On the Mechanism of Activation of L-Threonine Deaminase from Clostridium tetanomorphum by Adenosine Diphosphate

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SUMMARY

In order to clarify the mechanism of activation of L-threonine deaminase by adenosine diphosphate, the enzyme was purified about 700-fold from sonic extracts of Clostridium tetanomorphum, and kinetic and ADP-binding studies were carried out. Plots of reaction rates against L-threonine concentrations gave a sigmoid curve in the absence of ADP, whereas a hyperbolic curve was obtained in the presence of $10^{-3}$ M ADP. Double reciprocal plots were parabolic in the former and linear in the latter case.

Different pH profiles of the apparent $K_m$ and maximal velocity were observed with and without ADP. A linear Arrhenius plot was obtained in the absence of ADP, whereas a discontinuity in the slope was found in the presence of ADP. Values of the activation energy were calculated to be 13.9 and 11.4 kcal per mole over the temperature range of 4-37°C in the absence and presence of ADP, respectively.

The Michaelis constant for ADP as an activator was $2.3 \times 10^{-4}$ M at a threonine concentration of $10^{-3}$ M. No mutual effect of ADP and threonine on $K_m$ and $K_a$ was observed. Adenosine triphosphate offset activation of the enzyme by ADP, and increased the sigmoid nature of the rate-substrate concentration curve.

D-Threonine and semicarbazine inhibited the deaminase activity competitively toward L-threonine both in the presence and in the absence of ADP. p-Chloromercuribenzoate inhibited the enzyme competitively toward L-threonine in the absence of ADP, but noncompetitively in the presence of ADP.

The dissociation constant for ADP was estimated by equilibrium dialysis, was $3.0 \times 10^{-3}$ M, which agreed reasonably well with the value obtained from reaction kinetics. D-Threonine did not interfere with the binding of ADP.

On the basis of kinetic analysis, two substrate sites are postulated. A catalytic site with a dissociation constant for substrate ($K_a$) of $3.5 \times 10^{-3}$ M, and an activating site with a $K_a$ of $3.3 \times 10^{-2}$ M.

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During the course of a study on the metabolism of L-threonine by cell-free extracts of Clostridium tetanomorphum, evidence was presented that adenosine diphosphate specifically stimulated the deamination of L-threonine to α-ketobutyrate by threonine deaminase (1). The effect of ADP, which was most marked at low concentrations of the substrate, was ascribed to a decrease in the apparent $K_m$ of the enzyme for the substrate rather than to direct participation of ADP in the reaction. Subsequent studies revealed that ADP was bound by the enzyme protein and protected it against inactivation by dilution or heat (2).

In a teleological sense, ADP appeared to be a metabolic regulator of the anaerobic energy production in this microorganism during the catabolism of L-threonine (3).

Recently, similar phenomena have been reported from a number of laboratories and are usually referred to as "allosteric effect" (4), in which the enzyme activity in a key position of cellular metabolism is controlled by specific metabolites or nucleotides. Two kinetic features have generally been noticeable with the enzymes which are activated by allosteric effectors: first, a sigmoid curve of rate of reaction as a function of substrate concentration; second a change in the apparent $K_m$ for the substrate in the presence of the effector. The threonine deaminase also showed these two features, as will be described below. Some interpretations which have been reported to explain these features were based on the marked difference between the oxygen saturation curve of hemoglobin and that of myoglobin. In all these interpretations, the decrease in $K_m$ values was considered as the increase in affinity of the enzyme for the substrate in a direct sense. A possibility arises, however, that the decrease in $K_m$ can also be achieved without changing the actual affinity for the substrate, especially for the enzymes that have kinetics of an unusual type.

In this paper, we describe the results of kinetic studies and binding experiments with ADP-14C in order to clarify the mechanism of stimulation of the threonine deaminase by ADP. The experimental evidence is consistent with the following interpretations: (a) threonine is bound to the enzyme protein at two sites, one "catalytic" and the other "activating"; (b) ADP may be bound at a site which is distinct from the two sites for threonine ("ADP site") and (c) ADP functions in a manner similar to threonine at the activating site in activating the deaminase.

Indeed, ADP may stimulate the enzyme reaction without increasing the affinity of the enzyme for the substrate in a direct sense.
EXPERIMENTAL PROCEDURE

Materials—L- and d-Threonine were obtained from Tanabe Amino Acid Research Foundation. ADP was a gift of Takeda Chemical Industries, and ATP was a product of Sigma. Sodium α-ketobutyrate was purchased from Mann; pyridoxal-P, from Calbiochem; and Sephadex G-25 and DEAE-Sephadex A-50, from Pharmacia. ADP-8-3C (25.8 μC per mmole) was obtained from Schwarz and was purified by column chromatography on Dowex 1 (Cl-). All other chemicals were of analytical grade.

Growth of Organism—C. tetanomorphum (ATCC 3606) was grown for 15 hours at 37°C in 5-liter Erlenmeyer flasks in 4 liters of glutamate medium (3) containing 0.5% yeast extract, 1.5% polypeptide, 0.5% sodium glutamate, 0.005% thiglycolic acid, and 0.25% K₂HPO₄. Cells were harvested with a Sharples centrifuge at room temperature and stored at -15°C.

Assay—The activity of threonine deaminase was determined spectrophotometrically by measuring the formation of α-keto butyrate as its 2,4-dinitrophenylhydrazone. The standard assay system contained 100 μmoles of L-threonine, 200 μmoles of Tris-HCl (pH 8.4), 2 μg of pyridoxal-P, and the enzyme in a total volume of 1.0 ml. The reaction mixture was incubated at 37°C for 10 min, and the reaction was terminated by the addition of 1.0 ml of 1 N HCl. The α-keto acid formed was determined as the 2,4-dinitrophenylhydrazone at 410 μm by a modification of the method of Katuki et al. (6) with a Shimadzu Bausch and Lomb Spectronic 20 spectrophotometer. One unit of enzyme was defined as that amount producing 1 μ mole of α-keto butyrate per min under the standard conditions. Specific activity was expressed as units per mg of protein. Protein was determined from the absorption at 280 and 260 nm (7) with a Shimadzu DU spectrophotometer. Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer, with the naphthalene-dioxane system as a scintillator (8).

Binding Studies—For the determination of an average dissociation constant of ADP, equilibrium dialysis was carried out as follows. Visking dialysis tubing was soaked for several days in distilled water, which was changed periodically. Dialysis cellophane was made by milling rectangular grooves (1 × 4 × 0.5 cm) in polyvinyl chloride plates (5 × 6 × 1 cm) and pressing cellophane membrane between pairs of such plates. A thin film of silicone grease around the edge of the cellophane prevented capillary loss of solvent. Each pair of cells was fastened with bolts and nuts to hold the two halves of the container tightly together. To one compartment of each cell was added 0.30 ml of the enzyme solution (79 units, 2.8 mg of protein) in 0.03 M potassium phosphate, pH 7.0, and the protein was eluted with the above buffer. The solution was dialyzed overnight against a large volume of 0.05 M buffer. To this suspension was added 0.20 ml of a saturated solution of ammonium sulfate at pH 7.0 with stirring over a 30-min period at 0°C, and then an aliquot of 0.20 ml was placed on a column of Sephadex G-25 (0.3 cm × 8 cm) equilibrated with 0.1 M potassium phosphate, pH 7.0, and the protein was eluted with the above buffer solution at room temperature. After the first 14 ml, 4-drop fractions (about 0.16 ml) were collected into counting vials.

RESULTS AND DISCUSSION

Enzyme Purification

All procedures were carried out at 0–4°C.

Crude Extracts—Frozen cells (127 g) were suspended in 250 ml of 0.05 M potassium phosphate, pH 7.0, and were disrupted by sonic oscillation at 10 kc for 15 min. In all subsequent steps, potassium phosphate, pH 7.0, was used, which henceforth will be referred to simply as “buffer.”

Protamine Treatment—To 400 ml of crude extract were added 12 g of protamine sulfate, previously suspended in 40 ml of 0.5 M buffer, adjusted to pH 7 with KOH. After the suspension was kept overnight, the precipitate was removed by centrifugation at 8000 × g for 20 min.

Acetone Treatment—Two volumes of acetone, which had been kept at -20°C, were added to the supernatant solution with stirring over a 30-min period at -15°C, and the resulting precipitate was collected by centrifugation at 10,000 × g for 10 min and suspended in 300 ml of 0.05 M buffer. To this suspension were added 210 ml of a saturated solution of ammonium sulfate at pH 7.0. After stirring for 20 min, the precipitate was discarded, and 300 ml of the saturated solution of ammonium sulfate added to the resultant supernatant solution. After 30 min, the precipitate obtained was dissolved in 50 ml of 0.05 M buffer. The solution was dialyzed overnight against a large volume of 0.05 M buffer.

Ammonium Sulfate Treatment—To 74 ml of the above dialyzed solution were added 60 ml of a saturated solution of ammonium sulfate at pH 7.0 with stirring. After 20 min, the precipitate...
was removed by centrifugation. To the supernatant solution were added 30 ml of the saturated solution of ammonium sulfate. After 20 min, the precipitate was collected by centrifugation at 10,000 g for 15 min and dissolved in 15 ml of 0.05 M buffer.

First Chromatography on DEAE-Sephadex Column—A column of DEAE-Sephadex A-50 (6.7 cm² × 17.5 cm) was prepared and equilibrated with 0.05 M buffer. Through the column were passed 100 ml of the ammonium sulfate fraction, which had been dialyzed overnight against 0.05 M buffer. The column was washed with 200 ml of 0.1 M and then with 200 ml of 0.15 M buffer. Protein was eluted with a linear concentration gradient of buffer, from 0.15 M to 0.50 M. The mixing chamber contained 500 ml of 0.15 M buffer, and the reservoir had 500 ml of 0.50 M buffer. The flow rate was 50 ml per hour, and 15-ml fractions were collected. The enzyme, which was found in the 315- to 435-ml effluent volume, was collected and dialyzed overnight against 1 liter of 0.03 M buffer.

Second Chromatography on DEAE-Sephadex Column—The dialyzed enzyme solution was applied to a column of DEAE-Sephadex A-50 (2.6 cm² × 11.6 cm) which had been equilibrated with 0.05 M buffer. Elution was carried out with a linear concentration gradient of NaCl solution in 0.05 M buffer, from 0.1 to 0.4 M (mixing chamber, 250 ml of 0.1 M NaCl in 0.05 M buffer; reservoir, 250 ml of 0.4 M NaCl in 0.05 M buffer). The flow rate was 80 ml per hour, and 12-ml fractions were collected. The enzyme in the 124- to 192-ml effluent volume was collected and dialyzed against 1 liter of 0.05 M buffer overnight.

Hydroxylapatite Column—The dialyzed enzyme solution of the second DEAE-Sephadex fraction was applied again to a small column of DEAE-Sephadex A-50 (bed volume of 1.2 ml) and was eluted with 0.5 M buffer. After dialysis against 500 ml of 0.05 M buffer for 4 hours, 4.5 ml of the concentrated enzyme solution were passed through a column of hydroxylapatite (2.8 cm² × 1.8 cm) which had been equilibrated with 0.05 M buffer. Protein was eluted stepwise with 9 ml each of 0.05 M, 0.075 M, 0.10 M, and 0.125 M buffer. The two tubes of maximal specific activity were pooled. The combined fraction had a concentration of 0.075 M buffer. Protein was eluted with 0.5 M, was eluted with 0.4 M, and was eluted with 0.35 M buffer. The mixing chamber contained 214.0 ml of 0.1 M buffer; from 0.1 to 0.5 M buffer. After dialysis against 500 ml of 0.05 M buffer overnight, 250 ml of 0.15 M buffer, 200 ml of 0.4 M buffer, and 200 ml of 0.15 M buffer. The flow rate was 50 ml per hour, and 15-ml fractions were collected. The enzyme in the 124- to 192-ml effluent volume was collected and dialyzed against 1 liter of 0.03 M buffer overnight.

Typical results of enzyme purification are shown in Table I. Because the most purified enzyme preparation could not be obtained in appreciable amounts, most of the kinetic experiments and all binding studies described in this paper were performed with the partially purified preparations, which had a specific activity of 12 to 27 units per mg of protein. With these preparations, no evidence for degradation of α-ketobutyrate, ADP, or ATP was found under our assay conditions. A few kinetic experiments with the most purified preparation gave results identical with those obtained with the preparations of lower specific activities.

### Kinetic Properties

**Effect of Threonine**—When the rate of α-ketobutyrate formation was determined as a function of L-threonine concentration, a sigmoid curve was obtained instead of the usual hyperbolic one (Fig. 1). The rate of product formation was essentially zero until L-threonine concentration was raised to 10⁻³ M. Thereafter the rate increased slowly, with a half-maximal velocity at 3.7 × 10⁻² M L-threonine and a maximal velocity of 18.2 μmoles per min per mg of protein. When 10⁻³ M ADP was present in the reaction mixture, however, a hyperbolic curve was obtained, with a Kₘ of 3.5 × 10⁻⁴ M and a maximal velocity of 21.6 μmoles per min per mg of protein.

In the absence of ADP, a sigmoid curve was also observed at pH 7.1, 8.1, and 9.1, whereas a hyperbolic one was observed at pH 6.3 and 9.8. When L-serine, which could be utilized slightly by this enzyme, was used as the substrate instead of L-threonine, a sigmoid curve was also obtained, with an apparent Kₘ for L-serine of 3 × 10⁻¹ M. Again, in the presence of ADP, a normal curve was observed, with a Kₘ for L-serine of 2.2 × 10⁻³ M. EDTA at 10⁻⁴ M concentration had no effect on the relationships among rate, substrate, and activator. In addition, the rate, although normally measured by a single assay at 10 min, was shown to proceed linearly for 20 min, even at low concentrations of L-threonine. In view of these results, the sigmoid phenomenon does not appear to be due to the metal inhibitor or the instability of the enzyme at low concentrations of the substrate, but to the inherent nature of this enzyme.

**Curvature of Lineweaver-Burk Plot**—Double reciprocal plots showed a straight line in the presence of ADP, whereas a downward convex curve was observed in its absence (Fig. 2).

![Fig. 1. The rate of threonine deaminase reaction as a function of L-threonine concentration in the absence (O—O) and presence (—O) of 10⁻⁴ M ADP. The enzyme activity was determined with the standard assay system except that varying concentrations of L-threonine were used. The rate is expressed in micromoles of α-ketobutyrate formed per min per ml of reaction mixture with 10.7 μg of protein (specific activity, 13).](http://www.jbc.org/content/242/6/1141/F1.large.jpg)
reciprocal of the reaction rate was plotted against the square of the reciprocal of L-threonine concentration, the data followed a straight line at lower concentrations of threonine but an upward convex curve was obtained at high concentrations of threonine (Fig. 2). When another plot was employed, namely \( \frac{[V_{\text{max}}/v] - 1}{S} \) against \( 1/(S)^2 \) (where \( v \) represents reaction rate; \( V_{\text{max}} \), maximal velocity; and \( S \), substrate), a straight line was obtained with the concentrations of substrate examined (Fig. 3). Thus, the curved Lineweaver-Burk plot of Fig. 2 can be represented by Equation 2, which may suggest that there are two binding sites for substrate on the enzyme protein.

\[
\frac{1}{v} = 5.3 + \frac{1.95 \times 10^{-1}}{(S)} + 6.3 \times 10^{-1} \frac{1}{(S)^2}
\]  

\( (2) \)

Effect of pH on \( K_m \) and \( V_{\text{max}} \)—In order to obtain some information about the ionizing groups of the enzyme or the enzyme-substrate complex (10), the apparent values of \(-\log K_m\) and \(\log V_{\text{max}}\) at various pH values were determined, and are summarized in Fig. 4. \( K_m \) values vary with pH, as previously observed by Davis and Metzler with sheep liver threonine deaminase (11). These workers suggested that the uncharged amino group of the substrate anion combines with the enzyme, and that the free enzyme is reversibly converted to an inactive form by the loss of protons around pH 9.1 at 37°. Different pH profiles of maximal velocity were obtained with and without ADP, which may suggest that ADP influences the ionization of groups in the enzyme-substrate complex.

Effect of Temperature—The effect of temperature on the maximal velocity at pH 8.4 in the presence and absence of ADP is shown in Fig. 5. In the absence of ADP, a linear Arrhenius plot.

![Fig. 2. Lineweaver-Burk plots for L-threonine in the absence (—) and presence (O—O) of 10^{-5} M ADP. The data of Fig. 1 are plotted. Another plot, in which the reciprocal of the rate is related to the reciprocal of the square of L-threonine concentration, is also shown (X---X).](http://www.jbc.org/)

![Fig. 3. The rate of threonine deaminase reaction as a function of L-threonine concentration (S) in the absence of ADP. ([V_{\text{max}}/v] - 1)/(S) was plotted against 1/(S) from the results presented in Fig. 1. The \( V_{\text{max}} \) was obtained from the extrapolation of 1/v at high substrate concentrations in Fig. 2.](http://www.jbc.org/)

![Fig. 4. Effect of pH on the logarithm of the maximal velocity and the negative logarithm of the apparent \( K_m \) for L-threonine. The \( V_{\text{max}} \) and apparent \( K_m \) were calculated from Lineweaver-Burk plots in the absence (—) and presence (O—O) of ADP. The following concentrations of ADP were employed: 10^{-5} M at pH 8.4 and 9.1; 5 \times 10^{-2} M at pH 7.1; 10^{-4} M at pH 6.3, 9.5, and 9.8; 10^{-1} M at pH 10.4. The enzyme protein used was 10.7 \( \mu \)g (specific activity, 13).](http://www.jbc.org/)

![Fig. 5. Effect of temperature on the maximal velocity in the presence of 10^{-5} M ADP (O—O) and in its absence (—). Maximal velocity was obtained from a Lineweaver-Burk plot. The rate of product formation proceeded linearly with time over the temperature range tested. The enzyme protein used was 10.7 \( \mu \)g (specific activity, 13).](http://www.jbc.org/)
was found over the temperature range of 4-45°, and the calculated activation energy was 13.9 kcal per mole. It is interesting that, in the presence of ADP, a new slope was established above 37°. The energy of activation was calculated to be 11.4 kcal per mole over the temperature interval of 4-37°, and 5.5 kcal per mole in the temperature range of 37-45°. Thus, lowering the activation energy is one of the unique features of the activation of this enzyme by ADP. The $K_m$ value at 37° was about one-tenth as much as that at 4° in the absence of ADP. It was, however, one-half in the presence of ADP.

**Effect of ADP**—When the reaction rate was determined as a function of ADP concentration at a fixed threonine level ($10^{-3} M$), a curve of the Michaelis-Menten type was obtained, as shown in Fig. 6. The Michaelis constant of activation ($K_a$) was estimated to be $2.3 \times 10^{-5} M$ from this plot. In addition, plots of the reciprocal of the difference between reaction rates in the presence and absence of ADP against the reciprocal of ADP concentration ($12$) showed a bimodal character that yielded two $K_a$ values at higher concentrations of threonine (Fig. 7B). Below $10^{-2} M$ threonine, the bimodality was not observed (Fig. 7A). The bimodality was also observed at pH 7.1, 8.1, and 9.1.

**Mutual Effect of ADP and Threonine on $K_m$ and $K_a$**—With some other allosteric enzymes, the $K_m$ for the substrate decreases as the concentration of the added allosteric effector increases; conversely, an increase in the substrate concentration causes a gradual decrease in the $K_m$ for the allosteric effector (13, 14). In the present case, such a mutual effect could not be observed. The apparent $K_m$ value for threonine was essentially unaffected with ADP concentrations above $10^{-8} M$, as shown in Table II; however, the maximal velocity increased slightly from 15.2 to 22.7 pmoles per min per mg of protein. The Michaelis constant of activation ($K_a$) was also determined at varying concentrations of threonine and is summarized in Table III. The constants at high ADP concentrations ($K_{a2}$) were almost unchanged at various concentrations of threonine, although those at low ADP concentrations ($K_{a1}$) were dependent on the concentration of threonine.

**Inhibition Studies**—The enzyme activity was inhibited by a number of compounds (Table IV). ADP did not protect the enzyme from inhibition by these compounds. The mode of inhibition was also tested with D-threonine as a substrate analogue;
Fig. 8. Lineweaver-Burk plots for L-threonine in the presence of $5 \times 10^{-3}$ M d-threonine (■ — ■), in its absence (● — ●), in the presence of $5 \times 10^{-2}$ M d-threonine and $10^{-4}$ M ADP (T — T), and in the presence of $5 \times 10^{-3}$ M CMB and $10^{-2}$ M ADP (Δ — Δ) and $10^{-3}$ M ADP (O — O). Velocity is expressed in micromoles of α-ketobutyrate formed per min per ml of reaction mixture, which contained 2.1 μg of protein (specific activity, 13). As higher concentrations of L-threonine were used, straight lines were obtained by double reciprocal plots.

Effect of ATP on Reaction Kinetics—The sigmoid nature of the rate-substrate curve became more pronounced in the presence of ATP. At low concentrations of L-threonine the activity was lower in the presence of ATP than in its absence, whereas at high levels of substrate the activity was about 2 times greater in the presence of ATP (Fig. 10). ATP was found to offset activation of the enzyme by ADP, with an inhibition constant of $7 \times 10^{-4}$ M, when the enzyme activity was measured at $10^{-3}$ M threonine.

Table IV

| Inhibition experiments | Concentration | Relative activity*
<table>
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<tr>
<td>Reactant added</td>
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</tr>
<tr>
<td>d-Threonine</td>
<td>$5 \times 10^{-3}$</td>
<td>0.85</td>
</tr>
<tr>
<td>CMB</td>
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<td>0.55</td>
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<tr>
<td>p-Chloromercuriphenylsulfate</td>
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<td>Mersalyl acid</td>
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<td>HgCl₂</td>
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<td>Iodoacetic acid</td>
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<tr>
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<td>NH₄Cl</td>
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</table>

* Enzyme activity in the presence of the inhibitor relative to that in its absence. Standard assay conditions were used, with 2.1 μg of protein per tube.

Binding of ADP

Effect of ADP—The binding of ADP by the partially purified enzyme preparation has been reported (2). To rule out the possibility that ADP was bound by some protein other than the deaminase, the dissociation constant for ADP was measured by the method described in “Experimental Procedure.” From equilibrium dialysis, an average dissociation constant of the enzyme-ADP complex was calculated to be $3.0 \times 10^{-3}$ M (Fig. 11), which agrees reasonably well with the $K_a$ value of $2 \times 10^{-3}$ M obtained from reaction kinetics.² The above results indicate that without ADP, and an apparent $K_i$ of $2.3 \times 10^{-3}$ M with ADP, respectively. Hydroxylamine, a carbonyl reagent, also showed competitive inhibition.

CMB inhibited the deaminase strongly in a reversible manner. It was effective at a concentration as low as $10^{-4}$ M, its effect was not increased by preincubulation for 1 hour, and its inhibition was completely and instantaneously reversed by adding $10^{-4}$ M β-mercaptoethanol to the reaction mixture. As shown in Fig. 9, the effect of CMB was competitive with respect to L-threonine in the absence of ADP, with an apparent $K_i$ of $4 \times 10^{-3}$ M, whereas it was noncompetitive in the presence of ADP. The activation constant for ADP was not altered by the presence of $5 \times 10^{-3}$ M CMB. The interpretation of these findings will be discussed below.

Effect of CMB on Reaction Kinetics—CMB, as an —SH inhibitor; and semicarbazide, as a carbonyl reagent which might attack the carbonyl group of pyridoxal-P of the enzyme.

Fig. 9. Lineweaver-Burk plots for L-threonine in the presence of $10^{-3}$ M CMB (■ — ■), $5 \times 10^{-3}$ M CMB (Δ — Δ), and no CMB (● — ●), and in the presence of $5 \times 10^{-3}$ M CMB and $10^{-2}$ M ADP (Δ — Δ) and $10^{-3}$ M ADP (O — O). Velocity is expressed in micromoles of α-ketobutyrate formed per min per ml of the reaction mixture, which contained 2.1 μg of protein (specific activity, 13). As higher concentrations of L-threonine were used, straight lines were obtained by double reciprocal plots.

1 The abbreviation used is: CMB, p-chloromercuribenzoate.
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ADP is actually bound by the deaminase, although they do not unequivocally rule out a possibility that ADP is bound by some other protein with very similar dissociation constant for ADP.

Effect of Inhibitors—As the structure of ADP is distinct from that of threonine, it is hardly conceivable that the two compounds are bound at the same site. However, in order to obtain direct evidence, the effect of competitive inhibitors against L-threonine on the binding of ADP was investigated by the use of gel filtration.

Even when the competitive inhibitors such as n-threonine, CMB, semicarbazide, and hydroxylamine were included in the incubation mixture at concentrations well over the apparent $K_i$ values, the binding of ADP by the enzyme protein was not affected, as shown in Table V. On the other hand, $10^{-4}$ M ATP, which counteracted the activation by ADP, completely prevented the binding of ADP to the enzyme.

General Discussion

Kinetics in Absence of ADP—A concave curve of a Lineweaver-Burk plot is a characteristic feature of this threonine deaminase. A similar curvature was reported in the biosynthetic threonine deaminase from Escherichia coli (16), NAD-specific isocitrate dehydrogenase from yeast (15), and from Neurospora (17), thymidine kinase from E. coli (18), and aspartate transcarbamylase from E. coli (19). However, the biodegradative threonine deaminase from E. coli (14, 20) and that from animal tissue (11) gave a classical linear plot at all substrate concentrations tested.

A well-known example of the sigmoid curve is the oxygen saturation curve of hemoglobin, as already cited. Although hemoglobin is not an enzyme, several allosteric enzymes have been compared with it. For example, aspartate transcarbamylase of E. coli (19) was presumed to have several homologous active sites, but the attachment of 1 molecule of substrate to one site modifies the affinity of a different site for the second molecule of substrate. In those cases, the reaction obeys Hill's empirical equation for hemoglobin,

$$v = \frac{V_{\text{max}}(S)^n}{K_v + (S)^n}$$

and a plot of $\log [v/(V_{\text{max}} - v)]$ against $\log (S)$ (Hill's plot) gives a straight line with a slope of $n$, a parameter which is closely related to the average free energy of interaction of sites (21).

From Hill's plot for the clostridial deaminase, a value of 1.1 for $n$ was obtained as shown in Fig. 12. Since other allosteric enzymes, such as threonine deaminase and aspartate transcarbamylase of E. coli, have $n$ values between 1.4 and 2.8, the cooperative interactions between substrate sites are much weaker in this case than in other allosteric enzymes. A theoretical curve obtained from Equation 2 is also shown in Fig. 12, which shows that our data agree reasonably well with the theoretical curve.

The concave curve of a Lineweaver-Burk plot might also result from a substrate acting as an activator (22, 23). In the present case, the assumption was made that a single substrate could be bound to two distinct sites of the enzyme: one, the catalytically active site; the other, the activating site. Unless substrate was bound both at the activating and at the catalytic sites of the enzyme, the substrate at the catalytic site would not be degraded at all or perhaps would be broken down at a very slow rate.
The following equation can be derived.

\[
\begin{align*}
E + S &\rightleftharpoons ES_1, \quad K_1 \\
E + S &\rightleftharpoons ES_2, \quad K_2 \\
ES_1 + S &\rightleftharpoons ES_3, \quad \alpha K_1 \\
ES_2 + S &\rightleftharpoons ES_4, \quad \alpha K_1 \\
ES_4 & \rightarrow ES_3 + product
\end{align*}
\]

where \(ES_4\) represents the enzyme, with the catalytic site occupied by the substrate; \(ES_1\), the enzyme, with the activating site occupied by the substrate; and \(ES_3\), the activated form of the enzyme, which has sites occupied by substrate molecules. \(K_1\) and \(K_2\) are the dissociation constants for activating and catalytic sites, respectively, and \(\alpha\) characterizes any change in the dissociation constant by binding of one substrate molecule on the other site. The term \(k\) is the rate constant for the breakdown of \(ES_3\) to \(ES_1\) and product, which is assumed to be the rate-limiting step in the overall reaction. By assuming equilibrium conditions, the reciprocal rate expression is

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{\alpha(K_1 + K_2)}{(S)} + \frac{\alpha K_1 K_2}{(S)^2}
\]

which may be rearranged as

\[
\frac{V_{\text{max}}}{v} - 1 = \frac{\alpha K_1 + K_2}{(S)} + \frac{\alpha K_1 K_2}{(S)^2}
\]

If \([(V_{\text{max}}/v) - 1] (S)\) is plotted against \(1/(S)\), a straight line with the slope of \(\alpha K_1 + K_2\) will be obtained, and the intercept with the ordinate will be \(\alpha(K_1 + K_2)\), which is the apparent \(K_m\) obtained from a Lineweaver-Burk plot. As was shown in Fig. 3, a straight line was obtained in such a plot with an intercept of \(3.7 \times 10^{-3}\) M, which agrees well with the apparent \(K_m\) obtained by a Lineweaver-Burk plot (Fig. 2).

Assuming \(\alpha = 1\), \(K_1\) and \(K_2\) are calculated from the experimental data to be \(3.3 \times 10^{-3}\) M and \(3.5 \times 10^{-4}\) M, respectively.

**Kinetics in Presence of ADP**—In the presence of \(10^{-3}\) M ADP, normal kinetics was obtained with a \(K_m\) value for threonine of about one-tenth of that in the absence of ADP (Fig. 2). The binding experiments revealed that ADP was bound at a site distinct from the site for threonine. In order to simplify the calculations, reactions which were conducted under the following conditions only were analyzed. Since the dissociation constant \((K_4)\) for the substrate is \(3.3 \times 10^{-2}\) M, and the \(K_a\) for ADP is about \(2 \times 10^{-4}\) M, reactions conducted with threonine concentrations up to \(10^{-2}\) M and \(10^{-3}\) M ADP should be activated predominantly by ADP. Under these conditions, presumably only a small portion of the enzyme has both catalytic and activating sites occupied by threonine. Thus the mutual interaction between ADP and threonine sites need not be considered under these conditions, and the following sequence can be formulated.

\[
\begin{align*}
E + A &\rightleftharpoons EA_1, \quad K_4 \\
E + S &\rightleftharpoons ES_2, \quad K_2 \\
EA + S &\rightleftharpoons EAS_2, \quad K_4 \\
ES_2 + A &\rightlefth矛on 2, \quad K_4 \\
k' & EAS_2 \rightarrow EA + product
\end{align*}
\]

where \(K_a\) is the dissociation constant for ADP, and \(k'\) is the rate constant for the breakdown of \(EAS_2\), which is considered to be the rate-limiting step of the overall reaction. ADP is assumed to act like threonine, which is bound at the activating site. Since the rate of product formation from \(EAS_2\) is unquestionably slower than that of the formation from \(EAS_2\), the binding of ADP to the enzyme might be considered of major importance in allowing product formation. The reciprocal velocity expression is

\[
\frac{V_{\text{max}}}{v} = \left[1 + \frac{K_a}{(A)}\right] \left[1 + \frac{K_4}{(S)}\right]
\]

Thus the double reciprocal plot of \(1/v\) against \(1/(S)\) should be a straight line, and \(K_m\) might equal \(K_4\). With increasing concentrations of threonine up to \(10^{-2}\) M, the \(K_4\) value was estimated to be \(3.5 \times 10^{-3}\) M, which agrees well with the \(K_4\) value calculated from the results in the absence of ADP.

In the case of allosteric activation, the decrease in the \(K_m\) of the substrate has been attributed to an increase of affinity, which is due to an effector-induced conformational change in the enzyme protein. However, in our case, it is more attractive to assume that ADP and threonine at the activating site modify
the conformation of the enzyme protein and thereby allow the enzyme-substrate complex to break down more rapidly. The flexibility in enzyme proteins has been predicted by Koshland (24).

At concentrations of threonine up to $10^{-2} \text{m}$ in the absence of ADP, the catalytic site, which has a dissociation constant of the order of $10^{-4} \text{m}$, will first be filled. However, substrate breakdown is negligible because the activating site, which has a dissociation constant of about $10^{-2} \text{m}$, will not yet be occupied by threonine. Above $10^{-2} \text{m}$ threonine, the enzyme activity appears normally, as the activating site is beginning to be saturated. Because ADP is bound with the dissociation constant of the order of $10^{-6} \text{m}$, the enzyme is fully activated in the presence of $10^{-2} \text{m}$ ADP. Therefore, even at low concentrations of threonine, the rate of the reaction follows typical Michaelis-Menten kinetics, with a $K_m$ of the same value as $K_a$. As the $K_i$ value is one-tenth of $K_1$, the apparent $K_m$ value should decrease in the presence of ADP to one-tenth of its prior value. By this mechanism, therefore, the binding of the effector does not change the actual affinity of the enzyme for the substrate, although the $K_m$ may vary.

Allosteric Site—The following findings indicate that the allosteric site is distinct from the substrate sites: (a) mutual interaction was not observed between allosteric and substrate sites; (b) although D-threonine is considered from kinetic analysis to be bound at both substrate sites, it did not interfere with the binding of ADP; (c) CMB inhibited the enzyme competitively toward D-threonine in the absence of ADP, but noncompetitively in the presence of ADP (Fig. 9). If CMB were bound only at the substrate site, the activating site, or if it cut off the process of activation by binding of ADP, the catalytic site, which has a dissociation constant of about $10^{-6} \text{m}$, will not yet be occupied by threonine. Because ADP is bound with the dissociation constant of the order of $10^{-6} \text{m}$, the enzyme is fully activated in the presence of $10^{-2} \text{m}$ ADP. Therefore, even at low concentrations of threonine, the rate of the reaction follows typical Michaelis-Menten kinetics, with a $K_m$ of the same value as $K_a$. As the $K_i$ value is one-tenth of $K_1$, the apparent $K_m$ value should decrease in the presence of ADP to one-tenth of its prior value. By this mechanism, therefore, the binding of the effector does not change the actual affinity of the enzyme for the substrate, although the $K_m$ may vary.

Estimation of the dissociation constant ($K_a$) for ADP from kinetic data is often difficult. As described above we used a plot of the reciprocal of the difference in the reaction rate in the presence and absence of ADP against the reciprocal of ADP concentration. With low levels of L-threonine, $K_a$ can be obtained from such a plot. However, at a high concentration of L-threonine, another plot will be preferable.

When Equation 6 is subtracted from Equation 4, assuming $\alpha = 1$, the following equation can be obtained.

$$\frac{V_{\text{max}}}{v_0} - \frac{V_{\text{max}}}{v_a} = \frac{1 + K_a}{K_a} - \frac{1}{K_a} \left(\frac{S}{S} - \frac{A}{A}\right)$$

If the difference of $V_{\text{max}}/v$ in the absence and presence of ADP is plotted against $1/(ADP)$, a straight line with a slope of $-\frac{K_a}{1 + K_a/S}$ will be obtained. The intercept of the abscissa is $K_1/K_a(S)$. As $K_a, K_b$, and $(S)$ are known values, $K_a$ can be calculated from the experimental data. With this plot, almost the same values were obtained as those calculated from the above plot (Fig. 13 and Table II). Bimodality was also observed in this plot. The fact that the activation constant estimated from this kinetic treatment agrees reasonably well with that from the binding study suggests that above kinetic treatment is plausible.

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