\text{max}} - D \). Maximum likelihood estimators exist for \( \alpha \), \( \beta \), and \( \sigma^2 \) (variance) \((6)\).

Phosphate determination. The method of Murphy and Riley \((9)\) with modification was used for orthophosphate determination. \( \) (It is a highly sensitive method giving an optical density of 1.00 with 26.9 \( \mu \)g of P per 25 \( \mu \)l.) To 20.0 \( \mu \)l of water were added 1.0 \( \mu \)l of solution whose phosphate concentration was to be determined and 4.0 \( \mu \)l of the phosphate reagent of Murphy and Riley. The color produced was stable for 6 h and was read spectrophotometrically at 720 nm. This method is based on reduction of an ammonium molybdophosphate complex by ascorbic acid in the presence of antimony.

\( \beta \)-Glycerophosphate assay. Chemostat effluent samples, with cells removed by vacuum filtration, were treated initially with concentrated HNO\(_3\) to hydrolyze \( \beta \)-glycerophosphate; orthophosphate was then precipitated by treatment with 10% Mg(NO\(_3\))\(_2\), the residue was dissolved in 1.0 N HCl, and the orthophosphate concentration was determined.

Enzyme activity estimation with intact cells \((\text{in vitro})\). All assays were conducted at 37 C and at pH 5.2 to establish activity in the chemostat environment. Chemostat effluent cells were collected under vacuum on a membrane filter \((0.22 \mu \text{m}; \text{Millipore Corp.})\), resuspended in pH 5.2, 0.05 M Tris-maleate buffer to a concentration of about 10\(^4\) cells/mL, and then toluenized for 15 min to increase cell wall permeability \((0.02 \text{ ml of toluene per ml of cell suspension})\). A 1.0-ml amount of cell suspension was added to 3.0 ml of 0.33 M \( \beta \)-glycerophosphate in pH 5.2, 0.05 M Tris-maleate buffer. Aliquots were taken from the reaction mixture over a period of 17 min and placed directly into phosphate reagent. The final pH of this suspension \((\text{pH} < 1)\) was sufficiently low to completely inhibit enzyme activity. Accuracy was enhanced by removing cells \( (\text{centrifugation}) \) before spectrophotometric analysis.

Enzyme activity estimation with spheroplasts \((\text{in vitro})\). Assay conditions were the same as above with the exception of spheroplast formation. The enzyme is released when spheroplasts are formed \((6)\). This was accomplished as follows: to 0.75 ml of cell suspension of known concentration was added 0.01 ml of 0.2 M ethylenediaminetetraacetic acid (5 min) followed by 0.5 ml of 100 \( \mu \)g of lysozyme per ml \((15 \text{ min})\). The toluene treatment was omitted.

Enzyme \( K_m \) estimation. Assay conditions were those described above for in vivo enzyme activity estimation. \( \beta \)-Glycerophosphate concentration was varied from 0.05 to 0.25 M; activity could not be measured with concentrations below 0.05 M. Data were analyzed according to the Lineweaver-Burk procedure.

Cell counts. All cell counts were made with a Celsoscope III, Particle Data, Inc., Elmhurst, Ill.

\[ \text{RESULTS} \]

Maximum reproductive rates \((\mu_{\text{max}})\) were estimated with \( \beta \)-glycerophosphate and orthophosphate over a range of pH to determine the pH at which maximum reproductive rate becomes a function of the rate of hydrolysis of \( \beta \)-glycerophosphate by alkaline phosphatase. The results, reported in Table 1, suggest that this occurs at pH values between pH 5.5 and 5.2 inclusive. At pH values above pH 6.0 maximum reproductive rate estimates were the same for both substrates. At pH 5.0 the orthophosphate \((\text{control})\) maximum reproductive rate estimate was significantly less than at higher pH values. Maximum reproductive rates were also estimated at pH 5.2 with \( \beta \)-glycerophosphate concentrations ranging from 20 to 400 \( \mu \)M to determine whether maximum reproductive rate at pH 5.2 was influenced by \( \beta \)-glycerophosphate concentration. The estimates were equivalent. Mutant strain P1, unable to make normal alkaline phosphatase, was unable to metabolize \( \beta \)-glycerophosphate and grow at pH 5.2, whereas its growth with orthophosphate at pH 5.2 was unimpaired. This observation suggests that \( \beta \)-glycerophosphate hydrolysis at pH 5.2 in the chemostat is accomplished by alkaline phosphatase alone.

Population density in the equilibrium chemostat at pH 5.2 was estimated to be constant at about \( 1.5 \times 10^6 \) cells/ml at dilution rates ranging from 0.05 to 0.42 \( (\text{doubling time: 13.8 to 1.65 h})\).

Estimates of in vivo and in vitro alkaline phosphatase activity over the operating range of the chemostat \((\text{Fig. 1})\) suggest a linear decrease in specific activity with increasing dilution rate. The apparent \( K_m \) under these conditions was estimated to be 25 mM \((\text{Fig. 2})\).

\( \beta \)-Glycerophosphate concentration in the equilibrium chemostat was estimated to be less than 0.1 \( \mu \)g/ml \((\text{the sensitivity of our assay})\) at all dilution rates.

\[ \text{DISCUSSION} \]

Lazdunski et al. \((5)\) have proposed a Flip-Flop mechanism for alkaline phosphatase in which

| pH | Orthophosphate | \( \mu_{\text{max}} \) | \( t_d \) | \( \mu_{\text{max}} \) | \( t_d \) |
|----|----------------|-----------------|-------|-----------------|-------|
| 6.0 | 0.70 ± 0.05 | 0.99 | 0.81 ± 0.12 | 0.86 |
| 5.5 | 0.51 ± 0.06 | 1.35 | 0.76 ± 0.09 | 0.91 |
| 5.3 | 0.51 ± 0.05 | 1.35 | 0.85 ± 0.08 | 0.82 |
| 5.2 | 0.46 ± 0.05 | 1.50 | 0.78 ± 0.03 | 0.89 |

\[ * \text{Estimates are reported with 95% confidence intervals.} \]
the initial step is the phosphorylation of one of the two active sites of the free enzyme. Subsequent steps involve phosphorylation of the second active site with concurrent dephosphorylation of the first site to yield orthophosphate and the monophosphorylated derivative of the enzyme. This is the Flip-Flop step and is the primary mechanism at alkaline pH. At acid pH, another pathway becomes important. This is the formation of a diphosphorylated derivative of the enzyme. Subsequent dephosphorylation of the diphosphorylated derivative yields orthophosphate and the monophosphorylated derivative. The pH dependence for formation of the diphosphorylated derivative with organic substrates is sigmoidal ranging from about one phosphate covalently bound at alkaline pH to two phosphates covalently bound at pH 5.2 or lower. At pH 5.0 the binding constants, K1 and K2, for the two active sites differ by a factor of about 104 with organic substrates; and this anti-cooperativity increases with increasing pH. Thus, at alkaline pH the rate-limiting step is the Flip-Flop step, and at acid pH dephosphorylation of the diphosphorylated derivative is rate limiting (with high concentrations of substrate [5]).

Maximum growth rate (Table 1) in our chemostat system with β-glycerophosphate as phosphate source is reduced at pH 5.2 because the rate of hydrolysis of β-glycerophosphate by alkaline phosphatase is slower than the rate necessary to sustain the maximum rate of growth. We suggest that, at pH 5.2 and with the high concentrations of β-glycerophosphate obtaining throughout the period of μmax estimation, there is an accumulation of the diphosphorylated derivative of the enzyme. We suggest further that the dephosphorylation of this derivative is the reaction responsible for the reduced rate of hydrolysis of β-glycerophosphate under these experimental conditions.

In the equilibrium chemostat at pH 5.2 with β-glycerophosphate as phosphate source, the substrate concentration is below the level required to saturate the second active site of the enzyme under in vitro conditions (5). We suggest that at chemostat equilibrium most of the enzyme is present as the monophosphorylated derivative. Newly bound substrate results in formation of the diphosphorylated derivative rather than the Flip-Flop step because the rate of the former reaction is greater at pH 5.2. The rate-limiting reaction is then dephosphorylation of the diphosphorylated derivative. This conclusion is supported by two lines of experimental evidence. (i) Our μmax estimates conducted at pH 5.2 with β-glycerophosphate concentrations as low as 20 μM and as high as 400 μM were equivalent. (ii) Lazdunski et al. (5) have shown that at pH 4.5 (25 C) specific activity in vitro is almost independent of substrate concentration up to some critical value (1.0 mM) where activity increases considerably as a function of substrate concentration. (This critical value is consistent with our in vivo estimate of 25 mM for the apparent Km of alkaline phosphatase at pH 5.2, 37 C.) We suggest that these results are most easily explained by the hypothesis that at pH 5.2 the rate-limiting reaction for all substrate concentrations is dephosphorylation of the diphosphorylated derivative. Alternative
hypotheses based on the existence of different rate-limiting reactions at different substrate concentrations (such as Flip-Flop at very low substrate concentrations changing to dephosphorylation of the diphosphorylated derivative at higher substrate concentrations) would lead to expectations different from these observed results unless, of course, the rates of the different reactions were the same, and this seems unlikely.

The rate-limiting reaction in the equilibrium chemostat at pH 5.2 probably proceeds at the same rate at all dilution rates where chemostat equilibrium can be reached and maintained. This conclusion is in agreement with our observation of relatively constant equilibrium densities over a wide range of dilution rates.

Estimation of alkaline phosphatase activity (Fig. 1) was conducted at a high substrate concentration (0.25 M) where it is safe to assume that all of the enzyme existed in the diphosphorylated state. These estimates are therefore indicators of the maximum specific activity at pH 5.2 rather than the activity existing in the equilibrium chemostat where substrate concentrations are much lower. These estimates, however, should reflect conditions obtaining in the equilibrium chemostat, since the rate-limiting reaction is probably the same in both cases and the reactions proceed at different but probably constant rates over a wide range of dilution rates. With this in mind, we interpret the observed decrease in specific activity with increasing dilution rate as follows. We assume (i) that the concentration of alkaline phosphatase in the periplasm is relatively constant at about 0.1 mM (11), and (ii) that faster growing cells in a phosphate-limited chemostat are smaller than slower growing cells (13); smaller (faster growing) cells will therefore have less alkaline phosphatase and lower specific activity than larger (slower growing) cells. The difference between in vivo and in vitro estimates of activity (Fig. 1) is due, at least in part, to assay conditions. Both assays were conducted at the same substrate concentration, it was optimum for the in vivo assay and less than optimum for the in vitro assay.

This experimental system should facilitate at least two lines of experimentation. First, it should permit several direct tests of the Flip-Flop mechanism proposed by Lazdunski, including the measurement (with radioisotopes) of some of the specific reactions in vivo. Second, this system should prove useful as an experimental tool in molecular evolution since the environmental constraints are such that a positive selective pressure exists for mutants which enhance the rate of the growth-limiting reaction.

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