The Allosteric Threonine Deaminase of *Salmonella*. Kinetic Model for the Native Enzyme*

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ABSTRACT: Using a sensitive spectrophotometric assay it is demonstrated that the initial velocity data obtained with the allosteric threonine deaminase of *Salmonella typhimurium* is compatible with normal Michaelis–Menten kinetics. Product inhibition patterns show that there is an ordered liberation of products, α-ketobutyrate first, followed by ammonia. Allothreonine inhibits the enzyme competitively, perhaps by binding at the threonine site. The feedback inhibitor, L-isoleucine, produces complex effects on the slopes and intercepts of the double reciprocal plots when threonine is the varied substrate. At concentrations of isoleucine higher than 0.02 mM, the double reciprocal plots become curved.

The complex kinetics in the presence of isoleucine is considered to be very likely due to the inhibitor binding at an allosteric site and causing partial inhibition. The inhibition by isoleucine is partly relieved in the presence of L-norleucine, although by itself norleucine does not show any activating effect.

Ever since the early enzymological work of Umbarger (1956) and Yates and Pardee (1956) on regulatory or allosteric enzymes, a large body of information has accumulated on the kinetics of allosteric effects. While the physiological necessity for the presence of allosteric enzymes in cells of various kinds is immediately clear and has been greatly elaborated upon by various authors (Monod *et al.*, 1963; Umbarger, 1961, 1964), the mechanism of action of these enzymes is not very clear. The popular hypothesis at the moment is that of "subunit interactions," which has been discussed at length by Monod and co-workers (Changeux, 1964; Monod *et al.*, 1965). The essence of this hypothesis is that the allosteric proteins can exist in two (or more) reversible conformational states ("tight" and "relaxed"). Since the affinity of the substrate for the chemically identical binding sites in each of the two conformational states (K system) is assumed to be different, the model of Monod *et al.* (1965) provides an explanation for the sigmoid plots of initial velocity data frequently encountered with many allosteric enzymes (see Monod *et al.*, 1965). Further, the model also provides for the substrate–modifier interactions on the enzyme, and one of the important predictions in this regard is that in those allosteric enzymes, where the modifier affects the Michaelis constant of the substrate (K system of Monod *et al.*, 1965), the latter must exhibit "homotropic" interactions, *i.e.*, yield sigmoid plots of rate *vs.* concentration data in the absence of modifiers (provided that a major fraction (>90%) of the enzyme exists in the "T" form). Also, the effect of allosteric inhibitors would be, on the basis of this model, to increase the sigmoidity of the initial velocity plots, and the effect of activators would be to convert the plots to a rectangular hyperbola.

While the model of Monod *et al.* (1965) seems to fit observations made on deoxycytidine deaminase of animals (Scarano, 1964), aspartate transcarbamylase of *E. coli* (Gerhart and Pardee, 1964), deoxythymidine kinase (Okazaki and Kornberg, 1964) of *E. coli*, and some other enzymes (see Monod *et al.*, 1965), three lines of evidence suggest that it may not have universal applicability to allosteric enzymes. First, Atkinson *et al.* (1965) have pointed out that the substrate–substrate interactions (measured by Hill plots) of phosphofructokinase from liver fluke (Mansour and Mansour, 1962) and rabbit muscle (Passonneau and Lowry, 1962) and diphosphopyridine nucleotide (DPN)[1]-specific isocitrate dehydrogenase from yeast (Hathaway and Atkinson, 1963) and rat muscle are not affected by positive or negative modifiers. Second, using steady-state kinetic theory, Sanwal *et al.* (1964, 1965) and Chan *et al.* (1965) have shown that their kinetic data can very simply be interpreted on the basis of the presence of two different substrate binding sites (one regulatory or allosteric, and another active) on the surface of DPN-specific isocitrate dehydrogenase of *Neurospora* and *Aspergillus*. Third, a class of allosteric enzymes (other than "V" systems of Monod *et al.* (1965)) exists which shows the substrate–modifier interactions ("heterotropic" interactions), but in which substrate–substrate interactions ("homotropic" interactions) seem nonexistent. These enzymes are, glycogen synthetase (Traut and Lipmann, 1963), uridine diphosphate (UDP) D-

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[1] Abbreviations used: DPN, diphosphopyridine nucleotide; $DPNH_2$, reduced DPN; UDP, uridine diphosphate; TDP, thymidine diphosphate; CoA, coenzyme A.

glucose dehydrogenase (Neufeld and Hall, 1965), phosphorylase b (Madsen, 1964), thymidine diphosphate D-glucose pyrophosphorylase (Melo and Glaser, 1965), and L-glutamine D-fructose 6-phosphate transaminase (Kornfeld *et al.*, 1964).

For reasons of conformity, it is tempting to suggest that the seeming absence of homotropic substrate interactions in these "exceptional" enzymes may be due to several causes, some of the important ones being (1) that the enzyme preparations were wholly or partially "desensitized" during purification, or (2) that the assay methods were not sensitive enough to measure initial velocities in the critical ranges (where the substrate concentrations approached zero or infinity). On the other hand, it may very well be that the subunit interaction hypothesis is not applicable to these enzymes, and alternate plausible mechanisms must be looked into.

We describe below some observations of the "native" allosteric L-threonine deaminase of *Salmonella typhimurium*, and present a kinetic model in accord with the data. This model may also be applicable to some of the enzymes mentioned earlier.

Experimental Section

Organisms. The strains of *Salmonella typhimurium* used were: 447, in which the entire leucine operon was deleted and required leucine for growth; *C-19*, *ara 9*, which was constitutively derepressed for the enzymes of isoleucine–valine and leucine pathways; *leu* A_{124}, *ara 9*, which required leucine and lacked the α-keto-isovalerate–acetyl coenzyme A (CoA) condensing enzyme.

Media and Growth Conditions. The minimal medium contained in 1 l.: K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; sodium citrate·$5H_2O$, 0.47 g; $MgSO_4$ (anhydrous), 0.05 g; $(NH_4)_2SO_4$, 1.0 g. Glucose was added to this medium after autoclaving to a final concentration of 0.5% (except for strain *C-19* for which only 0.3% glucose was used). To derepress threonine deaminase in accordance to the principles of multivalent repression (Freundlich *et al.*, 1962) in strains *447* and *leu* A_{124}, a supplement of L-isoleucine, L-valine, and L-leucine was added to the minimal medium in the proportion 7:100:50 (μg/ml). The cells were grown with vigorous aeration for 14 hr at 30°.

Preparation of "Native" Threonine Deaminase. PREPARATION I. All operations were performed at 2–4° unless stated otherwise. Maximally derepressed cells (approximately 10-fold increase in threonine deaminase activity, compared to levels under repressed conditions) were harvested in a refrigerated centrifuge at 2°. The cells were washed once with 0.15 M NaCl and suspended in 0.25 M potassium phosphate buffer, pH 8.1, containing 0.05 mg/ml of pyridoxal phosphate, 10^{-4} M EDTA, 10^{-3} M β-mercaptoethanol, and 50 μg/ml of L-isoleucine (in accordance with the observations of Freundlich and Umbarger (1963) and Changeux (1963), isoleucine stabilized the enzyme). The suspension was sonicated for 4–5 min, and then centrifuged at 50,000 rpm in a Spinco ultracentrifuge for 90 min. After discarding the precipitate, the clear supernatant was fractionated by a cold saturated solution of ammonium sulfate (pH 8.0), containing 10^{-3} M β-mercaptoethanol. The precipitate appearing at 0.38 saturation was discarded, and that appearing between 0.38 and 0.48 saturation was dissolved in a small volume of 1.0 M phosphate buffer, pH 8.1, containing 10^{-4} M EDTA, 10^{-3} M β-mercaptoethanol, and 10 μg/ml of pyridoxal phosphate. To remove $(NH_4)_2SO_4$, the enzyme was dialyzed against frequent changes of the same buffer for 3 hr, and used immediately for kinetic experiments utilizing a coupled spectrophotometric assay. The ammonium sulfate precipitation step was necessary to rid the extracts of a strong $DPNH_2$ oxidizing activity. A purification of approximately 3-fold over the derepressed extracts was achieved by the above procedure. When it is considered, however, that the derepressed extracts have a specific activity about 10-fold higher than that of the normal cells, preparation I represents an over-all purification of about 30-fold.

PREPARATION II. For the colorimetric assay of threonine deaminase, and to compare data obtained with coupled spectrophotometric assay, a crude enzyme preparation was made essentially in the same way as preparation I, except that isoleucine was omitted from the suspension mixture used for sonication and the preparation was dialyzed after ultracentrifugation against 0.50 M phosphate buffer, pH 8.0, containing 10^{-4} M EDTA, 10^{-3} M β-mercaptoethanol, and 10 μg/ml of pyridoxal phosphate for 3 hr.

Assay of Threonine Deaminase. ASSAY 1. In this assay α-ketobutyrate was measured by the procedure of Friedemann and Haugen (1943), as modified by Sayre and Greenberg (1956). The final volume of the reaction mixture was 3.0 ml, and contained enzyme, 0.25 M phosphate buffer, pH 8.1, 10^{-4} M EDTA, 5 μg of pyridoxal phosphate, and various concentrations of L-threonine. The reaction time was 12 min and temperature was 30°. The reaction was terminated by adding trichloroacetic acid to a final volume of 5%. Suitable aliquots were then analyzed for α-ketobutyrate.

ASSAY 2. In this procedure, a coupled assay using rabbit muscle lactic dehydrogenase and $DPNH_2$ was utilized. Only enzyme preparation I was used in this assay because fractions other than this contained a strong $DPNH_2$ oxidizing activity. The assay mixture (pH 8.1) contained L-threonine, 10^{-4} M EDTA, 1 μg of pyridoxal phosphate, 0.1 mM freshly prepared $DPNH_2$, 0.4 mg of crystalline lactic dehydrogenase, 0.25 M potassium phosphate, and suitably diluted enzyme. Total volume of the reaction mixture was 3.0 and the temperature was 24–25°. Silica cuvets of 10-mm light path were used, and the oxidation of $DPNH_2$ was measured at 340 mμ in a Gilford Model 2000 recording spectrophotometer. At low substrate concentrations, the full-scale sensitivity of the recorder was selected between 0.1 and 0.2 absorbance unit. The velocity of the reaction was measured for 2 min (linear range) and is defined as the change in absorbance units per minute. To check any denaturation of the enzyme during ex-

periments, a standard reaction mixture containing 13.33 mM L-threonine was used. No loss in activity occurred at 0° for at least 3 hr under our experimental conditions.

ASSAY 3. The reaction mixture and conditions for this assay were the same as assay 1, except that the reaction was stopped by adding HCl to a final concentration of 0.1 N. Aliquots were then analyzed for ammonia by microdiffusion in Conway cells. The central well contained 1.5 ml of 0.01 N HCl. After diffusion, the contents of the central well were assayed for NH_3 by the sensitive colorimetric method of Crowther and Large (1956).

Reagents. Reduced DPN was a product of Boehringer Mannheim Corp., New York, N. Y. L-Threonine, L-norleucine, and L-valine were purchased from Sigma Chemical Co. They were checked for purity by paper chromatography. Crystalline rabbit muscle lactic dehydrogenase, obtained from Sigma, was extensively dialyzed before use to rid the preparation of $(NH_4)_2SO_4$. One milligram of the dialyzed enzyme converted about 100 μmoles of $DPNH_2$ to DPN per min at pH 8.0 in the presence of saturating amounts of α-ketobutyrate.

Kinetic Analysis. All kinetic data were processed according to Cleland (1963a) using an IBM 1620 digital computer. The raw data in duplicate or triplicate were first plotted in the reciprocal form to check unusual deviations from linearity. To avoid bias, least-square fits of the data were then made to an assumed initial velocity equation, using iterative procedures (Wilkinson, 1961; Cleland, 1963a). In general, the initial velocity data were fitted both to eq 1 and 2, assuming the curve to be a rectangular hyperbola

$$v = \frac{VS}{K + S} \tag{1}$$

or sigmoid

$$v = \frac{VS^2}{a + bS + S^2} \tag{2}$$

where v = velocity, V = maximal velocity, S = substrate concentration, b = an added constant, which is a measure of the sigmoid nature of the curve (when $b = 0$ the curve is maximally sigmoid, but when b is very large, eq 2 degenerates into eq 1).

In some cases the data were fitted to eq 3 when it appeared that they might be 2/1 functions.[2] The computer programs supplied the variance of fits and

$$v = \frac{V(S^2 + cS)}{a + bS + S^2} \tag{3}$$

[2] This refers to the highest power of substrate concentration that occurs in the numerator and denominator of the rate equation when written in a double reciprocal form. The curves given by this function are either concave up or concave down near the vertical axis (when $1/S$ value approaches zero), but become linear when $1/S$ value approaches infinity. The curves plot as, $y = (a + bx + cx^2)/(1 + dx)$.

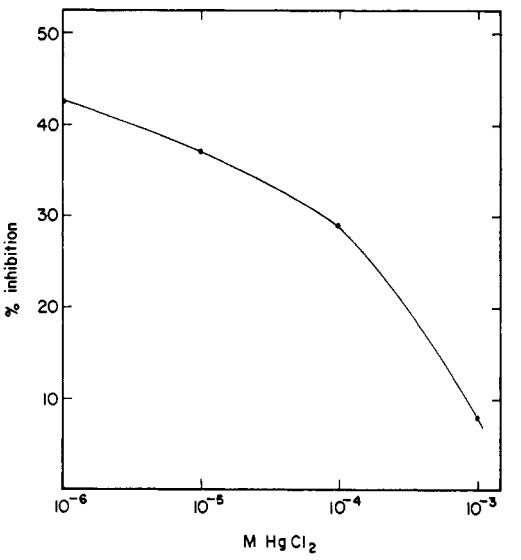


FIGURE 1: Effect of mercury on the desensitization of threonine deaminase to isoleucine inhibition. Enzyme preparation I from strain *C-19* was dialyzed against 1 M potassium phosphate buffer which did not contain β-mercaptoethanol and EDTA. Mercuric chloride was mixed with the enzyme (containing 1.8 mg of protein/ml) at zero time and brought up to 25° in 5 min. Aliquots were then assayed both in the presence of isoleucine (10^{-4} M) and in its absence by assay 2. A constant amount of L-threonine, 13.3 mM, was used throughout. The pH during mercury treatment was 8.0.

weighting factors for different kinetic constants. These were used for fitting replots of the slopes or intercepts against inhibitor concentrations. Least-square fits of the replots were made (using weighting factors) to a straight line, a parabola, a hyperbola, or a 2/1 function, when appropriate. Final kinetic constants with their standard errors were obtained whenever possible from fits to an over-all rate equation describing the observed type of inhibition or proper initial velocity pattern.

For linear competitive inhibition data were fitted to eq 4

$$v = \frac{VS}{K(1 + I/K_i) + S} \tag{4}$$

and, for linear noncompetitive inhibition to eq 5

$$v = \frac{VS}{K(1 + I/K_{is}) + S(1 + I/K_{ii})} \tag{5}$$

Results

Criteria for "Nativeness" of the Enzyme. Since one of the characteristic properties of most regulatory enzymes is the ease with which they are "desensitized" (Monod *et al.*, 1963, 1965) and lose their allosteric properties, it became of great importance to show that our enzyme

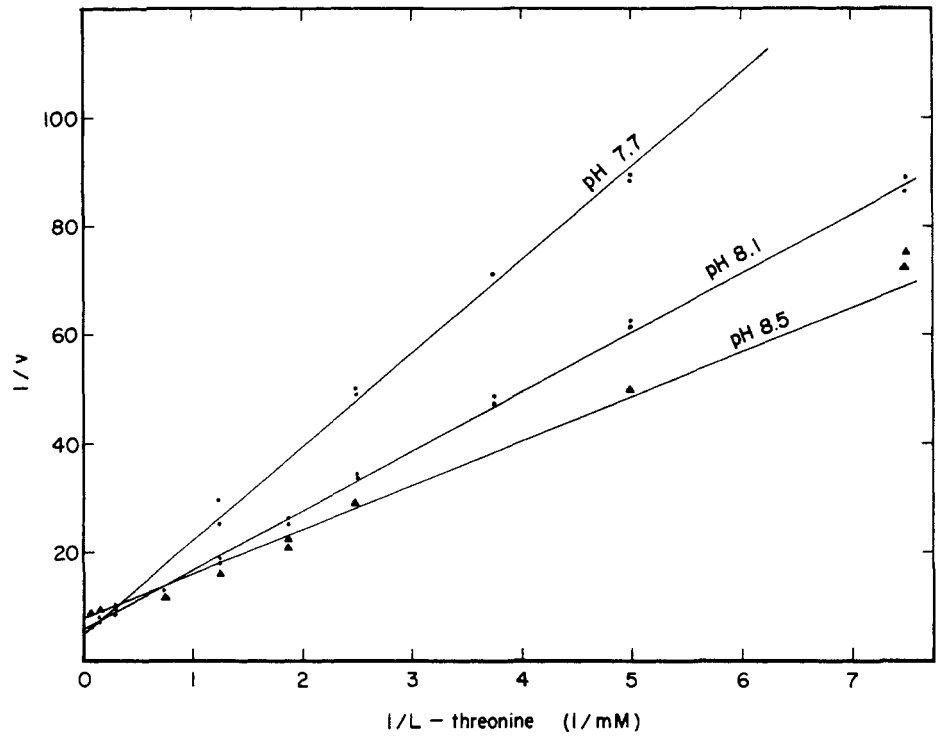


FIGURE 2: Double reciprocal plots of velocity *vs.* L-threonine at different pH values. Enzyme preparation I obtained from derepressed cells of strain 447 and assay 2 was used. The lines have been drawn from fits to eq 1. The V_{max} and K_m (mM) values are: pH 7.7, 0.21 ± 0.003 and 3.6 ± 0.16; pH 8.1, 0.18 ± 0.003 and 1.8 ± 0.09; pH 8.5, 0.13 ± 0.002 and 1.02 ± 0.05.

preparations were "native." Advantage was here taken of the observations already made by Freundlich and Umbarger (1963) with *Salmonella* enzyme and by Changeux (1961) with the threonine deaminase of *Escherichia coli* that "native" enzyme preparations can be desensitized to inhibition by isoleucine by mercury and heat treatment. Accordingly, enzyme preparation I was treated with $HgCl_2$ and its effect on the inhibition of enzyme activity by isoleucine was tested. The results of this experiment are presented in Figure 1. It will be noted that approximately 45% inhibition caused by 10^{-4} M L-isoleucine at a substrate concentration of 13.33 mM is reduced to less than 10% by treatment of the enzyme at higher concentrations of mercuric chloride. As noted by Changeux (1961) part of the enzyme activity is also lost by mercury treatment (about 25% in our experiments at the highest concentration of $HgCl_2$ tested, Figure 1). Since our enzyme preparations are susceptible to "desensitization," it follows that in all likelihood they represent "native" preparations. In all experiments reported later, the enzyme preparations before use were tested with 0.0165 mM L-isoleucine at an L-threonine concentration of 0.53 mM. Approximately 50% inhibition was always obtained.

Despite desensitization by mercury shown above, it may be possible that the enzyme preparations used in this work were a mixture of native and desensitized fractions. However, it has been shown by Changeux (1961) that the desensitized enzyme from *E. coli* has a higher K_m value as compared to the native enzyme. Were our enzyme preparations mixtures of two fractions with different K_m values, this would become apparent in the curvature of the double reciprocal plots near the vertical axis. However, in two experiments with enzyme preparation I and II, no unusual deviations from linearity were found when threonine was varied in initial velocity studies between the ranges of 0.13 and 53.2 mM (see below).

Initial Velocity Pattern. Using preparation I obtained from strain 447 and varying L-threonine between the ranges of 0.13 and 26.6 mM (0.1–15 K_m), the data obtained gave excellent fits to eq 1, but insignificant fits to eq 2. This can be clearly seen from Figure 2. At different pH values, the V_{max} and K_m changed (see legend to Figure 2), but the data still conformed to eq 1. This result was rather unexpected for the main reason that the allosteric threonine deaminase from the closely related bacterium, *E. coli* (Changeux, 1961), gives sigmoid plots of initial rate-concentration data at the optimum pH value of 8.1 and the curves become more sigmoid at pH 7.7 (Changeux, 1962). In view of the results reported for the *E. coli* enzyme, it was, therefore, considered plausible that the coupled spectrophotometric assay was either inherently unsuited for initial velocity studies, or else the enzyme of strain 447 had lost its allosteric properties due to the deletion of the leucine

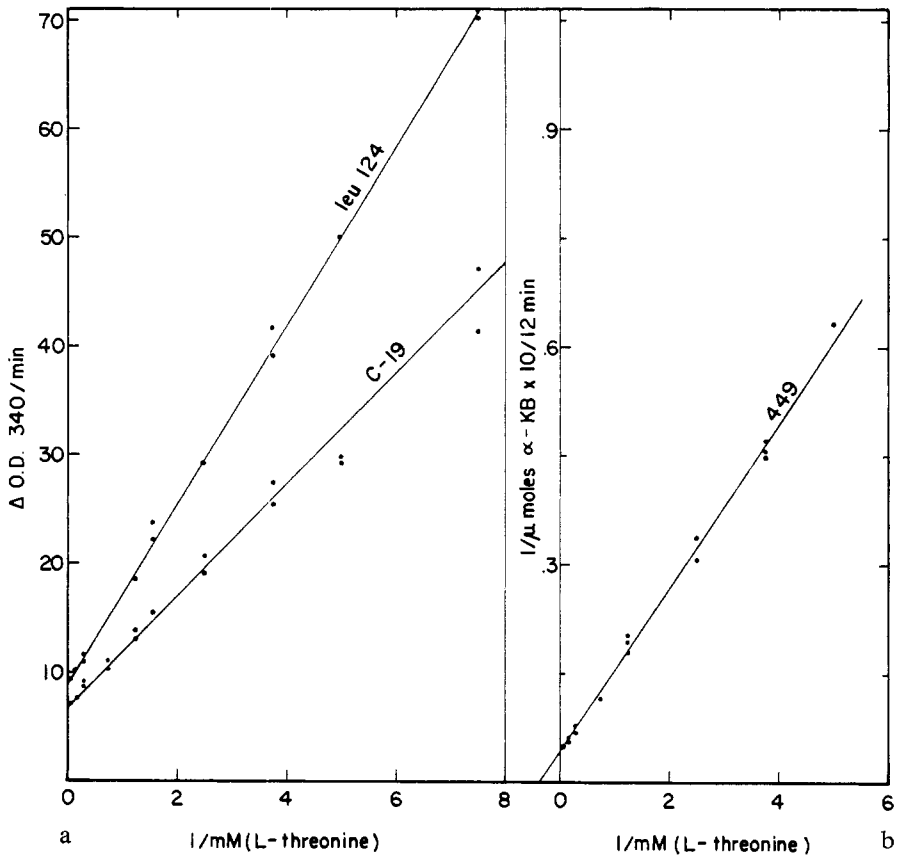


FIGURE 3: Double reciprocal plots of velocity *vs.* L-threonine obtained with enzyme preparations from different strains of *S. typhimurium*. (a) Enzyme preparation I from nutritionally derepressed cells of *leu* A_{124} and genetically derepressed cells of *C-19*. Assay 2 was used (pH 8.1). The V_{max} and K_m (mM) values are, for C-19, 0.14 ± 0.002 and 0.79 ± 0.04, and for *leu* A_{124}, 0.1 ± 0.001 and 0.91 ± 0.027 (obtained from fits to eq 1). (b) Enzyme preparation II from nutritionally derepressed cells of strain 449. Assay 1 was used (pH 8.1). The line is drawn from fits to eq 1. The K_m and V_{max} values are 2.9 ± 0.18 and 24.5 ± 0.49, respectively.

operon, or due to the methods used for derepression of threonine deaminase.

Both of these possibilities could be rejected on the basis of two experiments. In one experiment, enzyme preparation II instead of preparation I from strain 447 was used for initial velocity studies (assay 1). The data again conformed to eq 1 (Figure 3b). In the second experiment, enzyme preparation I from the genetically derepressed mutant *C-19* and nutritionally derepressed cells from *leu* A_{124}, both studied by assay 2, again gave perfectly linear double reciprocal plots (Figure 3a).

Product Inhibition Studies. It has been reported that the threonine deaminase from yeast cells (Holzer *et al.*, 1964) is activated at high levels of substrate by ammonia, a product of the enzymic reaction. In order to test this for *Salmonella* enzyme and to compare the native enzyme with the desensitized one later, product inhibition studies were made. When NH_4^+ was used at various concentrations and L-threonine was varied, competitive inhibition was obtained (Figure 4). The replot of slopes against inhibitor concentrations was linear. With assay 3 and α-ketobutyrate as the product inhibitor, noncompetitive inhibition was obtained (Figure 5). The replots of slopes and intercepts against α-ketobutyrate concentrations were also linear.

The data obtained so far suggest that the products are released in an ordered sequence, α-ketobutyrate first followed by ammonia. The rate equation for this mechanism using Cleland's nomenclature (Cleland, 1963b) is of the form

$$v = \frac{V_1\left(A - \frac{PQ}{K_{eq}}\right)}{K_a + A + \frac{K_{ia}P}{K_{ip}} + \frac{K_aQ}{K_{iq}} + \frac{K_aPQ}{K_pK_{iq}} + \frac{AP}{K_{ip}}} \quad (6)$$

where A is the concentration of threonine, P is the concentration of α-ketobutyrate, Q is the concentration of ammonia, K_a, K_p, and K_q are Michaelis constants for A, P, and Q; K_{ip} and K_{iq} are inhibition constants for P and Q; and K_{ia} is the dissociation constant of A. Thus, when threonine (A) is varied and ammonia (Q) is the

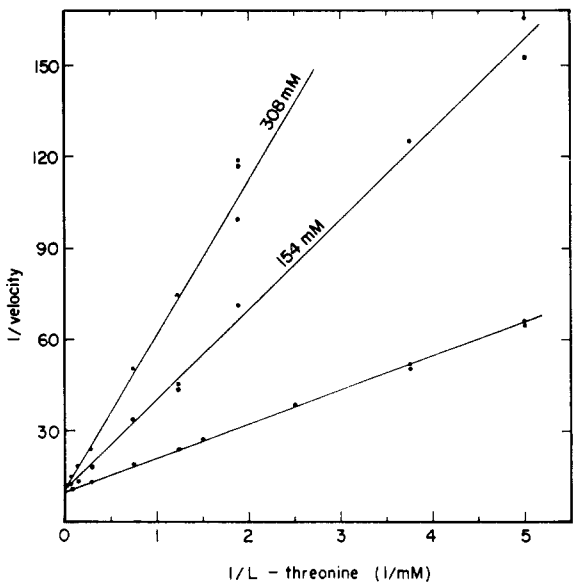


FIGURE 4: Product inhibition of threonine deaminase by ammonia, with L-threonine as the variable substrate. Individual lines are drawn from fits to eq 1. Preparation I from strain 447 and assay 2 was used (pH 8.1). Figures above the lines refer to the concentration of NH_4^+. Fresh preparations of NH_4Cl were used.

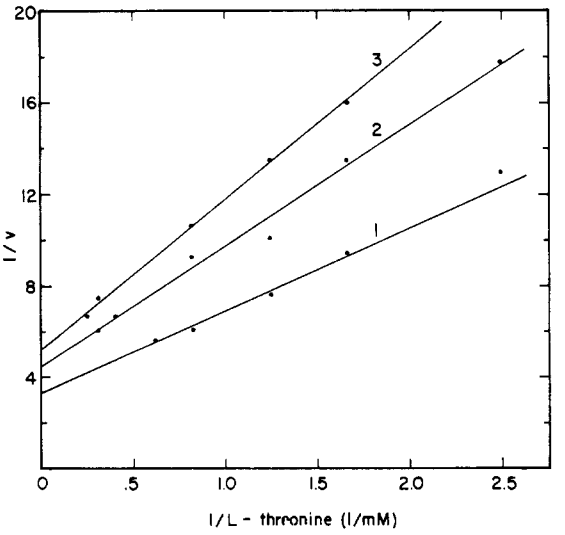


FIGURE 5: Product inhibition of threonine deaminase by α-ketobutyrate. Individual lines are drawn from fits to eq 1. Preparation I from strain 447 and assay 3 was used (pH 8.0). The concentrations of inhibitor used were, 1 = no inhibitor, 2 = 40 mM, and 3 = 80 mM.

inhibitor, linear competitive inhibition is expected (eq 7).

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{Q}{K_{iq}}\right)\frac{1}{A} + \frac{1}{V} \qquad (7)$$

When α-ketobutyrate (P) is the inhibitor, the initial velocity equation becomes (eq 8)

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}P}{K_aK_{ip}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{P}{K_{ip}}\right) \qquad (8)$$

The various kinetic constants which could be evaluated from our data are given in Table I.

Inhibition of Activity by Allothreonine. It has been shown by Changeux (1962) in *E. coli* and Freundlich and Umbarger (1963) in *S. typhimurium* that the allosteric threonine deaminase is inhibited by allothreonine. In Figure 6, we present data which show that allothreonine inhibits the enzyme competitively. The replot of slopes against inhibitor concentration is also linear, which shows that allothreonine is a dead-end inhibitor, and probably combines with the threonine binding site and blocks the addition of the substrate. A K_i value of 0.07 mM can be calculated from over-all fits to eq 5.

Inhibition of Activity by Isoleucine. Since the early work of Umbarger (1956) and Umbarger and Brown (1957), it has repeatedly been shown that isoleucine serves as a powerful feedback inhibitor of allosteric threonine deaminase (Freundlich and Umbarger, 1963; Changeux, 1961). Changeux (1961) and Monod *et al.* (1963) postulated that isoleucine binds at a site different from the active site. The remarkable feature of the inhibition of threonine deaminase by isoleucine (Figure 7) is that up to an inhibitor concentration of 0.0165 mM the data conform to eq 1 but begin to deviate significantly at higher concentrations of the inhibitor. Thus, at a concentration of 0.0396 mM isoleucine (Figure 7), the double reciprocal plot shows a marked curvature upwards at low substrate concentrations. Fits of this line to eq 2 or 3 turned out to be insignificant. The intercept values at different isoleucine concentrations (lines 1–3 in Figure 7) show small but statistically significant differences (within the limits of precision of the data). This difference in intercept values has been consistently noted in four separate experiments. It is considered likely, therefore, that inhibition by isoleucine is not really competitive. The replot of intercepts (lines 1–4 in Figure 7) against isoleucine concentrations

TABLE I: Some Kinetic Constants of Threonine Deaminase.

Constant	mM
K_a	0.92 ± 0.07[a]
K_{ia}	1.59 ± 0.30
K_{ip}	148 ± 28
K_{iq}	95 ± 15
K_i (allothreonine)	0.07 ± 0.009

[a] This is an average value from ten different experiments.

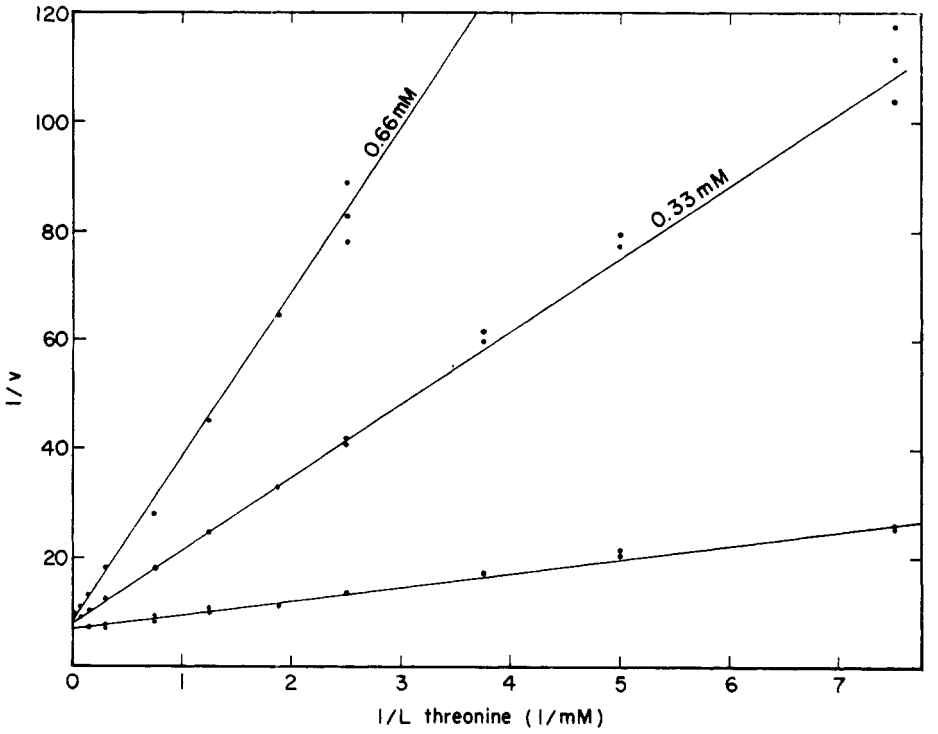


FIGURE 6: Inhibition of threonine deaminase activity by DL-allothreonine. All lines are drawn from fits to eq 1. Preparation I from strain 447 and assay 2 was used (pH 8.1).

(Figure 8) gives indications of a hyperbolic intercept activation, although meaningful fits cannot be made to this function owing to the large standard errors of some estimates. When the slopes of lines from Figure 7 (up to 0.0264 mM isoleucine) are plotted against isoleucine concentrations, the resulting curve is also found to be markedly nonlinear (Figure 8). This curve can neither be fitted to a parabola nor to a 2/1 function. As discussed later these results are likely to be due to isoleucine acting as a partial inhibitor and also combining at the active center.

Effect of Activators on Threonine Deaminase. The enzymes from *E. coli* (Changeux, 1963) and *S. typhimurium* (Freundlich and Umbarger, 1963) have both been shown to be activated by L-norleucine and L-valine. Using both preparations I and II and assay procedures 1 and 2, we have been unable to demonstrate any activating effect of norleucine or valine up to a concentration of 5 mM. Concentrations higher than 5 mM cause slight inhibition.

Although we were not able to demonstrate activation by L-norleucine, it must bind on the enzyme surface, as is readily apparent from Figure 9. When a constant amount of L-threonine (3.3 mM, unsaturating) is used and the concentrations of isoleucine and norleucine are varied in the assay mixture, the inhibition caused by isoleucine is relieved to a considerable extent by norleucine. The relief of inhibition, however, is not complete, and this may be due to the fact (see below) that norleucine while capable of excluding isoleucine from its binding site is not able to prevent a dead-end combination of the inhibitor at the active site.

Protection of the Enzyme Activity by Isoleucine. All of the data we have presented so far are easily explicable, if it is demonstrated that the enzyme exists primarily in a conformational state (state R, see Monod *et al.*, 1965) which does not bind isoleucine. To test this point, we investigated the effect of isoleucine on the thermal inactivation of the enzyme. Both in the absence and the presence of isoleucine, the inactivation of the enzyme followed first-order kinetics at 30°. The rate constant of inactivation

$$k = \frac{1}{t} \ln (x_0/x') \qquad (9)$$

where x_0 is enzyme activity at zero time, and x' is the activity remaining after time t, when plotted against the concentration of isoleucine (Figure 10), yielded a hyperbola. The "protection" constant (which under certain conditions may equal the dissociation constant; Burton, 1951) calculated from Figure 10 is 0.003 mM. It is clear that the enzyme must exist in a conformational state which is easily accessible to isoleucine.

Discussion

The nature of most kinetic deductions is such that they are only suggestive of a mechanism, but are not

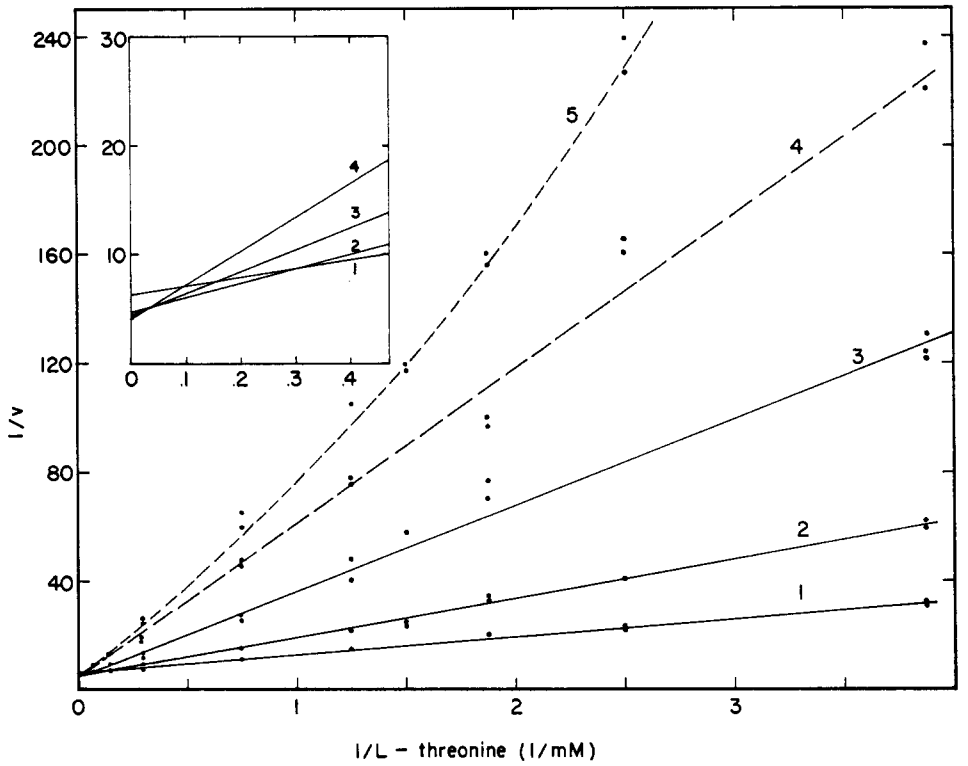


FIGURE 7: Inhibition of threonine deaminase activity by L-isoleucine. Solid lines 1, 2, and 3 have been drawn from fits to eq 1. The standard errors of the kinetic constants for these lines are in the range of 5–10%. Dotted lines 4 and 5 are fitted by eye. The standard errors of estimates, assuming fits to eq 1, are, for line 4, $K = 8.9 \pm 2.7$, $V = 0.24 \pm 0.04$, and for line 5, $K = 28.7 \pm 13.4$, $V = 0.44 \pm 0.16$. Preparation I from strain 447 and assay 2 was used. The concentrations of L-isoleucine are: 1 = 0, 2 = 0.0066 mM, 3 = 0.0165 mM, 4 = 0.0264 mM, and 5 = 0.0396 mM. The inset shows intercepts of the plots on an expanded scale.

proof for it. Ideally, before a kinetic model is presented, all other likely models should also be examined and excluded as far as possible. It was with this in mind that the work presented here was undertaken with the allosteric threonine deaminase of *Salmonella*. This regulatory enzyme from the closely related *E. coli* was not only the first one of its kind studied (Umbarger, 1956; Umbarger and Brown, 1957), but has also served as a basis for the generalized kinetic model of allosteric effects proposed by Monod *et al.* (1965).

A study of the literature shows that three main types of allosteric enzymes can be distinguished on the basis of initial velocity data in the presence and absence of allosteric effectors.

(1) One class consists of enzymes which yield sigmoid curves for rate–concentration data in the absence of effectors. This class has been referred to as the K system by Monod *et al.* (1965). In the presence of activators the rate–concentration curves become nearly hyperbolic, while in the presence of inhibitors they become more sigmoid (see Monod *et al.*, 1965).

(2) The second class of enzymes yields initial velocity data which conform to a rectangular hyperbola. The allosteric effectors do not change the shape of the curve, but affect only the maximal velocity ("V" system of Monod *et al.*, 1965).

(3) The third class of enzymes is similar to class 2, except that in the presence of allosteric effectors the maximal velocity does not seem to change, although, frequently, in the presence of allosteric inhibitors the rate–concentration curves become sigmoid (Neufeld and Hall, 1965; Madsen, 1964; Melo and Glaser, 1965; Kornfeld *et al.*, 1964).

Since the kinetics of threonine deaminase presented here have many points in common with the kinetics of enzymes belonging to class 3, we restrict our discussion and comparison only to this group of enzymes. Alternative models have already been presented for some enzymes belonging to class 1 (Sanwal *et al.*, 1964, 1965; Sanwal and Stachow, 1965; Atkinson *et al.*, 1965). For the enzyme described here, the results are not compatible with the subunit interaction hypothesis for the following reasons. It can be seen from Figure 10 that the protection of the enzyme against thermal inactivation by isoleucine follows an adsorption isotherm, very much like the threonine deaminase of *E. coli* (Changeux, 1964). In terms of Monod's model (Monod *et al.*, 1965) this suggests that the allosteric constant in eq 10 is

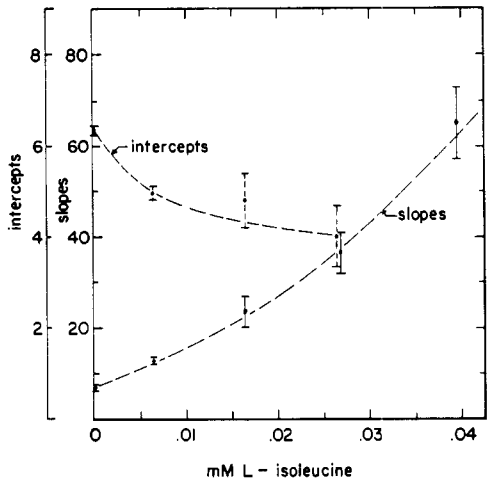


FIGURE 8: Replots of slopes and intercepts from Figure 7 against L-isoleucine concentrations.

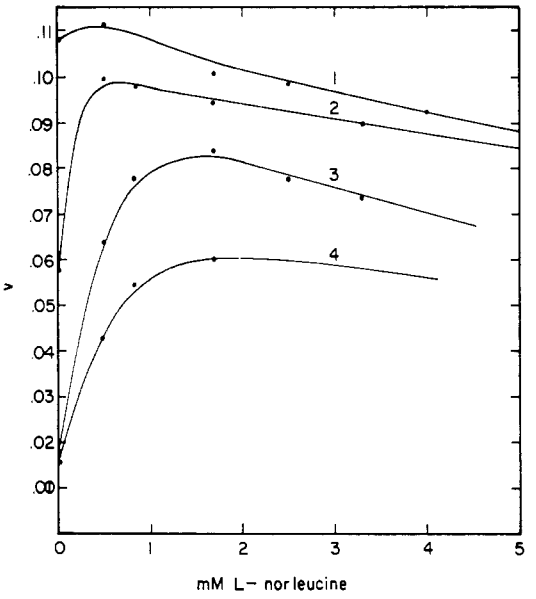


FIGURE 9: The activation of threonine deaminase by L-norleucine in the presence of various concentrations of L-isoleucine. Preparation I from derepressed cells of *leu* A_{124} and assay 2 was used (pH 8.1). All assay mixtures contained 3.3 mM L-threonine. The activator, inhibitor, and substrate were added simultaneously. The concentrations of isoleucine are, 1 = 0, 2 = 0.0066 mM, 3 = 0.033 mM, 4 = 0.0495 mM.

large (*i.e.*, the enzyme is present mostly in the T form and has very small affinity for threonine).

$$Y_f = \frac{Lc\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}}{L(1 + c\alpha)^n + (1 + \alpha)^n} \qquad (10)$$

where Y_f is the saturation function, L = allosteric equilibrium constant for the two conformational states $R \rightleftharpoons T$; c = ratio of the dissociation constants (K_R and K_T) of a ligand (A) bound in state R and T; $\alpha = A/K_R$; n = number of binding sites for A. Under such conditions, the so-called homotropic interactions of the substrate should be very much evident, *i.e.*, the rate–concentration curves should be sigmoid. Equation 10 has the disadvantage that the constants are thermodynamic equilibrium constants and are only related in a special way to the kinetic constants. However, in terms of the latter, the initial velocity equation, for a mechanism (I) of the kind

$$E_T + S \underset{k_2}{\overset{k_1 S}{\rightleftharpoons}} E_RS \qquad (I)$$

$$E_RS + S \underset{k_4}{\overset{k_3 S}{\rightleftharpoons}} E_RSS \xrightarrow{k_5} \text{products}$$

where E_T = enzyme in T form, E_R = enzyme in R form, is given by eq 2 or, in the double reciprocal form, by eq 11

$$\frac{1}{v} = \frac{a}{V}\left(\frac{1}{S}\right)^2 + \frac{b}{v}\left(\frac{1}{S}\right) + \frac{1}{V} \qquad (11)$$

where a and b are combinations of constants. In mechanism I, it is assumed that all of the enzyme is present in E_T form, the release of products only occurs after the central complex E_RSS is formed, and the binding of S to E_T is a slow step.

In any case, according to both equations (10 and 11), nonlinearity of the double reciprocal plots should become apparent when initial velocity data are gathered over a wide range of substrate concentrations; this has been done in our work and, furthermore, the data have been statistically analyzed to avoid any bias. It may be noted, parenthetically, that the form of eq 11 does not change if more than one molecule of the substrate binds to the enzyme in each of the two states, as long as one assumes that, in each of the given states of the protein, binding of one substrate molecule is independent of the binding of any other.

Assuming with certain justification now that there is no interaction of subunits during substrate binding, the nonlinearity of the double reciprocal plots in the presence of high concentrations of isoleucine (Figure 7) must be explained. Were we to assume that the allosteric constant in eq 10 was very small for the enzyme or that the $R \rightleftharpoons T$ equilibrium was strongly in favor of R state, the nonlinearity of the plots (Figure 7) could be taken as evidence for subunit interactions, and this could also explain the absence of activation by L-norleucine and L-valine. This, however, is not the case, as we have seen earlier (Figure 10).

Since the threonine deaminase of *Salmonella* can be desensitized to inhibition by isoleucine (Figure 1) much like that of *E. coli* (Changeux, 1961), the possibility

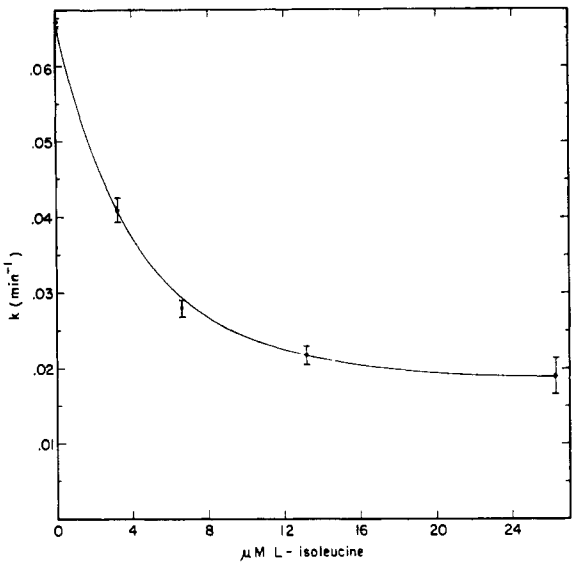
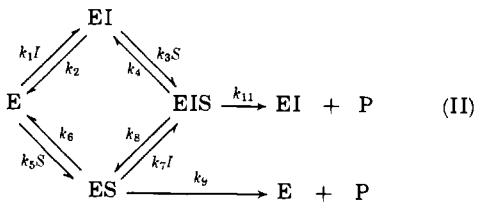


FIGURE 10: The thermal inactivation of threonine deaminase in the presence of L-isoleucine. Enzyme preparation I from strain *C-19* was dialyzed against 1 M potassium phosphate buffer (pH 8.0) which did not contain β-mercaptoethanol and EDTA. The protein solution was diluted 10 times at zero time in a solution of L-isoleucine (equilibrated at 30°) to give the desired concentration of isoleucine. The final concentration of the protein was 0.23 mg/ml and buffer concentration was 0.1 M phosphate (pH 8.0). At various times aliquot were removed, and remaining activity was measured by assay 2.

suggests itself that the isoleucine binding site is distinct from the substrate binding site (Monod *et al.*, 1963). If it is assumed that the E–isoleucine complex can still bind threonine and such a complex releases products (mechanism II), the initial velocity equation derived by King



(II)

and Altman's (1956) method has the form

$$\frac{v}{E_t} = \frac{\text{numerator}}{\text{constant} + aS + bS^2 + cSI + dS^2I + eSI^2 + fI + gI^2} \quad (12)$$

where I is the concentration of isoleucine, S is the concentration of threonine, and a, b, ... g are combinations of rate constants. The numerator is

$$k_9[k_2k_5(k_8 + k_4)S + k_3k_8(k_1I + k_5S)S] + k_{11}[k_5k_7(k_2 + k_3S)SI + k_1k_3(k_6 + k_7I)SI] + k_9k_{11}[(k_1k_3I + k_2k_5)S] \quad (13)$$

In the absence of the inhibitor, eq 12 reduces to

$$v = \frac{k_9E_tS}{(k_6 + k_9)/k_5 + S} \quad (14)$$

which is identical with eq 1.

Since in eq 13 there are terms of S^2 in the numerator, it is expected that as more inhibitor is added the double reciprocal plots will become increasingly nonlinear (as the importance of the alternate pathway increases). From Figure 7 it can be seen that curvature is already apparent at an isoleucine concentration of 0.0264 mM. Equations 12 and 13 predict that the double reciprocal plots will be 2/1 functions except in the absence of inhibitor or when inhibitor is saturating, and the intercepts of these plots will be a hyperbolic function of inhibitor concentration. The slope replots (initial or asymptote slopes) will be a more complex function of inhibitor concentration, but the curve will also have a horizontal asymptote. The data in Figures 7 and 8 conform only partially to this pattern; the intercept replot (Figure 8) is hyperbolic (the activation of intercepts results from the numerator inhibition constant being higher than the denominator inhibition constant; Cleland, 1963c), but the slope replot appears parabolic (Figure 8) instead of tending to reach a plateau. This suggests that there may be dead-end combination of isoleucine at the active site with EI of mechanism II. The slopes of the asymptotes of the 2/1 functions predicted by eq 12 would then be 3/2 functions, which is reasonably consistent with the data of Figure 8. This would also explain why norleucine is incapable of overcoming the isoleucine inhibition (Figure 9). Combination of norleucine at the allosteric site could prevent isoleucine binding and the resulting changes at the active site, but could not prevent dead-end combinations of isoleucine at the active site.

In contrast to isoleucine, allothreonine seems to be a competitive dead-end inhibitor, perhaps causing inhibition by steric blocking of the active threonine site. This conclusion is supported by the linearity of the replot of slopes against allothreonine concentrations.

The mechanism of activation of the enzyme by norleucine in the presence of isoleucine is obscure. It is possible that norleucine is able to exclude isoleucine from its binding site, but whether this is due to steric hindrance or due to allosteric changes cannot be ascertained at the present time.

The simple model that emerges as a result of the present study is that the threonine deaminase of *S. typhimurium* presumably has two types of sites, one regulatory and another catalytic. If there is more than one catalytic site, it is clear from our data that binding

of substrate to each one of these is perhaps independent of binding of any other (or, in other words, there is presumably no interaction). The binding of allosteric inhibitor, isoleucine, possibly occurs on the regulatory site. This binding changes the catalytic properties of the active centers. Whether this is brought about by conformational changes of the protein cannot be ascertained from purely kinetic data. Also, it is impossible to decide whether the two kinds of sites postulated before are present on the same subunit or two different types of subunits.

It will be noted that the results presented here are at variance with those obtained by Changeux (1961) for threonine deaminase of *E. coli*, and in some details, with those of Freundlich and Umbarger (1963) for *S. typhimurium*. Two main discrepancies are immediately apparent: one, the sigmoid plots of initial velocity data for *E. coli* enzyme, and, second, the activation of the enzyme from *E. coli* and *Salmonella* by norleucine and valine in the absence of added isoleucine. Our comparative experiments with *E. coli* extracts (unpublished), however, do not differ substantially from those presented in this work, and the cause of this discrepancy remains to be investigated.

While the model we have proposed for threonine deaminase seems applicable to enzymes belonging to class 3, discussed before, it is possible that it may, after suitable modifications, be applicable to class 2 enzymes (V system of Monod *et al.*, 1965) also. Following the earlier work of Botts and Morales (1953) and Hearon *et al.* (1959), Frieden (1964) has recently discussed numerous conditions under which the rate equation of type 12 is reduced to a simpler form. The analysis presented by Frieden (1964) taken together with the basic theory of slope and intercept variation for bi- or multireactant enzymic mechanisms developed by Cleland (1963b,c) should prove very useful for kinetic analysis of allosteric enzymes.

Acknowledgments

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A New Adenylate Deaminase from Red Marine Alga *Porphyra crispata**

Jong-Ching Su, Chien-Chung Li, and Catherine Chongling Ting

ABSTRACT: A new type of adenylate deaminase has been extensively purified from the red marine alga *Porphyra crispata*. The enzyme deaminates 5′-adenylic acid, adenosine diphosphate, adenosine triphosphate, diphosphopyridine nucleotide, and adenosine at rates decreasing in that order. It showed different optimal pH for different substrates: the larger the molecular weight of the substrate, the lower the optimal pH. Calcium, magnesium, and barium ions enhance the rate of reaction. Manganese ion activates the enzyme at acidic pH values; however, at neutral and alkaline pH values, it acts on the enzyme as an inhibitor. *p*-Mercuribenzoate is also inhibitory. The enzyme preparation is free from other adenylate deaminases; this is indicated by the findings that the ratio of activities toward the different substrates at different purification stages or after different partial inactivation treatments remained constant.

Adenylate deaminases are a group of enzymes which catalyze the hydrolytic removal of the 6-amino group of adenylate compounds. The first discovery of an enzyme of this category dates back to as early as 1928 (Schmidt). It is the myosin-associated, highly specific 5′-adenylic acid deaminase found in striated muscle. This enzyme has been extensively studied by a number of investigators in the years following (Kalckar, 1947; Humphrey and Webster, 1951; Nikiforuk and Colowick, 1956), yet crystallization of it has not been achieved until 1957 (Lee). Adenylate deaminases with similar properties have been found in various animal tissues (Weil-Malherbe and Green, 1955; Abood and Romanchek, 1955; Mendicino and Muntz, 1958; Zydowo, 1959; Yoshizumi, 1959; Pennington, 1961; Askari, 1963) and microorganisms (Hochster and Madsen, 1959, Fujiwara and Spencer, 1962). Presence of a somewhat similar enzyme in the particulate fraction of pea seed has also been reported by Turner and Turner (1961). The nonspecific fungal adenosine deaminase, which was originally reported by Mitchell and McElroy (1946), has been further purified and characterized by Kaplan *et al.* (1952).

During the course of investigation on adenosine triphosphate (ATP)[1] utilization by the cell-free extract of the red sea weed *Porphyra crispata*, the authors found in the reaction mixture a product with an electrophoretic mobility (pH 3.5) greater than that of ATP. On considering the dissociation properties of purine nucleotides, we soon suspected it to be ITP. Indeed, the spectral data of this spot further confirmed what we assumed was true. Later, it was found that the activity of this deaminase mainly existed in the soluble fraction, and it showed much greater activity toward 5′-AMP than toward ATP. A survey of the substrate specificity of the enzyme indicated that it could be a new type adenylate deaminase. In this paper are presented the procedure for the extensive purification of the enzyme and some properties of the purified enzyme.

Materials and Methods

Sea weed used in this work was collected from the sea shore near Keelung, Taiwan, from September 1964 to February 1965. Most of the nucleotide derivatives used were commercial products. ADP, tri- (TPN),

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[1] The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; 5′-AMP, adenosine 5′-monophosphate; 2′-AMP, adenosine 2′-monophosphate; 3′-AMP, adenosine 3′-monophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ADPR, adenosine diphosphate ribose; IMP, inosine monophosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; FAD, flavin adenine dinucleotide; GSH, reduced glutathione; BAL, 2,3-dimercaptopropanol; PCMB, *p*-mercuribenzoate.