REACTION VELOCITY RESPONSE OF THE ESCHERICHIA COLI BIOSYNTHETIC L-THREONINE DEAMINASE TO RAPID CHANGES IN SUBSTRATE AND MODIFIER LIGAND CONCENTRATIONS*

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SUMMARY: It has been determined that the biosynthetic L-threonine deaminase from Escherichia coli responds rapidly to a sudden increase in the concentration of either L-isoleucine or L-valine but, in the presence of L-isoleucine, exhibits a hysteretic response to a rapid increase in the level of the substrate, L-threonine. This effect is observed only at L-threonine concentrations much higher than that required for saturation of the catalytic site.

Frieden has recently defined hysteretic enzymes as those that respond slowly in terms of some kinetic characteristic to rapid changes in ligand concentrations (1). A large number of enzymes fall into this category. Some are regulatory enzymes that are important in metabolic regulation. Frieden suggests that the hysteretic properties of these enzymes may serve an important function in buffering against rapid changes in metabolite levels in pathways that utilize common intermediates or in which there are multiple branch points.

One example of a hysteretic enzyme is the L-threonine deaminase from Bacillus subtilis (1, 2, 3). This enzyme exhibits a slow reaction velocity response to both its inhibitor, L-isoleucine and its activator, L-valine. The subtilis enzyme also responds slowly to a rapid increase in the substrate level in the presence of L-isoleucine. The work reported here was undertaken to determine if the E. coli L-threonine deaminase exhibits similar hysteretic responses.

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METHODS

Growth of Organism. An E. coli K12 derivative, strain T1R8, isolated by Szentermai and Umbarger (4), which is resistant to the growth inhibiting effects of thiaisoleucine and exhibits constituitively derepressed levels of L-threonine deaminase, was used as the enzyme source.

The cells were cultivated on a solid agar surface which contained Difco valine assay medium, 10 gm per liter, Davis and Mingioli minimal salts (5), 2.0% Difco bacto-agar, and 0.5% glucose. The agar was prepared in flat Wearever aluminum cake pans (17 x 23 x 2-3/8 in.) and inoculated by spreading 2 mls. of a fully grown nutrient broth culture over the agar surface. These cultures were incubated at 37°C for 14 hours and harvested by scraping the cells from the agar surface with an 8 inch rubber window squeegee.

Preparation of Enzyme. The enzyme samples used throughout this study were at least 30% pure as determined by disc gel electrophoresis. The crude extracts were obtained from cultures of E. coli, strain T1R8, (4) by ultrasonic oscillation in 0.1 M potassium phosphate, pH 7.5, containing 1×10^{-4} M L-isoleucine and 1×10^{-3} M mercaptoethanol. The cell debris was removed by centrifugation at 27,000 x g for 15 minutes and the supernatant solution was decanted. This solution was heated to 55°C for 5 minutes and immediately chilled to 0°C. The resultant precipitate was removed by centrifugation and the supernatant fluid was brought to 45% saturation with solid ammonium sulfate. The precipitate was redissolved in an equal volume of the same buffer and again heated to 55°C for 1.5 minutes, and immediately chilled to 0°C. The precipitated protein was removed by centrifugation and the enzyme solution was dialyzed for several hours in a large volume of .02 M potassium phosphate, pH 6.9, containing 1×10^{-4} M L-isoleucine, 1×10^{-3} M mercaptoethanol, 1×10^{-4} M pyridoxal-5'-monophosphate, and 1×10^{-3} M EDTA. The

dialyzed enzyme solution was then adsorbed on a calcium phosphate column (brushite form) and eluted with a gradient from .02 M to .1 M potassium phosphate, pH 6.9, containing the same additives as the previous dialyzing buffer. The active fractions from this column were collected and pooled. The pH of the pooled fractions was adjusted to pH 7.5 and the sample was concentrated by vacuum dialysis and frozen for use the next day in the kinetic analyses. The kinetic parameters of the enzyme remain constant throughout this procedure.

Enzyme Assays. During the purification procedure, L-threonine deaminase activity was monitored by measuring the α-ketobutyrate formed as the 2, 4-dinitrophenylhydrazone derivative as described by Friedemann and Haugen (6). The reaction mixture contained per ml: potassium phosphate, pH 8.0, 100 μmoles; ammonium chloride, 100 μmoles; and L-threonine, 80 μmoles. The reactions were started with the addition of enzyme and terminated after incubation at 37°C for 5, 10 and 15 minutes by transferring an appropriate portion of the reaction mixture directly into 3 ml of 0.025% 2, 4-dinitrophenyl hydrazine dissolved in 1 N HCl.

The continous assay used was a modification of the coupled assay of Maeba and Sanwal (7). The reaction mixture contained per ml: potassium phosphate, pH 8.0, 100 μmoles; NH_4Cl, 100 μmoles; freshly prepared NADH, .2 to .3 μmoles; beef heart lactic dehydrogenase (LDH), .4 mg; and L-threonine, L-isoleucine and L-valine as indicated. The rate of oxidation of NADH in 1.0 ml quartz cuvettes (10 mm light path) was followed at 340 mμ using a Gilford model 2000 recording spectrophotometer at 37°C. No NADH oxidation could be detected in a reaction mixture lacking only L-threonine and a linear relationship between the steady state reaction velocity and the enzyme concentration was demonstrated.

RESULTS

Effect of Preincubation with L-isoleucine. When L-threonine is added to a reaction mixture containing enzyme but no L-isoleucine the reaction velocity proceeds linearly with time until the NADH concentration becomes limiting. If, however, the enzyme is preincubated for a short time in the presence of L-isoleucine a slow response is observed in which the reaction velocity slowly increases until a steady state rate is reached (Figure 1a). That is, a hysteretic response is observed when the concentration of the substrate is suddenly increased to high levels in the presence of the inhibitor, L-isoleucine.

Reaction Velocity Response to a Rapid Change in L-isoleucine Concentration. In order to determine whether or not the response of the reaction velocity to a sudden increase in the concentration to L-isoleucine was rapid or slow, a reaction was initiated with L-threonine. L-isoleucine was added later to the progressing reaction (Figure 1b). If the response were slow, a gradual decrease in the reaction velocity would have been expected. Instead, the rate was immediately decreased and, in fact, the subsequent rate after the L-isoleucine addition increased owing to the slow activating effect exerted by the L-threonine in the reaction mixture.

Reaction Velocity Response to a Rapid Change in L-valine Concentration. In this experiment the enzyme was preincubated with L-isoleucine and the reaction was initiated with L-threonine. When the reaction was about one-third of the way through its normal lag phase a high concentration of L-valine was added. If the response to the activating effects of L-valine were slow, an exaggerated curvature of the reaction velocity plot would be expected. Instead, the velocity was immediately adjusted to the new rate (Figure 1c) indicating that the activating effect of L-valine is exerted rapidly.

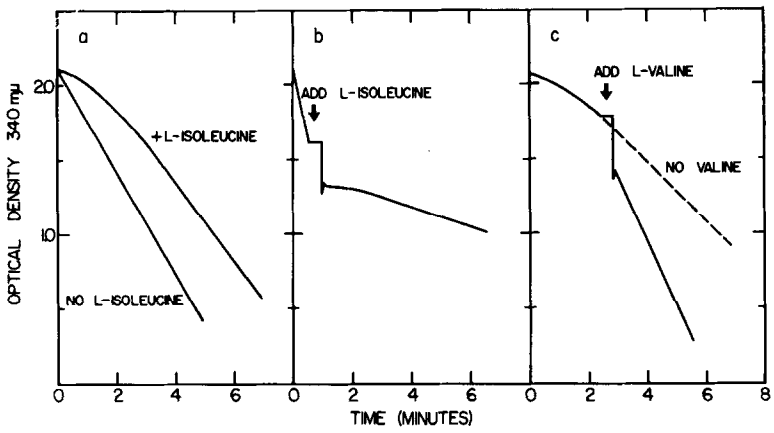

Figure 1. (a) The upper curve illustrates the course of a reaction in which the enzyme was preincubated in a 1.0 ml reaction mixture containing 0.1 μmoles of L-isoleucine for 5 minutes at 37°C. The reaction was initiated with the addition of 80 μmoles of L-threonine. The lower curve represents the same reaction in the absence of L-isoleucine. (b) A 1.0 ml reaction was initiated with the addition of 20 μmoles of L-threonine and 0.2 μmoles of L-isoleucine was added at the time indicated by the arrow. (c) A 1.0 ml reaction mixture containing enzyme and 0.1 μmoles of L-isoleucine was initiated with the addition of 40 μmoles of L-threonine, 1 μmole of L-valine was added at the time indicated by the arrow. The dotted line indicates the course of the same reaction in the absence of the added L-valine.

DISCUSSION

The biosynthetic L-threonine deaminase from Bacillus subtilis can be properly defined as hysteretic (1, 2, 3). This enzyme, unlike the L-threonine deaminase from E. coli, shows a slow reaction velocity response to rapid concentration changes of each of its site-specific ligands. The addition of either L-threonine or L-valine to the enzyme-isoleucine complex results in a time dependent increase of the reaction velocity and the addition of L-isoleucine to either the enzyme-substrate or enzyme-valine complex results in a time dependent decrease of the reaction velocity. It has been demonstrated for the B. subtilis enzyme that these slow responses are a consequence of a slow, ligand induced isomerization rate between the active and inhibited states of the enzyme (2, 3). It is possible that the activating effect exhibited by L-

threonine is due to its binding at an L-valine specific site on the enzyme thereby mimicking the activating effect of the physiological activator, L-valine. The same situation might occur with the E. coli L-threonine deaminase, except that the isomerization rate from the inactive to the active state is rapid when the normal activator, L-valine, is complexed with the enzyme and slow only when the L-valine analog, L-threonine, occupies the activator site. It has been shown here that the isomerization rate in the reverse direction (i.e. from the active to the inactive state) is also rapid with the E. coli enzyme. Therefore, since the substrate concentrations required to elicit a reaction velocity response are far in excess of that required for catalytic saturation and because the endproduct effectors, L-isoleucine and L-valine, do not elicit a hysteretic response, it appears doubtful that the hysteretic enzyme concept relating to the regulation of metabolic processes is applicable to the L-threonine deaminase in E. coli.

A detailed analysis of the presteady state kinetic behavior of the E. coli L-threonine deaminase is currently in progress to determine the mechanism of isomerization between the various enzyme states and to confirm that the time dependent activation exerted by L-threonine on the enzyme-isoleucine complex is wrought through its interaction with the enzyme at an L-valine specific activator state.

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