FORMATION OF BIODEGRADATIVE THREONINE DEAMINASE IN ESCHERICHIA COLI

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Studies were carried out on the formation of biodegradative L-threonine deaminase (EC 4.2.1.16) in resting cells of Escherichia coli. The results obtained are as follows: (1) The enzyme level was increased by adding L-threonine together with yeast extract to the cell suspension. (2) In the course of the enzyme formation, the addition of chloramphenicol or glucose to the medium resulted in instantaneous cessation of the enzyme synthesis. (3) Yeast extract could be partially replaced by casamino acids. A mixture of amino acids whose composition is similar to that of milk casein was also effective. (4) Omission of L-serine from the mixture caused an almost complete loss of the activity, indicating that L-serine as well as L-threonine was essentially required for the enzyme formation. (5) Other amino acids were also needed for the enzyme synthesis, although individual omission of them had a slight influence compared with that of L-serine.

The biodegradative L-threonine deaminase (EC 4.2.1.16) from Escherichia coli is known to be activated by adenosine 5′-monophosphate. The enzyme contains firmly bound pyridoxal phosphate as a cofactor. It is distinct from the biosynthetic threonine deaminase of the same microorganism in respect to a lack of end product inhibition by L-isoleucine (1). The enzyme has been purified to homogeneity (2, 3) and its enzymatic properties have been investigated in detail (1-12).

This work is concerned with conditions of the synthesis of the enzyme. Evidence is presented that the enzyme is induced in resting cells by addition of L-threonine and yeast extract, and that yeast extract can be replaced by a mixture of amino acids, containing L-serine. Preliminary accounts of this work have been published (13, 14).
EXPERIMENTAL

1. Growth of organism. E. coli W was grown at 37°C for about 15 hr with shaking in a synthetic medium containing 0.77% K2HPO4, 0.33% KH2PO4, 0.011% MgSO47H2O, 0.11% (NH4)2SO4, and 0.22% glucose in a total volume of 20 ml. Five milliliters of the culture were transferred to 100 ml of the fresh medium, and cultivation was carried out at 37°C. Cells were harvested at the end of the logarithmic phase of growth and washed with 15 ml of 0.04 M potassium phosphate buffer (pH 7.4). The washed cells (0.5 g in wet weight) were suspended in about 6 ml of the same buffer.

2. Incubation of resting cells. The standard reaction mixture for the induction experiments contained, in a final volume of 1.0 ml, 50 μmoles of potassium phosphate (pH 7.4), 50 μmoles of L-threonine, 1% yeast extract, and 0.2 ml of the above cell suspension. The absorbance of the reaction mixture at 540 nm was 3 OD units at zero time and did not show an appreciable change during the incubation up to 2.5 hr, indicating that cell growth did not proceed in such a heavy cell suspension. After the incubation, aliquots were withdrawn and a few drops of toluene were added. After standing in ice bath for more than 1 hr with occasional shaking, 0.1 ml aliquots were analyzed for threonine deaminase activity. Omission of L-threonine from the above reaction mixture caused marked decrease in the activity for the enzyme induction.

3. Enzyme assay. The activity of threonine deaminase was determined spectrophotometrically by measuring the formation of α-ketobutyrate as its 2,4-dinitrophenylhydrazone as previously described (6). The standard assay system contained 100 μmoles of potassium phosphate (pH 7.4), 100 μmoles of L-threonine, 10 μmoles of AMP, 10 μmoles of L-isoleucine, and the enzyme (0.1 ml of the toluenized cell suspension) in a total volume of 1.0 ml. Incubation was performed at 37°C for 10-15 min. One unit of enzyme was defined as the amount that produced 1 μmole of α-ketobutyrate per minute. The assay was linear with respect to both time and amount of enzyme added under the experimental conditions.

4. Materials. L-Threonine, L-serine and other amino acids were provided by the Kyowa Hakko Kogyo Company, Ltd. (Tokyo). AMP was provided by the Takeda Research Laboratories, Takeda Chemical Industries, Ltd. (Osaka). Yeast extract was obtained from the Daigo Eiyo Kagaku Company, Ltd. (Osaka). All other chemicals were of reagent grade.

RESULTS

1. Formation of threonine deaminase in resting cells

Figure 1 shows a time-dependent change of the enzyme activity during incubation of cells with a medium containing L-threonine and yeast extract. The enzyme
activity increased with incubation time after a lag period of about 30 min.

The induction did not take place when 100 μg of chloramphenicol were added per ml of the medium at the beginning of the induction (Fig. 2). Addition of the antibiotic in the course of the induction process rapidly arrested the production of the enzyme. These results indicate that the enzyme formation involves de novo protein biosynthesis.

The biodegradative threonine deaminase is known to be repressed by glucose (1). The glucose effect was clearly demonstrated in this induction system using the resting cells as shown in Fig. 3. The enzyme formation was markedly and instantaneously repressed upon addition of glucose.

The effect of yeast extract at various concentrations was examined on the enzyme induction. The activity of enzyme formation increased with an increase in concentration of yeast extract up to a level of 1% (Fig. 4). An inhibitory effect was, however, observed above this concentration.

2. Effect of various amino acids

Preliminary experiments with ignition, molecular sieve and ion exchange
Fig. 2. Influence of chloramphenicol on the induction of threonine deaminase. Chloramphenicol in a final concentration of 100 μg per ml, was added either at the beginning of the incubation or in the course of the enzyme formation. Results with chloramphenicol (○); results with control (●).

Fig. 3. Effect of glucose on the induction of threonine deaminase. Glucose was added in a final concentration of 13 mM at the designated time. Results with glucose (○); results with control (●).

chromatography indicated that the active principle(s) in yeast extract may be an organic, low molecular and amphoteric electrolyte(s). It was, therefore, presumed that one or more amino acids in yeast extract is responsible for the
Fig. 4. Effect of concentration of yeast extract on the induction of threonine deaminase. Cells were incubated for 1.5 hr in the induction mixture containing 50 mM L-threonine and yeast extract at various concentrations as indicated.

Fig. 5. Effect of casamino acids on the induction of threonine deaminase. Casamino acids were added at the designated levels with or without yeast extract (0.2 or 1%) to the incubation mixture containing 50 mM L-threonine.

inducing activity. Figure 5 shows that the activity of yeast extract is partially replaced by casamino acids. In addition, the enzyme level increased with an increase in concentration of casamino acids when yeast extract was present at
a low concentration (0.2%), which did not give a maximum level of enzyme induction. Addition of casamino acids exhibited no effect when 1% yeast extract was present, as shown in the same figure. These results strongly suggest that amino acids, which are constituents of yeast extract, play an important role in the formation of the enzyme.

In order to know what kinds of amino acid are required for the enzyme induction, the effect of a mixture of various amino acids was examined, the composition of which is the same as that of cow milk casein (15), except for the omission of L-threonine. The mixture contained 270 μg of glycine, 300 μg of L-alanine, 720 μg of L-valine, 920 μg of L-leucine, 610 μg of L-isoleucine, 1,130 μg of L-proline, 500 μg of L-phenylalanine, 34 μg of L-cystine, 280 μg of L-methionine, 120 μg of L-tryptophan, 410 μg of L-arginine, 310 μg of L-histidine, 820 μg of L-lysine, 710 μg of L-aspartic acid, 2,240 μg of L-glutamic acid, 630 μg of L-serine, and 630 μg of L-tyrosine in a total amount of about 10 mg. The mixture in an aqueous solution was added to the incubation mixture containing 0.2% yeast extract. The activity of the mixture and effect of omission of individual amino acid are shown in Table 1. Except for L-alanine, L-valine, L-leucine, L-phenylalanine, and L-

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<tr>
<th>Omission</th>
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<td>Control</td>
<td>0.19&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> A mixture of amino acids whose composition was the same as that described in the text was added to the incubation mixture containing 50 mM L-threonine and 0.2% yeast extract.

<sup>b</sup> The enzyme level in the absence of amino acids except for L-threonine, which was used as a control.

serine, omission of each amino acid exhibited no effect. The most marked effect was observed when L-serine was omitted from the mixture and the enzyme level decreased to the level of control. Omission of various amino acids did not show a significant effect except for L-serine. In addition, effects of omission of amino acids other than L-valine and L-leucine were not reproducible in different experiments. For example, in a separate experiment, L-alanine and L-phenylalanine showed little effect, while L-proline, L-cystine, L-methionine, L-tryptophan, and L-
**Fig. 6.** Effect of L-serine on the induction of threonine deaminase. L-Serine was added at indicated levels to the incubation mixture containing 50 mM L-threonine. Results with 0.2% yeast extract (●); results without yeast extract (○).

**Fig. 7.** Effect of amino acid-mixture on the induction of threonine deaminase in the presence of L-threonine and L-serine. A mixture of amino acid at various concentrations in the same composition, except for omission of L-serine, as that described in Table 1 was added to the incubation mixture containing 50 mM L-threonine and 50 mM L-serine. The levels of enzyme formed were compared with that formed with 50 mM L-threonine and 50 mM L-serine plus 0.2% yeast extract (dashed line).
glutamic acid exhibited an appreciable effect upon omission. Thus, effects of amino acids other than L-serine varied to a considerable extent, presumably depending upon the pool size of each amino acid in the cell. In contrast, the effect of L-serine appeared to be quite consistent.

The results obtained above suggest that L-serine is an active principle in yeast extract. However, as shown in Fig. 6, the enzyme formation did not take place even when L-serine was added to the induction mixture, when yeast extract was absent from the mixture. In contrast, L-serine exerted a stimulatory effect on the enzyme formation in the presence of a low level of yeast extract. The induction increased almost linearly with an increase in L-serine up to 10 mM.

It was also found that, as shown in Fig. 7, yeast extract could be replaced by the amino acid-mixture, the composition of which was the same as that described above, except for omission of L-serine. In the presence of 50 mM L-threonine and 50 mM L-serine, the enzyme induction was enhanced as the amount of the amino acid-mixture was increased. A maximum level was obtained with 0.08% of the amino acid-mixture. Preliminary inspection of the kind and the quantity of the effective components in the amino acid-mixture indicated that at least several kinds of amino acid were involved, though in trace amounts.

Correlation of the varied concentrations of L-threonine and L-serine in the presence of 0.1% amino acid-mixture was studied. First, the effect of varied concentrations of L-serine was examined in the presence of 25, 50, and 100 mM L-

![Fig. 8. Relationship between concentrations of L-threonine and L-serine required for the induction of threonine deaminase. L-Threonine (or L-serine) at a fixed concentration (25, 50 or 100 mM) and L-serine (or L-threonine) at varied concentrations were added to the incubation mixture containing 0.1% of a mixture of amino acids in the same composition as that in Fig. 7. Results with L-threonine (A) and L-serine (B) at fixed concentration: 25 mM (x), 50 mM (o), 100 mM (●).]
threonine. Second, the effect of varied concentrations of L-threonine was examined in the presence of 25, 50, and 100 mM L-serine. As shown in Fig. 8, the results indicated that L-threonine or L-serine alone was ineffective for the enzyme induction, unless the other amino acid was present. For example, when the concentration of one amino acid was as low as 25 mM, a maximum level of induction was attained by the other amino acid at a similar concentration. When the concentration of one amino acid was 50 mM or above, the concentration of the other amino acid required for the maximum induction was as low as about 20 mM.

DISCUSSION

Regulatory mechanisms have been recently reported concerning the activity of biodegradative threonine deaminase. TOKUSHIGE and NAKAZAWA (16) proposed that α-aminocrotonate and α-aminoacrylate, intermediates of the α,β-elimination reaction of L-threonine and L-serine, respectively, inactivate the enzyme by forming a covalent linkage with a sulfhydryl group of the enzyme protein in the absence of AMP. SHIZUTA et al. (17) reported that α-ketobutyrate, a final product of the enzyme reaction, promotes the dissociation of the enzyme into inactive subunits. AMP acts in an antagonistic manner maintaining the active tetrameric form. FELDMAN and DATTA (18) demonstrated that the so-called glucose effect is caused by pyruvate, a catabolite of glucose. In turn, the enzyme dissociates into subunits, followed by oxidation of sulfhydryl group of the enzyme protein in conjunction with the keto acid, which inactivates the enzyme. AMP and sulfhydryl compounds counteract the effect of the keto acid.

In the present investigation it was demonstrated that the formation of biodegradative threonine deaminase was instantaneously arrested upon addition of chloramphenicol. This observation indicates that the apparent induction of the biodegradative threonine deaminase by specific amino acids involves de novo protein biosynthesis, rather than conversion of inactive forms into active enzyme.

As well reviewed recently by PARDEE (19) and KABACK (20), membrane transport could be a limiting step for incorporation of individual amino acid into bacterial cells. In fact, levels of intracellular L-threonine and L-serine appeared to be as low as 0.09 and 0.04 mM, respectively, as PIPERNO and OXENDER reported (21). In the case of β-galactosidase in E. coli, the concentration of isopropyl-thio-β-D-galactoside required for the enzyme induction was in the order of 1-0.01 mM, as PARDEE and PRESTIDGE reported (22). In contrast with the above informations, combinations of as high as 50 mM L-threonine and L-serine at similar concentrations still exhibited a marked synergistic effect on the enzyme induction in the present work.

It is still unknown whether both L-threonine and L-serine are specifically required as the true inducers or only one of the two amino acids is needed. In other words, the following possibility cannot be excluded at present: L-threonine
and L-serine act as structural analogues and not as dual inducers and, because of the transport barriers in the membrane, combination of the two amino acids results in increase in the intracellular concentration of the inducer amino acid. Roles of the amino acids other than L-threonine and L-serine present in the amino acid-mixture are also uncertain at present.

In addition to L-threonine and L-serine, cyclic adenosine 3',5'-monophosphate also acts as an effective regulator of the enzyme synthesis as SHIZUTA and HAYAISHI reported (22).

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