Threonine Deaminase from *Escherichia coli*

I. PURIFICATION AND PROPERTIES*

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**SUMMARY**

A procedure has been devised for the purification of the biosynthetic L-threonine deaminase (L-threonine hydrolase, deaminating: EC 4.2.1.16) from a genetically derepressed mutant, strain TIR8, of *Escherichia coli* K-12. The procedure produces a purification of 400-fold relative to derepressed enzyme levels and 4000-fold relative to repressed levels. The purity of the final enzyme preparation has been determined by sodium dodecyl sulfate gel electrophoresis and by sedimentation equilibrium experiments.

The native enzyme, molecular weight 204,000, is a tetramer composed of four apparently identical subunits, molecular weight 51,000. When the native enzyme is resolved of its cofactor, pyridoxal 5'-monophosphate, catalytically inactive apodimers, molecular weight 102,000, are formed. Addition of pyridoxal 5'-monophosphate to the apodimers promotes reassociation to catalytically active tetramers.

The kinetic data of the L-threonine deaminase reaction are similar with the pure enzyme and with partially purified preparations. L-Isoleucine is a competitive allosteric inhibitor. The enzyme exhibits cooperative homotropic interactions with respect to the substrate only in the presence of L-isoleucine. These interactions are reversed by L-valine.

The enzyme exists in a pH-dependent equilibrium between two distinct and catalytically active species; a tetramer sensitive to L-isoleucine inhibition and a dimer insensitive to L-isoleucine inhibition. The tetramer is favored at pH values from 6.0 to 7.5 while at pH values from 8.5 to 10.0 the enzyme is present almost exclusively as the dimer. At pH 8.0 a mixture of the two is present.

Among the many regulatory enzymes which have been studied, the biosynthetic L-threonine deaminase from *Escherichia coli* K-12 has perhaps had a greater influence on the concepts of biological control than any other. In 1956, Umbarger showed that this enzyme from *E. coli*, the first enzyme in the biosynthetic pathway leading to the ultimate formation of L-isoleucine, was inhibited effectively by the end product of this pathway (1). This was the first demonstration of the now familiar phenomenon of end product inhibition which has since been shown for virtually every biosynthetic pathway. Furthermore, the formation of the concept of allosteric as a regulatory mechanism, proposed by Monod et al. in 1963 (2) and formalized by Monod et al. in 1965 (3), was based, at least partially, on kinetic data obtained from the *E. coli* K-12 L-threonine deaminase by Changeux (4). Until now, however, although this enzyme has been purified from other bacterial sources (5–10), it has not been purified from *E. coli*. Therefore, it has not been possible heretofore to study the physical properties of this enzyme which has played such a historically significant role in shaping our view of cellular regulatory mechanisms. These studies are now possible. The present study presents the procedure for the purification of the biosynthetic L-threonine deaminase from *E. coli* K-12 and some of its basic physical parameters.

**EXPERIMENTAL PROCEDURE**

Cultivation of Cells—*E. coli* strain TIR8, which produces constitutive levels of threonine deaminase as a result of a mutational altered isoleucyl-tRNA synthetase (11) was used as a source of the enzyme. The cultivation of the cells was as previously described (12).

Enzyme Assays—The reaction was followed at pH 8.0 by measuring the α-ketobutyrate formed as the phenylhydrazone derivative (13) or by a modification of the coupled assay of Maeba and Sanwal (14) as previously described (12, 15). A unit of threonine deaminase activity is defined as the amount of enzyme forming 1 μmole of product (α-ketobutyrate) per min.

**Sodium Dodecyl Sulfate Gel Electrophoresis**—Sodium dodecyl sulfate gels were performed according to the procedure described by Weber and Osborn (16). For the estimate of the subunit molecular sodium dodecyl sulfate gels bovine serum albumin, catalase, ovalbumin, and hexokinase were included as standard markers.

Sucrose Density Gradients—Linear 5-ml sucrose gradients, 5 to 20%, were prepared in 50 mM Tris-HCl buffer at the indicated pH values (at 25°C) containing 1.0 mM L-isoleucine, 1.0 mM EDTA, and 0.5 mM dithiothreitol. Sucrose gradients for detecting actively catalyzing enzyme at pH 8.5 were prepared in the same...
buffer containing 80 mM L-threonine. Purified enzyme (53.4 μg in 0.01 ml) was diluted to 0.10 ml with the above buffer at the appropriate pH, and the entire 0.1-ml sample was layered on the surface of the sucrose gradient. The sucrose gradients were centrifuged at 20° for 5 hours at 50,000 rpm in an SW 50.1 rotor in a Beckman L2-65B preparative ultracentrifuge. Fractions of 8 drops were collected by puncturing the bottom of the tube and assays were performed immediately.

**Sedimentation Experiments**—A Spinco model E ultracentrifuge equipped with Rayleigh interference optics and an RTIC temperature control unit was used for the sedimentation equilibrium experiments. Either a standard double sector centerpiece or a six-channel Yphantis centerpiece and sapphire windows were used. The AN-D rotor was used. Fringe patterns were recorded on Kodak II-g spectrographic plates. Measurement of fringe displacement against radial distance were made with a Gaertner microcomparator. The system was judged to be at equilibrium when the fringe displacement at the solution–fluorocarbon junction remained constant for a period of 5 to 10 hours as described by Kawahara and Tanford (17). Column height and rotor speed were adjusted to enable the use of the meniscus depletion method developed by Yphantis (18). Molecular weights were calculated using a partial specific volume of 0.739 ml per g determined by differential sedimentation equilibrium experiments in deuterium oxide and water according to the method of Edelstein and Schachman (19).

**Protein Measurement**—Concentration of protein was estimated either by the method of Lowry et al. (20) or by the absorbance at 280 nm.

**Chemicals**—Amino acids, dithiothreitol (Cleland’s reagent), and pyridoxal 5’-monophosphate were obtained from Calbiochem. Ultrapure guaniidine hydrochloride was purchased from Heico and used without further purification. DEAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals. Calcium phosphate gel was obtained from the California Biochemical Corp. Agarose A-1.5 was purchased from Bio-Rad Laboratories. All other chemicals were reagent grade.

**RESULTS**

**Enzyme Purification**

**Preparation of Crude Extract**—Approximately 50 g of cells, wet weight, were harvested and washed three times in 100 mM potassium phosphate buffer, pH 7.3, containing 1 mM L-isoleucine and 0.5 mM dithiothreitol. The cells were suspended in 200 ml of the same buffer and disrupted in the cold by sonic oscillation for 25 min with a Branson Sonifier. The cell debris was removed by centrifugation at 27,000 × g for 15 min. The supernatant solution was decanted and centrifuged at 50,000 rpm, for 30 min at 5° in a T160 rotor in a Beckman model L2-65B preparative ultracentrifuge. The supernatant solution containing the enzyme was stored at −20°. The frozen extract retains at least 95% of its initial activity even after storage for several weeks. The crude extracts usually contain 3,000 units with a specific activity of 0.4 to 0.6.

**Heat Steps and Ammonium Sulfate Precipitation**—Several crude extracts were combined and heated to 55° for 0.5 min and immediately chilled to 0°. The resultant precipitate was removed by centrifugation and the supernatant fluid was brought to 45% of saturation with solid ammonium sulfate. The precipitate was redissolved in an equal volume of 100 mM potassium phosphate buffer, pH 7.3, containing 1 mM L-isoleucine and 0.5 mM dithiothreitol, and again heated to 55° for 0.5 min, and immediately chilled to 0°, and centrifuged. The enzyme solution of 75 to 100 ml was dialyzed overnight against 4 liters of 20 mM potassium phosphate buffer, pH 7.3, containing 1 mM EDTA, 1 mM L-isoleucine, and 0.5 mM dithiothreitol. This treatment increased the specific activity 5-fold with 95% recovery of enzyme activity. The dialyzed enzyme was either frozen or applied immediately to a DEAE-Sephadex A-50 column. All subsequent steps in the purification procedure were carried out at 4°.

**Chromatography on DEAE-Sephadex A-50**—The dialyzed enzyme from the previous step was placed on a DEAE-Sephadex A-50 column, 5 × 50 cm, equilibrated with 20 mM potassium phosphate buffer, pH 7.3, containing 1 mM EDTA, 1 mM L-isoleucine, 0.5 mM dithiothreitol, and 100 mM KCl. The protein was eluted with a linear gradient consisting of 2 liters of the above buffer in the mixing chamber and 2 liters of the same buffer made 400 mM with respect to KCl in the reservoir. L-Threonine deaminase was eluted near the end of the gradient. Fractions with a specific activity greater than 30 were pooled and concentrated by vacuum dialysis.

**Adsorption on Calcium Phosphate Gel**—The concentrated DEAE-Sephadex pool was dialyzed overnight against 100 mM potassium phosphate buffer, pH 6.8, containing 1 mM L-isoleucine, 1 mM EDTA, and 0.5 mM dithiothreitol. The dialyzed enzyme was diluted to 100 ml with the same buffer, mixed gently for 10 min with 10 ml of calcium phosphate gel, and centrifuged. An additional 10 ml of calcium phosphate gel were added to the supernatant fluid, mixed, and centrifuged. The two pellets were combined and the enzyme was eluted with two 100-ml washes of 10 mM potassium phosphate buffer, pH 7.3, containing 1 mM EDTA, 1 mM L-isoleucine, and 0.5 mM dithiothreitol. The two supernatant fractions were combined and concentrated to 2.0 ml by vacuum dialysis.

**Gel Filtration on Agarose**—The concentrated enzyme from the calcium phosphate step was passed through an agarose A-1.5 column (1.5 × 115 cm) equilibrated with 100 mM potassium phosphate buffer, pH 7.3, containing 1 mM EDTA, 1 mM L-isoleucine, and 0.5 mM dithiothreitol, and eluted with the same buffer. The enzyme eluted in a volume of about 25 ml and the pooled fractions were concentrated by vacuum dialysis. The pure enzyme has a specific activity of 210. The purification procedure is summarized in Table I.

**Criteria of Purity**—The final enzyme preparation obtained from the purification procedure described above was judged to be greater than 95% pure by sodium dodecyl sulfate gel electrophoresis (Fig. 1) and by the linearity of the fringe displacements versus radial distance measurements obtained from the sedimentation equilibrium experiments (Fig. 2).

**Determination of Partial Specific Volume**—The partial specific volume of the native enzyme was determined by differential sedimentation equilibrium experiments in deuterium oxide and water according to the method of Edelstein and Schachman (19).

| Table I | Purification of threonine deaminase |
|---------|-----------------------------------|
| Fraction | Total protein | Total activity | Specific activity | Yield of activity | Purification |
|         | mg           | units         | units/mg         | %               | -fold |
| Crude extract | 16,800 | 8,100 | 0.48 | 100 | 1 |
| Heat steps | 3,300 | 7,900 | 2.36 | 95 | 5 |
| DEAE-Sephadex | 65.5 | 9,290 | 45.0 | 95 | 94 |
| Calcium phosphate | 14.0 | 2,160 | 164 | 27 | 820 |
| Agarose | 4.3 | 905 | 210 | 11 | 440 |
Polyacrylamide gel electrophoresis of sodium dodecyl sulfate-treated purified \( \text{L-threonine deaminase} \). Electrophoresis was from top to bottom. The gel contained 17.5 \( \mu \text{g} \) of enzyme.

**Fig. 2 (right).** Sedimentation equilibrium experiments with purified \( \text{L-threonine deaminase} \). A, native enzyme, 200 \( \mu \text{g} \) per ml, in 50 mm potassium phosphate buffer, pH 7.5, prepared in water containing 1.0 mm EDTA, 1.0 mm \( \text{L-isoleucine} \), and 0.5 mm dithiothreitol, centrifuged at 16,000 rpm, \( d \ln f / dr^2 = 2.30 \), molecular weight = 203,800. B, enzyme, 200 \( \mu \text{g} \) per ml, in 50 mm potassium phosphate buffer, pH 7.5, prepared in deuterium oxide containing 1.0 mm EDTA, 1.0 mm \( \text{L-isoleucine} \), and 0.5 mm dithiothreitol, centrifuged at 16,000 rpm, \( d \ln f / dr^2 = 3.02 \), molecular weight = 203,800. C, enzyme, 200 \( \mu \text{g} \) per ml, dialyzed against 50 mm potassium phosphate, pH 7.5, containing 6 \( \times \) guanidine hydrochloride and 100 mm \( \beta \)-mercaptoethanol, centrifuged at 26,000 rpm, \( d \ln f / dr^2 = 1.40 \), molecular weight = 51,800. D, enzyme, 200 \( \mu \text{g} \) per ml, dialyzed against 50 mm Tris-HCl, pH 8.5, containing 1.0 mm EDTA and 5 mm dithiothreitol, centrifuged at 24,000 rpm, \( d \ln f / dr^2 = 3.49 \), molecular weight = 102,600.

A partial specific volume of 0.719 ml per g in concentrated guanidine hydrochloride was estimated by introducing a correction term which reduces the corresponding value in dilute salt solution by 0.02 ml per g (22).

**Molecular Weight Determinations**—The molecular weight of the native enzyme was determined to be 203,800 from sedimentation equilibrium experiments (Fig. 2A). Dialysis of the enzyme for 18 hours against 6 \( \times \) guanidine hydrochloride and 100 mm \( \beta \)-mercaptoethanol dissolved in 50 mm potassium phosphate buffer, pH 7.5, resulted in the formation of subunits of molecular weight of 51,800 (Fig. 2C). A subunit molecular weight of 53,000 was obtained from sodium dodecyl sulfate gel electrophoresis performed with known molecular weight markers (Fig. 3). Thus, the native enzyme is a tetramer composed of four apparently identical subunits.

When the native enzyme is resolved of its cofactor, pyridoxal 5'-monophosphate, by dialysis against 50 mm Tris-HCl buffer, pH 8.5, containing 1 mm EDTA and 5 mm dithiothreitol (23)
Determination of the molecular weight of the L-threonine deaminase subunit in sodium dodecyl sulfate gels. Standards of known molecular weight: bovine serum albumin, 68,000; catalase, 57,000; ovalbumin, 43,000; and hexokinase, 36,000.

Effect of pyridoxal 5'-monophosphate concentration on the rate of L-threonine deaminase reaction. Pyridoxal 5'-monophosphate was resolved from the purified enzyme as described in the text. The rate of α-ketobutyrate formation was determined using the colorimetric assay.

Catalytically inactive apodimers of molecular weight equal to 102,600 are formed (Fig. 2D). Addition of pyridoxal 5'-monophosphate to a solution of these apodimers promotes tetramer formation and restores catalytic activity.

Affinity of Enzyme for Pyridoxal 5'-Monophosphate—Purified threonine deaminase was resolved of pyridoxal 5'-monophosphate by dialysis against 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and 5 mM dithiothreitol. The rate of the L-threonine deaminase reaction was measured as a function of pyridoxal phosphate concentration (Fig. 4). The \( K_m \) of the enzyme for the cofactor as determined by this method is \( 7 \times 10^{-5} \) M.

Catalytic Properties of Purified Enzyme—The steady state kinetic properties of the biosynthetic L-threonine deaminase from E. coli K-12 do not differ significantly from data reported by others obtained from partially purified or crude extract enzyme preparations (4). Plots of initial velocity versus substrate concentration follow a normal adsorption isotherm (Fig. 5). In the presence of low L-isoleucine concentrations a sigmoidal curve indicating cooperativity is seen (Fig. 5). This cooperative effect is eliminated by L-valine (Fig. 6).

Effect of pH

Catalysis and L-Isoleucine Inhibition—The effect of pH on catalysis and inhibition by L-isoleucine was examined. Catalytic activity increases with increasing pH from pH 6.5 to 8.0 and plateaus at maximal values from pH 8.0 to 10.0 (Fig. 7). Inhibition of enzyme activity by L-isoleucine is greatest from pH 6.5 to 8.0, but from pH 8.0 to 10.0 the enzyme becomes progressively resistant to inhibition (Fig. 7).
FIG. 7. The effect of pH on the L-threonine deaminase reaction. The reaction mixture contained 100 mM potassium phosphate buffer adjusted to the indicated pH with HCl, 100 mM NH₄Cl, and 80 mM threonine. The colorimetric assay was used. ○, without L-isoleucine; ●, 1 mM L-isoleucine.

FIG. 8. Effect of pH on sedimentation rate of purified L-threonine deaminase in sucrose gradients (see text for details).

Molecular Weight—The sedimentation rate of L-threonine deaminase was examined in sucrose gradients at pH values from 6.5 to 10.0. The enzyme present in the sucrose gradient fractions was assayed in the standard pH 8.0 reaction mixture. At pH 7.5 or 8.5 the enzyme sediments as a single species with sedimentation coefficients of 9.6 and 7.3, respectively (24) (Fig. 8). At pH 8.0 the enzyme sediments with a major peak and a shoulder, with sedimentation coefficient values intermediate between those seen at pH 7.5 and 8.5 (Fig. 8). At pH 6.5 or 7.0 the enzyme sediments as a single peak coincident with that seen at pH 7.5, and at pH 9.0 the enzyme sediments as a single peak coincident with that seen at pH 8.5.

Since it has been determined previously that the native enzyme exists as a tetramer at pH 7.5 (Fig. 2A) it was presumed that the lightest molecular weight species observed in the sucrose gradient experiments was a dimer. In order to confirm this conclusion the molecular weight of the enzyme was determined in the analytical ultracentrifuge by sedimentation equilibrium at pH 8.5 employing the same buffer conditions as those used in the sucrose gradient experiments. Under these conditions the enzyme is indeed present in the dimer form and exhibits a molecular weight of 102,000. These pH effects are reversible; the dimer isolated from the pH 8.5 sucrose gradients (Fig. 8) is sensitive to inhibition by L-isoleucine when assayed at pH 8.0.

We considered the possibility that the dimer detected at high pH is catalytically inactive, and is converted to an active tetramer when assayed at pH 8.0 in a reaction mixture containing L-threonine. Accordingly, sucrose gradients were prepared in a pH 8.5 reaction mixture solution in order to determine the molecular weight of actively catalyzing L-threonine deaminase. Following the centrifugation the amount of product, α-ketobutyrate, present in the fractions was determined. It can be seen (Fig. 9) that the catalytically active species present at pH 8.5 sediments as the dimer. Therefore, the enzyme can exist in either of two distinct and catalytically active molecular weight species which differ in their sensitivity to L-isoleucine inhibition. The two forms of the enzyme are in rapid equilibrium and this equilibrium is markedly dependent upon pH.

DISCUSSION

The purification procedure described here permits the isolation of milligram quantities of pure biosynthetic L-threonine deaminase from E. coli K-12. These amounts of enzyme are sufficient for determining its physical properties and ascertaining its kinetic parameters. The enzyme requires both L-isoleucine and dithiothreitol for stability. As with the L-threonine deaminase from Salmonella typhimurium (25) β-mercaptoethanol is a poor substitute for dithiothreitol. The purified enzyme is catalytically indistinguishable from that present in crude extracts.

This study has revealed many similarities between the E. coli and S. typhimurium L-threonine deaminases. They appear to be similar in subunit composition, and both form catalytically inactive apodimers when pyridoxal 5'-monophosphate is resolved from the enzyme by alkaline dialysis (23). The enzymes exhibit no apparent catalytic differences with respect to L-isoleucine and L-valine binding, and for the influence of pH on catalysis and L-isoleucine inhibition (25). Our findings differ from an early report indicating that the E. coli K-12 L-threonine deaminase exhibits homotropic interactions with respect to its substrate in the absence of the inhibitor, L-isoleucine (4).

At low pH, the E. coli L-threonine deaminase is subject to inhibition by L-isoleucine and is present as a tetramer; at high pH the enzyme is resistant to inhibition by L-isoleucine and is...
present as a dimer. It is possible that the L-isoleucine sensitivity and the molecular weight interconversions are related in mechanism; i.e., the relative sensitivity to L-isoleucine inhibition at a given pH reflects the ratio of dimer to tetramer present. Alternatively, the pH effects may be exerted independently, but in parallel. The protonation of essential groups on the enzyme may influence inhibitor binding, as suggested for the S. typhimurium L-threonine deaminase (25). It has been shown with a number of multimeric proteins that high pH levels favor dissociation by increasing the repulsion between subunits (26).

The similarities observed between the \textit{E. coli} and \textit{S. typhimurium} L-threonine deaminases are not unexpected in view of the fact that these are very closely related bacterial species. However, the \textit{E. coli} enzyme seems to be slightly larger in molecular weight (204,000 versus 194,000), and is distinguishable antigenically.\footnote{R. O. Burns, personal communication.}

Our interest in the \textit{E. coli} enzyme stems from the fact that \textit{E. coli} presents a superior genetic system for testing current theories of gene regulation. Hatfield and Burns (27) proposed a model suggesting a repressor function for the immature form of the \textit{S. typhimurium} L-threonine deaminase in the regulation of the \textit{ile} operon. Pledger and Umbarger (28) have recently described a single mutation in \textit{E. coli} K-12 which simultaneously:

(a) renders L-threonine deaminase insensitive to its end product inhibitor, L-isoleucine, (b) alters the pattern of regulation of the enzymes of the isoleucine-valine biosynthetic pathway normally controlled by multivalent repression (29), and (c) results in the loss of the ability of the \textit{ile} C gene product, the acetohydroxy acid isomerase, to be induced by its substrates (30). These pleiotropic effects of a single mutation strongly support a central role for L-threonine deaminase in the mechanism of multivalent repression (27). However, this mutation has been mapped (28) at a site on the \textit{E. coli} chromosome which is separated from the structural gene for L-threonine deaminase by the operator locus of the \textit{ile} operon described in 1963 by Ramakrishnan and Adelberg (31). Therefore, Pledger and Umbarger (28) have entertained the idea that this mutation does not lie in the structural gene for L-threonine deaminase but marks the site of a new regulatory locus. Recent results from this laboratory have shown that the mutant L-threonine deaminase, purified by the procedure reported here, retains the feedback-resistant property exhibited by the enzyme in crude extracts. Consequently, the site of this pleiotropic mutation must reside in the structural gene for L-threonine deaminase. It follows, therefore, that the "operator" mutations originally described by Ramakrishnan and Adelberg (31) are also mutation sites within the structural gene for L-threonine deaminase.

Experiments are currently in progress in this laboratory, utilizing the purified preparations of the biosynthetic L-threonine deaminase from \textit{E. coli} K-12, to elucidate the role of L-threonine deaminase in the molecular mechanism of the multivalent repression of the genes involved in the biosynthetic pathways of the branched chain amino acids.

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