# A TIME-DEPENDENT ACTIVATION OF THREONINE DEAMINASE*

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Biosynthetic threonine deaminase, the first enzyme in the pathway specific for the formation of isoleucine, has been known since its first description to exhibit "abnormal" kinetic properties (1). The enzyme in crude microbial extracts usually, but not always, exhibits such properties and can be rendered "normal" by the addition of valine (2). Extracts showing normal kinetic properties are rendered abnormal by isoleucine, the endproduct inhibitor of threonine deaminase. It thus seems likely that the enzyme might be poised in an "inactive" or "active" state by the relative amounts of isoleucine and valine contaminating the crude extract. An alternative explanation, suggested by Cennamo (3) raises the possibility that the abnormal kinetic data may be an artifact due to lower stability of threonine deaminase at lower threonine concentrations.

The experimental results on which the above suggestion was based were obtained with a wild type strain of Salmonella typhimurium in which the enzyme activity was relatively repressed and an indirect assay was employed. In order to determine whether inactivation might have been the cause of the apparent abnormal kinetic data in earlier experiments, extracts of the "derepressed" mutant, strain CV-19 (4), of S. typhimurium have recently been examined. It was therefore possible to employ direct (colorimetric) product determinations after short time periods. It was found that, contrary to the suggestion of Cennamo (3), there was an activation rather than an inactivation

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of threonine deaminase which occurred at low substrate concentrations.

## EXPERIMENTAL

Organisms and preparation of extracts. Crude extracts of S. typhimurium strain CV-19 were prepared by ultrasonic oscillation in 0.05 M potassium phosphate, pH 7.0, containing 1.2 x $10^{-4}$M isoleucine. Extracts of a wild type Bacillus subtilis strain were prepared by oscillation in 0.02 M potassium phosphate, pH 7.2, containing $10^{-4}$M pyridoxal phosphate and $10^{-3}$M mercaptoethanol (stock buffer). The activity was stable in cells stored or harvested in the cold. However, after disruption, the activity in the crude extract was stable in the stock buffer at 20C but lost activity at 4C.

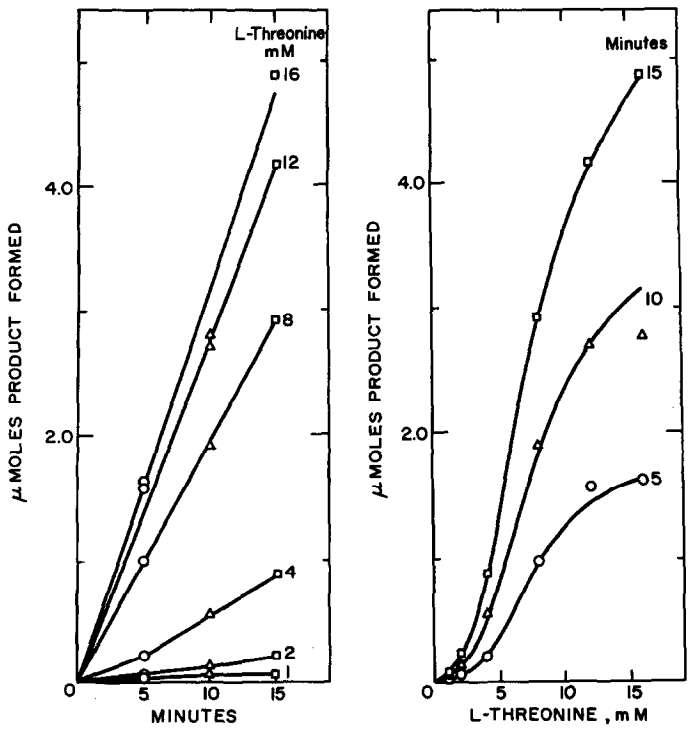


Fig. 1. Effects of time and substrate concentration on threonine deamination with a S. typhimurium extract. Each 1.0 ml reaction mixture contained Tris-HCl, 0.1M, pH 8.0; ammonium chloride, 0.1M; pyridoxal phosphate, 0.1mM; crude extract protein, 10 mg; threonine as indicated. Samples removed for analysis at time indicated. Temperature 37C.

Effect of time and extract concentration on the deamination of threonine by S. typhimurium. Fig. 1 shows the results obtained using a crude extract with which the reaction proceeded at a linear rate for at least 15 minutes in the presence of 8, 12 or 16 mM L-threonine. At lower concentrations, the rate was not linear but actually increased with time. This effect was observed in most extracts. However, the substrate concentration at which activation could be observed was variable.

When the same data were plotted against substrate concentration, sigmoid curves, typical for this enzyme, were obtained. If extracts were used with which, even at low substrate concentrations, there was nearly a linear rate of product appearance, the extent of sigmoidicity was much less apparent. In some cases, the data provided good fits to a hyperbola.

Since this variation might have been due to different proportions of valine and isoleucine, in the various extracts, the question of activation by substrate was examined with crystallized B. subtilis threonine deaminase.

Threonine deamination by this enzyme is shown in Fig. 2. In contrast to the crude Salmonella enzyme, when threonine was added to the B. subtilis enzyme in a reaction mixture lacking isoleucine, the reaction rate was linear throughout the course of the reaction (curve D). However, when threonine was added to an enzyme preincubated for several minutes in a reaction mixture containing isoleucine, a gradual acceleration of the reaction was observed (curve A). When the reaction was initiated by the addition of enzyme to a reaction mixture containing threonine and isoleucine, a gradual deceleration of the reaction was observed (curve B). The same deceleration of the reaction was observed when isoleucine was added after the reaction had been initiated (curve C).

It is suggested that the enzyme exists in an "active" state in its native form and is reversibly inactivated by the inhibitor, isoleucine. Furthermore, it has been observed that this inactivation process itself is slow.

That the activation observed with the coupled assay system was an activa-

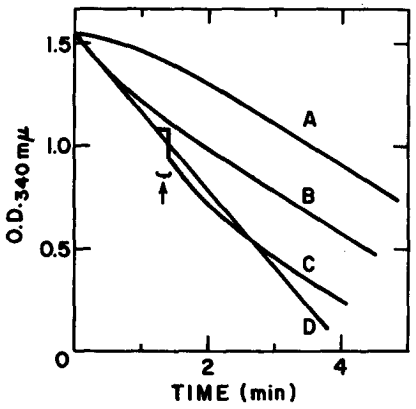


Fig. 2. Patterns of threonine deamination with B. Subtilis enzyme. A 0-60% ammonium sulfate precipitate (pH 7.0) of the crude extract was desalted on a Sephadex G-25 column and applied to a column of brushite (6). Activity was eluted with a 0.02 to 0.2M linear concentration gradient of the stock buffer. This step rendered the enzyme cold stable and the fractions were collected at 4C. Active fractions were concentrated and applied to an ECTEOLA-cellulose column (7). The activity was eluted with a 0.05 to 0.6M linear concentration gradient of the stock buffer. The enzyme was crystallized according to the method of Jakoby and Kohn (8).

Curvettes (0.5 ml) contained: potassium phosphate, 0.1M, pH 8.2; lactic dehydrogenase, 0.2 mg; DPNH, 0.2 mM; threonine, 40 mM; enzyme, 0.0125 μg; and, where indicated below, isoleucine, 25 μm. Temperature: 37C.

| | 5 min. Preincubation | Added at 0 time |
|---|---|---|
| A | Enz, ile | Thr |
| B | Thr, ile | Enz |
| C | Enz | Thr, ile at ↑ |
| D | Enz | Thr |

tion of threonine deaminase itself, and not an artifact of the coupled assay system, was confirmed with two independent, direct assays. In one, [U-$^{14}$C] threonine was used as the substrate and the formation of labelled product was followed. In the other, the amount of product formed during the course of the reaction was measured as its dinitrophenylhydrazone. Both of these direct assays showed an activation period of the same duration as that observed using the coupled assay when the enzyme, substrate, and inhibitor concentrations were the same.

In the absence of isoleucine, a normal substrate saturation curve, adequately described by a simple hyperbolic function (5), was obtained. In the

presence of various concentrations of isoleucine, steady state kinetic data (obtained by measuring rates achieved after the activation of preincubated enzyme was complete) yielded a family of sigmoid curves. These curves could be fitted quite well by the equation described by Monod, Wyman, and Changeux (6) for a K system enzyme. Their model assumes that the enzyme can undergo a free isomerization (an allosteric transition) between the active and inactive form and precludes any mechanism involving a ligand specific induced transition. However, it should be noted that the equations describing the allosteric model are concerned only with the steady state kinetic behavior of the enzyme and do not deal with the mechanism of the transition. In fact, the steady state situation does not reveal whether the transition is induced or spontaneous. In order to decide between these possibilities the presteady state kinetic behavior of the enzyme must be examined.

A detailed study in collaboration with Dr. W. J. Ray Jr. of the presteady state kinetics of this enzyme is currently in progress. Of particular interest is the observation that the rate of activation is related to the concentration of the positive effector, threonine, but not to that of the negative effector, isoleucine. It is difficult to explain this observation by any mechanism other than one involving a ligand specific induced transition.

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