Derepression of Alkaline Phosphatase in Escherichia coli by p-Fluorophenylalanine

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p-Fluorophenylalanine (FPA) causes a 100-fold increase in alkaline phosphatase in Escherichia coli B, strain PR1 at 30 C in minimal medium that contains excess inorganic phosphate (1.92 × 10^{-1} M). Little increase in alkaline phosphatase synthesis occurs under these conditions at 22 C. [This strain is known to have a mutation in a regulator gene (R_3) that, in the absence of FPA, permits derepression of alkaline phosphatase synthesis at 37 C, but not at 30 C or below.] In contrast, E. coli B3 (the strain from which E. coli B strain PR1 was derived) is not derepressed at 30 C by FPA. ^4C-FPA is incorporated into bacterial proteins. Temperature-shift experiments (30 C→22 C) in the presence of FPA are consistent with the following mechanism. FPA is incorporated into the genetically altered R_3 protein at 30 and 22 C. This further alteration due to the incorporation of analogue makes the R_3 protein inactive at 30 C, but active at 22 C.

Alkaline phosphatase synthesis is controlled by three regulator genes (R_1, R_{sa}, and R_{sb}) in Escherichia coli (9, 11). Mutations in any of these loci can derepress alkaline phosphatase synthesis in media that contain excess phosphate. The protein product of the R_{sb} region has been isolated by Garen and Otsuji (11); the R_{sa} protein increases when alkaline phosphatase increases and decreases when alkaline phosphatase decreases. In addition, suppressor mutations that act at the level of messenger ribonucleic acid (mRNA) translation into protein (1, 2, 9, 12, 19, 20) suppress constitutive mutations in R_1, R_{sa}, and R_{sb}, indicating that all three regulator genes specify proteins (9). The studies of Gallant and Stapleton (5–7) with a temperature-sensitive R_3 phosphatase-constitutive mutant (E. coli B, strain PR1) provide further indications that R_3 specifies a protein. This mutant produces alkaline phosphatase at high temperature, but not at low temperature in media that contain excess phosphate. [It has not been determined whether the mutation in this strain (E. coli B, strain PR1) is at the R_{sa} or R_{sb} locus.] The wild type (E. coli B3) does not produce the enzyme at either temperature in media that contain excess phosphate. Furthermore, treatment of the mutant with inhibitors of protein synthesis (chloramphenicol or canavanine) elevates alkaline phosphatase synthesis at a temperature (35 C) at which partial derepression occurs (7).

In previous studies, low concentrations of DL-p-fluorophenylalanine (FPA) induced the synthesis of capsular polysaccharide in E. coli K-12 (strain AB259) and caused derepression of several enzymes involved in capsular polysaccharide synthesis (13). The results were consistent with the following model: FPA is incorporated into the protein product of a regulator gene and alters it sufficiently to allow derepression of several enzymes of polysaccharide synthesis. Therefore, the present study was undertaken to examine the effect of FPA on the regulation of alkaline phosphatase synthesis.

Studies with wild-type E. coli K-12 (strains K-10 and W3747) and E. coli B3 demonstrated that FPA did not derepress alkaline phosphatase synthesis. E. coli B, strain PRI was then examined, since it was possible that the product of the temperature-sensitive R_{3} gene (which was likely to be a mutationally altered protein) could be affected at concentrations of FPA that would not affect synthesis or activity of other proteins. Thus, it was assumed that a protein with a mutationally altered primary sequence of amino acids might be inactivated more readily upon incorporation of amino acid analogue than most other "normal" cell proteins. A preliminary report of the present work has been published (Kang and Markovitz, Federation Proc. 25:338, 1966).
E. coli B3 and E. coli B strain PRI were obtained from J. Gallant. E. coli B3 is a thymineless mutant of E. coli B; strain PRI is a 5-bromouracil-induced mutant of E. coli B3. Cells were grown in tris(hydroxy-methyl)aminomethane (Tris)-glucose medium (3) supplemented with thymine (10 \( \mu \text{g/ml} \)) and 1.92 \( \times 10^{-3} \) M potassium phosphate unless otherwise indicated.

For derepression of enzyme synthesis, FPA was added to exponentially growing cells to a final concentration of 8 \( \times 10^{-3} \) M when optical density (OD) at 600 \( \text{nm} \) approached 0.05, except where so stated. Alkaline phosphatase was measured and expressed essentially as described by Garen and Levinthal (10). Samples of cultures were centrifuged, resuspended in 0.1 M Tris (pH 7.5), and treated with toluene (approximately 0.05 ml toluene per ml of culture) for 30 min at 30 C. Enzymatic assays were done in a total volume of 3.0 ml that contained 2.25 \( \mu \text{mol} \) 5-nitrophenyl phosphate, 0.85 M Tris (pH 8.0), and 0.5 ml of toluenized cell suspension. The increase of OD at 410 \( \text{nm} \) (absorption maximum of 5-nitrophenol) was followed with a cylindrical tube 1.16 cm in diameter at room temperature (approximately 23 C; Bausch & Lomb Spectronic-20 colorimeter). Cell turbidity was measured as the OD at 540 \( \text{nm} \) with the Spectronic-20 colorimeter as above (data of Fig. 1) and at 600 \( \text{nm} \) with a cylindrical tube 1.6 cm diameter (Coleman Jr. colorimeter) for all other experiments. Enzymatic activity was expressed as the increase of OD per minute at 410 \( \text{nm} \), and the specific enzymatic activity was calculated as units of enzyme activity per unit of cell turbidity at 600 \( \text{nm} \) (Table 1) or at 540 \( \text{nm} \) (Fig. 1).

For the measurement of net protein synthesis in the presence of FPA (Fig. 2), L-leucine-4,5-\( ^{3} \text{H} \) (10\(^{-3} \) M final concentration, 1 \( \mu \text{c} / \mu \text{mole} \)) was added to a 10-ml cell suspension containing 8 \( \times 10^{-3} \) M FPA when the OD at 600 \( \text{nm} \) was 0.05. At intervals, a 0.2-ml portion was added to an equal volume of 10% trichloroacetic acid, and the mixture was heated at 90 C for 15 min. The precipitate (protein plus cell wall fraction) was deposited quantitatively on a membrane (Millipore Corp., Bedford, Mass.) by filtration and subsequent washing with 5.0 ml of 5% trichloroacetic acid. The simultaneous measurement of incorporation of FPA and leucine was accomplished by the addition of leucine-4,5-\( ^{3} \text{H} \) and FPA-3-\( ^{14} \text{C} \) (final concentration of 5 \( \times 10^{-4} \) M and 1 \( \times 10^{-4} \) M, respectively; both had a specific activity of 1 \( \mu \text{c} / \mu \text{mole} \)) to a 10-ml cell suspension. Portions (1 ml) were treated as indicated above to measure incorporation into protein and cell wall fraction.

Radioactivity was determined in a Tri-Carb liquid scintillation spectrometer with 0.65% 2,5-diphenyloxazole-0.010% 1,4-bis-2-5phenyl-oxazolyl -benzene in toluene.

FPA and p-nitrophenyl phosphate were purchased from Calbiochem, Los Angeles, Calif. L-Leucine-4,5-\( ^{3} \text{H} \) and FPA-3-\( ^{14} \text{C} \) were purchased from New England Nuclear Corp., Boston, Mass., and Calbiochem, respectively.

### RESULTS AND DISCUSSION

Experiments with strain PRI indicated that derepression of alkaline phosphatase synthesis and adequate growth would occur at 30 C in media that contained excess phosphate (1.92 \( \times 10^{-3} \) M KH\(_{2}\)PO\(_{4}\)) and 8 \( \times 10^{-3} \) M FPA. The data in Table 1 demonstrate that a 100-fold increase in specific enzymatic activity can be obtained by growth of strain PRI, but not by the parent (strain B3), under these conditions. Both strains were derepressed by growth in limited phosphate (Table 1), in confirmation of earlier studies (4). The fact that FPA caused derepression of alkaline phosphatase synthesis in E. coli B strain PRI (a strain with a temperature-sensitive \( R_{1} \) locus), but not E. coli B3, indicates that the effect of FPA is related to the mutationally altered product of the \( R_{1} \) locus.

The kinetics of FPA-induced alkaline phosphatase synthesis at 30 C are presented in Fig. 1. Derepression began at approximately 50 min (0.4 generations) in this experiment, at which point the generation time also changed from 130 to 180 min. When a twofold excess of L-phenylalanine (with respect to FPA) was added at 100 min, repression started abruptly 40 min (0.25 generations) later (the generation time also changed from 180 to 160 min within 20 min after addition of L-phenylalanine).

Preliminary results showed that the differential rate of alkaline phosphatase synthesis (\( \Delta \) alkaline phosphatase/\( \Delta \) OD of culture) produced by

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conditions</th>
<th>Cell growth (OD 600 ( \text{nm} ))</th>
<th>Specific enzymatic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRI</td>
<td>-FPA</td>
<td>0.42</td>
<td>0.07</td>
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<tr>
<td></td>
<td>+FPA</td>
<td>0.20</td>
<td>6.8</td>
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<tr>
<td>B3</td>
<td>-FPA</td>
<td>0.39</td>
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<td></td>
<td>+FPA</td>
<td>0.28</td>
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\* Excess phosphate (1.92 \( \times 10^{-3} \) M KH\(_{2}\)PO\(_{4}\)) was present in all tubes; FPA, when present, was at a concentration of 8 \( \times 10^{-3} \) M. Exponentially growing cells were diluted so that OD at 600 \( \text{nm} \) was 0.02 and then grown for 14 hr at 30 C. Specific enzymatic activities were determined and expressed as described in Materials and Methods. Results are the average of two experiments.

\* As positive controls, strains PRI and B3 were grown on limited phosphate (3.2 \( \times 10^{-3} \) M KH\(_{2}\)PO\(_{4}\)) without FPA. Specific enzymatic activities were: strain PRI 3.2; strain B3 2.7.

\*
FPA at 22 C was 5 to 10% of that at 30 C. The difference in the differential rate of alkaline phosphatase synthesis at these two temperatures could be explained if the differential rate of incorporation of FPA into protein were much lower at 22 C as compared with 30 C. However, double labeling experiments demonstrated that 14C-FPA incorporation into protein (as measured by 3H-leucine incorporation) was not detectably different from that at 22 C. Thus, the difference in alkaline phosphatase synthesis at these two temperatures is not explained by a temperature-dependent change in the differential rate of 14C-FPA incorporation into the total protein fraction.

There was a striking difference in the FPA-induced differential rate of alkaline phosphatase synthesis at 30 C as compared with 22 C. Therefore, temperature-shift experiments in both directions were undertaken. Figure 2 shows the results of shifts from 30 to 22 C. The most significant point is the following. During derepression at 30 C, a shift to 22 C resulted in an immediate reduction in the differential rate of alkaline phosphatase synthesis.

Since FPA-induced derepression was slight at 22 C, the effect of previous growth in FPA at 22 C on subsequent derepression at 30 C could be examined. The results are presented in Fig. 3a and b. When cells were placed in FPA at 30 C, derepression of alkaline phosphatase synthesis began in approximately 60 min. However, when cells were grown for 150 min in FPA at 22 C and then shifted to 30 C, derepression of alkaline phosphatase synthesis began within 5 min (Fig. 3a and b).

The results of the temperature-shift experiments may be explained by the following mechanism. FPA is incorporated into the genetically altered R₃ protein at 30 and 22 C. This further alteration due to the incorporation of analogue makes the R₃ protein inactive at 30 C, but active at 22 C. R₃ protein that contains FPA and is active at 22 C can be inactivated rapidly by shifting the temperature to 30 C. Other mechanisms not involving incorporation of FPA into the R₃ protein are considered as follows. (i) FPA is not incorporated into the R₃ protein but specifically inhibits the differential rate of synthesis of the R₃ protein at 30 and 22 C; the amount present is sufficient for repression at 22 C, but not at 30 C. (ii) FPA is not incorporated into the R₃ protein but increases the rate of degradation of the R₃ protein at 30 and 22 C; (a) the amount present is sufficient for repression at 22 C, but not at 30 C, or (b) synthesis is very rapid at 22 C.

If the last alternative is correct, preincubation at 22 C in FPA should not affect the lag in syn-
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$\text{FIG. 3a. Effect of temperature shift (22 C} \rightarrow \text{30 C)}$
on derepression of alkaline phosphatase synthesis by FPA. FPA ($8 \times 10^{-5}$ M) was added to cells growing exponentially (OD 600 $m_A = 0.05$) at 22 C. At the times indicated by the numbers above the arrows, samples were transferred to 30 C. The numbers above each curve of enzyme activity indicate the time (minutes) at which that sample of cells was shifted from 22 to 30 C. The data on the culture incubated at 30 C from zero-time were obtained in a separate experiment.

Fig. 3b. Effect of growth of cells at 22 C with FPA on the length of lag for derepression after temperature shift (22 → 30 C). A plot of the log of alkaline phosphatase activity versus time (data from Fig. 3a) yields a series of straight lines for the cultures shifted from 22 to 30 C (at some time after the shift). These lines intercept the line obtained for the culture at 22 C throughout the experiment. The intercepts yield estimates of the length of lag.

thesis of alkaline phosphatase when a culture is shifted to 30 C. In fact, the lag is reduced from 60 to 5 min (Fig. 3a and b). Mechanisms i and iia both require that less R$_2$ protein be needed at 22 C as compared with 30 C for the repression of alkaline phosphatase synthesis. We have measured the differential rate of alkaline phosphatase synthesis in varying concentrations of inorganic phosphate (limiting to excess) at 22 and 30 C (in the absence of FPA) and find that the differential rate is dependent on phosphate concentration but independent of temperature (30 and 22 C).

Gallant and Stapleton found a twofold higher differential rate of alkaline phosphatase synthesis at 30 C as compared with 22 C in excess phosphate with the same strain (5). This difference in results may be accounted for by difficulties in enzymatic activity assays on crude extracts with very low specific enzymatic activities. The simplest explanation of these results is that the amount of R$_2$ protein is approximately equal at 22 and 30 C. Sadler and Novick (18) have provided support for this assumption in the β-galactosidase system. If the differential rate of alkaline phosphatase synthesis is a measure of the amount of R$_2$ protein and if FPA were not incorporated into the R$_2$ protein (i and iia), then we would expect the differential rate of alkaline phosphatase synthesis to be equal at 22 and 30 C in the presence of FPA (just as it is in the absence of FPA; see above). In fact, the differential rate is reduced 5- to 10-fold on shifting from 30 to 22 C. Therefore, mechanisms i and iia seem unlikely, but are not eliminated.

Gallant and Stapleton showed that rapid appearance of R$_2$ protein activity can occur at lower temperatures in strain PR1 (6). Therefore, the rapid cessation of FPA-induced alkaline phosphatase synthesis on shifting from 30 to 22 C can be explained either by rapid de novo synthesis of R$_2$ protein at 22 C or reactivation of existing R$_2$ protein at 22 C. On the basis of this mechanism, the delay in re-establishment of repression after phenylalanine addition to PR1 derepressed by FPA at 30 C (Fig. 1) would be a measure of the time required for synthesis of sufficient phenylalanine-containing R$_2$ protein to stop alkaline phosphatase synthesis at 30 C. There is some precedent for a mechanism based on FPA incorporation. Although FPA incorporation into alkaline phosphatase in place of phenylalanine does not alter the activity or stability of this enzyme (17), incorporation of other analogues into some proteins leads to alteration in stability. For example, incorporation of fluorotyrosine in β-galactosidase yields an enzyme that is enzymatically active but thermolabile (14, 16).

Gallant demonstrated that a shift of strain PR1 from 37 C in media that contained excess phosphate, where rapid alkaline phosphatase synthesis was taking place, to 25 C resulted in "immediate re-establishment of repression" (4). Gallant and Stapleton (5, 6) preferred to explain this effect as a rapid resynthesis of R$_2$ gene product which was more rapid at lower temperature. However, their results (4-7) are equally compatible with the reactivation of existing R$_2$ gene product at the lower temperature (below 30 C).

Genetic studies of partial diploid strains of E. coli K-12 led to the suggestion that the product of a regulator gene (R$_2$) that controls several
enzymes involved in capsular polysaccharide synthesis is a protein composed of subunits (15), and growth in FPA derepresses several of these enzymes (13). Results obtained by Sadler and Novick (18) in kinetic experiments on haploid and homozygous partial diploid strains suggest that the i gene product that controls \( \beta \)-galactosidase synthesis is a protein composed of subunits. They have also suggested a plausible explanation for a regulator gene product that displays temperature-sensitive synthesis during growth. Gallant (4) has demonstrated that the \( R_2 \) mutation in strain PR1 is of this type. The active "repressor" is a polymer, itself thermostable, but assembled from subunits (monomers) which are thermostable" (18). Our temperature-shift experiments on alkaline phosphatase synthesis in the presence of FPA suggest that the FPA containing \( R_2 \) protein is relatively stable when it is in an active (22 C) form, but is rapidly inactivated when exposed to a temperature of 30 C. If we assume, for the moment, that active corresponds to oligomers and inactive to monomers, then we can provide a reasonable explanation for the \( R_2 \) temperature-sensitive synthesis regulator gene mutant (strain PR1). It may be a mutant in which assembly of inactive monomers into active oligomers takes place at low, but not at high temperature. [Using a strain that contains \( \beta \)-glycosidase (super-repressed at 30 C, but constitutive for \( \beta \)-galactosidase after growth at 43 C), we have demonstrated a 25-fold increase in the differential rate of \( \beta \)-galactosidase synthesis due to growth in FPA at 35 C. An isogenic \( i^+ \) strain was not derepressed under these conditions (Kang, Rockey, and Markovitz, Federation Proc. 26:678, 1967).] Alternatively, if the relevant protein in the temperature-sensitive synthesis mutant is not composed of subunits, then it may be a mutant able to form an active tertiary structure at low, but not at high temperature.

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LITERATURE CITED