Threonine Deaminase from *Salmonella typhimurium*

RELATIONSHIP BETWEEN REGULATORY SITES

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Kinetic analysis of the biosynthetic threonine deaminase, EC 4.2.1.16, from *Salmonella typhimurium* yields hyperbolic substrate saturation curves in the absence of, and higher order substrate saturation curves in the presence of, L-isoleucine. L-Valine reverses this effect of L-isoleucine by restoring the hyperbolic substrate saturation curves. The inhibition of enzyme activity and the reversal of valine stimulation is a function of a second order concentration of L-isoleucine, whereas antagonism of inhibition is a function of first order concentration of valine. The antagonistic effects on enzyme activity of L-isoleucine and of L-valine appear as competitive in diagnostic plots. Threonine deaminase possesses two L-isoleucine binding sites \( K_d = 3.6 \mu M \) and one L-valine binding site \( K_d = 36 \mu M \); the binding of these ligands appear competitive. Exclusion of L-valine requires the binding of 2 molecules of L-isoleucine whereas binding of a single L-valine molecule prevents the binding of 2 L-isoleucine molecules. Cooperative binding of L-isoleucine is not observed under any of the conditions tested.

Two cases, expressed in terms of modified Adair equations and based upon the assumption that L-threonine also serves as an activator ligand which binds to the L-valine site, are presented. Case I states that liganding of the activator site must precede substrate-binding at the active site, and Case II states that the activator site liganding is required solely for reactivation of the L-isoleucine-inhibited enzyme. Analysis of kinetic data by a curve-fitting process suggests that Case II describes the relationship between the activator site and the L-isoleucine sites. An enzymatically inactive derivative of threonine deaminase, prepared by reduction with borohydride, binds isoleucine and valine in a manner similar to native holoenzyme. Binding of L-threonine and L-valine to the derivatized enzyme is competitive. The \( K_d \) for threonine binding is 3 mM, which is in excellent agreement with the \( K_d \) determined by the curve fitting process. It is concluded that the modulation of threonine deaminase activity is wrought by interaction between inhibitor sites and an activator site rather than inhibitor and active sites and that induced transitions rather than concerted transitions more adequately describe the underlying regulatory principle.

Isoleucine biosynthesis in *Salmonella typhimurium* as well as in a number of other microorganisms and plants is initiated by the conversion of L-threonine to \( \alpha \)-ketobutyrate and ammonia (1). The enzyme which catalyzes this reaction is termed biosynthetic L-threonine deaminase (L-threonine hydrolyase, deaminating; EC 4.2.1.16), and its activity is modulated in both a negative and positive manner. Isoleucine, a negative effector of biosynthetic L-threonine deaminase activity, is responsible for two related effects noted in kinetic analysis:

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Relatively high concentrations of this end product completely inhibit enzyme activity, whereas lower concentrations induce high order substrate saturation curves. It is noteworthy that substrate saturation curves appear hyperbolic in the absence of the negative effector L-isoleucine. L-Valine, which is physiologically related to L-isoleucine by virtue of partially shared biosynthetic pathways, is a positive effector of threonine deaminase activity. It has been known for some time that L-valine is capable of reversing the isoleucine-induced apparent "homotropic cooperativity" of the substrate sites in threonine deaminase (2).

Previous work has shown that biosynthetic threonine deaminase from *S. typhimurium* is composed of four apparently identical polypeptide chains and the native tetramer contains two pyridoxal 5'-monophosphate residues and presumably two active sites (3). There is ample evidence to suggest that the active and the inhibitor sites are separate and consequently

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inhibition is wrought by an allosteric rather than isoeoteric mechanism (4). With the exception of one preliminary report no information is available with regard to the relationship between the "valine site" and either the active sites or the isoleucine sites (5). The work described in this paper is an attempt to correlate kinetic data with observations obtained by direct ligand-binding analysis and to determine the relationship which exists among the three types of ligands which stereospecifically bind to threonine deaminase, namely substrate, negative, and positive effectors.

MATERIALS AND METHODS

Bacterial Strains—Threonine deaminase was purified from Salmonella typhimurium ilv E-231, an isoleucine auxotroph kindly supplied by Dr. M. Freundlich. This organism produces little if any transaminase B, the product of the ilv E gene.

Growth of Bacteria—S. typhimurium ilv E-231 was grown in a minimal medium which contained 0.086 M KH₂PO₄, 0.047 M KHPO₄, 0.016 M (NH₄)₂SO₄, 1.2 mM MgSO₄, 0.38 mM L-isoleucine, and 23 mM glucose. Eight 12-liter carboys were inoculated with 200 ml of an overnight nutrient broth culture of S. typhimurium ilv E-231 and incubated at 37° with forced aeration for 24 hours, after which the cultures were harvested and treated as described below.

Enzyme Assay—Threonine deaminase was assayed as previously described (3), or by direct measurement of product absorbance at 235 nm using a Gilford 2200 spectrophotometer (6).

Enzyme Purification—Threonine deaminase was purified as previously described (3) except that an initial ammonium sulfate precipitation was introduced. Crude extract was diluted to 10 mg per ml of protein with standard buffer (3) containing 0.05 M pyridoxal 5'-monophosphate. Ammonium sulfate was added slowly in the cold to achieve 35% saturation. The mixture was allowed to stir for 30 min then centrifuged at 10,000 x g for 60 min. The sediment was discarded and the supernatant liquid brought to 60% saturation with ammonium sulfate, stirred for 30 min, and centrifuged at 10,000 x g for 60 min. The supernatant fluid was discarded and the precipitate was dissolved in a minimum quantity of standard buffer containing pyridoxyl 5'-monophosphate. This preparation which is stable to overnight storage at 4° was desalted through a column of Sephadex G-25 which had been equilibrated with standard buffer containing pyridoxyl 5'-monophosphate. The desalted enzyme preparation was then carried through the previously described purification procedure. Enzyme purity was monitored with sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Ligand Binding—Ligand binding to threonine deaminase was measured by equilibrium dialysis and ultrafiltration. Ease of operation and frugal utilization of pure enzyme made ultrafiltration (7) the method of choice for most measurements, however, the results were verified by equilibrium dialysis in every case tested. Ultrafiltration membranes (Diaflo XM-20) were purchased from Amicon Corp., Lexington, Mass. Enzyme and ligand were allowed to incubate at room temperature for 10 min prior to filtration in 0.05 M potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.5 mM EDTA. The reaction volume was 0.5 ml of which 0.45 was transferred to the filtration apparatus by Hamilton syringes. The filters were back-washed after filtration at 40 psi of N₂ with 5 ml of ethylene glycol to remove adhered filtration solution. The filters were then counted in 15 ml of a solution containing 1 liter of Triton, 2 liters of toluene, 12 g of 2,5-diphenyloxazole (PPO), and 0.6 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) scintillation fluid using a Beckman LS-233 liquid scintillation system. Equilibrium dialysis was performed at room temperature according to the technique described by Changeux et al. (8). Samples were removed for counting at 4 hours and 8 hours, to be certain equilibrium had been obtained; the 4 and 8 hour readings were identical for each ligand concentration.

Preparation of NaBH₄-reduced Enzyme—Threonine deaminase (5 mg per ml) was dialyzed against standard buffer without isoleucine to remove pyridoxal 5'-monophosphate. Solid NaBH₄ was added directly to the enzyme solution until all color disappeared. The reduced enzyme was dialyzed against 250 volumes of standard buffer without isoleucine. If activity remained at this point the NaBH₄ treatment was repeated. The reduction was monitored spectrophotometrically by the decrease in absorption at 410 nm which is characteristic of the aldimine bond between pyridoxal 5'-monophosphate and a primary amine, and the subsequent increase in absorption at 315 nm which is characteristic of pyridoxal enzymes (9).

Computer Analyses—A Digital Equipment Corp. pdp 11 (Maynard, Mass.) digital computer was used. All linear plots were derived from at least mean square analysis of the data.

Chemicals—Radioactive amino acids were purchased from Schwarz/Mann, Orangeburg, N. Y., or New England Nuclear, Boston, Mass. Ammonium sulfate was Baker's reagent grade. Pyridoxal 5'-monophosphate and dithiothreitol were purchased from Pabst Laboratories, Inc., Milwaukee, Wisc. All other chemicals were reagent grade and were used without further purification.

RESULTS

Properties of Threonine Deaminase from S. typhimurium ilv E-231—The biosynthetic L-threonine deaminase purified from strain ilv E-231 and from strain leu A-124 (10), the standard strain previously employed in this laboratory, are indistinguishable. Comparison of the enzymes with respect to amino acid composition, molecular weight of constituent polypeptide chains, tryptic peptide patterns, and pyridoxal 5'-monophosphate content revealed no differences. The tryptophan content of the enzyme, not previously reported, determined by the method of Bencze and Schmid (11) and by reaction of the carboxymethylated protein with O-nitrophenylsulfenyl chloride (Eastman) according to the method of Scoffone et al. (12) is 5 residues per polypeptide chain.

The extinction coefficient (E₅₅₀ cm) of the native enzyme was determined using amino acid analysis to quantify the protein. The value obtained, 6.4, is significantly different from the one previously reported from this laboratory. The extinction coefficient reported here was obtained with enzyme from both strain leu A-124 and strain ilv E-231 suggesting that the original determination obtained gravimetrically (10) is wrong, probably owing to impurities in the preparation employed. Enzyme concentrations were determined for the present purposes by employing an E₅₅₀ of 6.4.

Effect of L-Isoleucine on Kinetics of Threonine Deaminase Reaction—Fig. 1 depicts the initial velocity of the threonine deaminase reaction as a function of substrate concentration in the presence and absence of L-isoleucine. These results demonstrate that the rate of the reaction has a dependence upon a first order and higher order concentration of threonine in the absence and presence of isoleucine, respectively. This and

Fig. 1. Reaction velocity of purified L-threonine deaminase as a function of substrate concentration. The enzyme was dialyzed against 10° volumes of 0.05 M potassium phosphate, pH 7.5, containing 0.5 mM EDTA and 0.5 mM dithiothreitol to remove L-isoleucine. The enzyme was assayed by direct spectrophotometric measurement of product (A₅₅₀) at 22°. []—[] no addition; O—O, 1.5 μM L-isoleucine; Δ—Δ, 3 μM L-isoleucine; ▲—▲, 6 μM L-isoleucine.

1G. H. Luginbuhl, personal communication.
The enzyme is first order in ligand concentration. The extrapolated number of binding sites is 2 per native tetramer. These results show that in the presence of L-isoleucine and L-valine, the concentration dependence of inhibition by isoleucine remains second order even in the presence of the activating ligand, L-valine. It should be emphasized that this stimulatory effect of L-valine is demonstrable only in the presence of the inhibitor, L-isoleucine, and the levels of L-valine employed here have no effect on the activity of noninhibited enzyme.

Fig. 3 shows the results of a kinetic analysis which was designed to demonstrate the order-dependence of valine concentration on the reversal of isoleucine inhibition. Concentrations of L-isoleucine and L-threonine were selected to permit a second order even in the presence of the activating ligand, L-valine. It should be emphasized that this stimulatory effect of L-valine is demonstrable only in the presence of the inhibitor, L-isoleucine, and the levels of L-valine employed here have no effect on the activity of noninhibited enzyme.

Subsequent kinetic analyses, as well as the ligand-binding experiments to be described, were performed using the same conditions of pH, ionic strength, and temperature to allow more adequate comparison of kinetic and binding data.

**L-Isoleucine Inhibition of Threonine Deaminase Activity and Its Reversal by L-Valine**—Fig. 2 depicts the effects of L-isoleucine and L-valine on threonine deaminase activity. This analysis suggests that inhibition of enzyme activity depends upon [isoleucine]$^i$. These results show that in the presence of L-valine higher concentrations of L-isoleucine are required for maximum inhibition. The inset in Fig. 2 demonstrates that the concentration dependence of inhibition by isoleucine remains second order even in the presence of the activating ligand, L-valine. It should be emphasized that this stimulatory effect of L-valine is demonstrable only in the presence of the inhibitor, L-isoleucine, and the levels of L-valine employed here have no effect on the activity of noninhibited enzyme.

Fig. 3 shows the results of a kinetic analysis which was designed to demonstrate the order-dependence of valine concentration on the reversal of isoleucine inhibition. Concentrations of L-isoleucine and L-threonine were selected to permit a 50% inactivation of enzyme activity and the effect of valine on reversal of inhibition was assessed. The results show that reversal of isoleucine inhibition appears to bear a dependence on a first order concentration of L-valine.

**Binding of L-Isoleucine to Native Threonine Deaminase**—The binding of L-isoleucine to threonine deaminase was determined by equilibrium dialysis and by ultrafiltration. Isoleucine was removed from enzyme solutions prior to use by dialysis against 10$^4$ volumes of 0.05 M potassium phosphate, pH 7.5, containing 0.5 mM EDTA and 0.5 mM dithiothreitol. Fig. 4 shows the amount of L-isoleucine bound to the enzyme as a function of L-isoleucine concentration. These results, which are typical of several independent experiments, show that binding depends on a first order of ligand concentration. The dissociation constant calculated from these data is 3.59 ± 0.29 μM and the extrapolated number of binding sites is 2 per native tetramer.

**Binding of L-Valine to Native Threonine Deaminase**—Binding of the activator ligand, L-valine, to threonine deaminase was measured by experiments similar to those described for L-isoleucine. Fig. 5 shows that the amount of valine bound to the enzyme is first order in ligand concentration. The dissociation constant for valine binding is 26.2 ± 3.6 μM and the extrapolated number of sites per tetramer is 1. This observation was confirmed with several sources of L-[14C]valine and [3H]valine all of which were shown to be radiochemically pure by amino acid analysis. The radioactive valine used in these studies was fully deaminated by L-amino acid oxidase (Worthington) and would not serve as substrate for D-amino acid oxidase.

**Interaction of Isoleucine and Valine Binding Sites**—The kinetic data presented in Figs. 1, 2, and 3 as well as results from several other laboratories (2–4, 13) show that isoleucine and valine are mutually antagonistic with regard to threonine deaminase activity. These kinetic data appear to be consistent with the results of the binding analysis in that two isoleucine sites allow a second order concentration requirement for inhibition of enzyme activity and the single valine site limits the concentration dependence of reversal of inhibition by this ligand to first order. In order to obtain additional insight into
FIG. 5. L-Valine binding to L-threonine deaminase. Binding of L-[¹⁴C]valine to L-threonine deaminase was measured by (▲) equilibrium dialysis and (●) ultrafiltration as described under "Materials and Methods" and Fig. 4. Equilibrium dialysis cells (0.3 ml on each side) contained 2.41 mg per ml of enzyme versus 130,000 cpm of L-[¹⁴C]valine with varying concentrations of L-valine. Ultrafiltration wells contained 0.318 nmol of L-threonine deaminase, 425,000 cpm of L-[¹⁴C]valine plus varying concentrations of L-[¹⁴C]valine. Binding was performed at 22°C.

The relationship between the binding of these ligands, isoleucine binding in the presence of valine was measured. The results of this experiment which are shown in Fig. 6 demonstrate that isoleucine binding remains a first order function of ligand concentration even in the presence of valine. Furthermore, the binding appears to be strictly competitive. The results of a reciprocal binding (i.e., L-valine binding in the presence of L-isoleucine) also revealed what appeared to be strict competitive inhibition; these results are shown in Fig. 7. It is clear from these results that a single residue of L-valine bound to the enzyme is sufficient to exclude 2 residues of L-isoleucine. The data depicted in Fig. 6 do not in themselves reveal whether 1 or 2 residues of L-isoleucine are required to prevent valine binding. These results show, however, that L-valine does not induce cooperative binding of L-isoleucine. In order to determine whether exclusion of valine from the enzyme requires the liganding of 1 or 2 molecules of L-isoleucine the following experiment was performed. Varying concentrations of L-isoleucine were added to solutions containing constant amounts of L-threonine deaminase and radioactive L-valine, these were filtered and the amount of L-valine released from the enzyme (calculated by subtracting the radioactive counts bound to the enzyme in the presence of L-isoleucine from those bound to the enzyme in the absence of L-isoleucine) was plotted as a function of L-isoleucine concentration. Fig. 8 shows the results of this experiment and indicates that L-valine is excluded from the enzyme as a function of L-isoleucine concentration, i.e., 2 residues of L-isoleucine must bind to the enzyme in order to prevent the binding of a single L-valine molecule.

Relationship between Isoleucine and Valine Sites—Consideration of kinetic properties together with the results of the ligand-binding studies leads to two models to explain the relationship among the stereospecific sites which exist on threonine deaminase. If the complexities of precise mechanisms are not considered and if it is assumed that the site on threonine deaminase which is recognized by virtue of L-valine binding is an activator site which can also bind L-threonine, then rather straightforward and simple relationships among the inhibitor (L-isoleucine), activator (L-valine), and active sites can be imagined. Two cases could exist. Case I would require that L-threonine or L-valine bind to the activator site prior to substrate (L-threonine) binding at the active site. Case II would require that liganding of the activator site be necessary solely for overcoming inhibition by L-isoleucine.
therefore at the substrate levels used for typical kinetic obtained for Case I using $K_s = 4.5$ for L-valine (0.03 mM) then the activator site of the enzyme typical substrate concentrations, apparent first order substrate would be saturated at a much lower (-100 times) L-threonine saturation curves are observed only when the $K_s$ is at least two substrate. For example, if the dissociation constant of L-threo- nine for the activator site ($K_a$) is the same as that determined constant and $K_s$ is varied. These plots demonstrate that at greater than one. The observation that kinetic analysis are close to the value of $K_s$ suggest that the experimental possess a dependence upon an order of L-threonine concentra- tion.

According to Case I the threonine deaminase reaction would possess a dependence upon an order of L-threonine concentration greater than one. The observation that kinetic analysis (Fig. 1) does not reveal a high order substrate saturation curve except in the presence of L-isoleucine does not of necessity rule out that activity is a function of a high order concentration of substrate. For example, if the dissociation constant of L-threo- nine for the activator site ($K_a$) is the same as that determined for L-valine (0.03 mM) then the activator site of the enzyme would be saturated at a much lower (~100 times) L-threonine concentration than would the active site ($K_m = 4.5$ mM), and therefore at the substrate levels used for typical kinetic analyses the high order substrate concentration dependence could be obscured. The following relations (14) derived from the Adair equation (15) can be used to describe Cases I and II and to distinguish between them.

\[
N_a = \frac{K_a'(s)}{1 + K_a'(s)}
\]

where $N_a$ is the fraction of activator sites liganded, $K_a'$ is the association constant for the activator ligand (threonine), and $s$ is threonine concentration, and

\[
N_v = \frac{K_v'(s) + 2K_v'(s)^2}{1 + K_v'(s) + K_v'(s)^2}
\]

Since threonine deaminase possesses two active sites $K_1' = 2K_2'$ and $K_2' = 1/2 K_1'$, and

\[
N_v = \frac{2K_v'(s) + 2K_v'(s)^2}{1 + 2K_v'(s) + K_v'(s)^2}
\]

where $N_v$ is the number of active sites liganded per enzyme molecule and $K_v'$ is the association constant of threonine for the active site. Since the limit of Equation 3 is two and the fractional saturation of the enzyme $N_v$, is to be considered then

\[
N_v = \frac{K_v'(s) + 2K_v'(s)^2}{1 + 2K_v'(s) + K_v'(s)^2}
\]

and the velocity ($v$) of the reaction is related to the maximum velocity ($V_{max}$) by

\[
v = \frac{N_v}{N_v + \frac{1}{V_{max}}}
\]

Equation 5 describes Case I, i.e., when liganding the activator site is a prerequisite for catalysis. This same equation describes the pattern of substrate saturation in the presence of L-isoleucine according to Case II; in the absence of L-isoleucine, however, Equation 1 need not be considered and Equation 3 describes Case II. These equations can be employed to compute $K_a$ from the substrate saturation data for the inhibited reactions shown in Fig. 1. As was mentioned above, the value of $K_a$ distinguishes Case I from Case II because the lack of apparent cooperativity in the noninhibited substrate curve requires that $K_a$ be much lower than $K_m$. The following relationship was used to determine $K_a$.

\[
K_{ob} = K_a[1 + \left(\frac{1}{K_1}\right)^2]
\]

This relationship is derived from the competitive nature of inhibitor and activator binding and the observation that two isoleucine molecules are required to exclude a single activator molecule; $K_{ob}$ was determined by a computer-aided curve fitting process and $K_a$ is the dissociation constant of isoleucine calculated from the data in Fig. 4. The $K_a$ values which best fit Curves B, C, and D in Fig. 1 are 1.37, 1.51, 1.32 mM, respectively. These values which are reasonably consistent and are close to the value of $K_a$ suggest that the experimental results are consistent with Case II rather than Case I.

This contention is borne out by Fig. 9 which shows theoretical plots of Case I where $K_{ob}$ which is assumed to equal the experimentally determined $K_m$ (4.5 mM) for threonine, is held constant and $K_a$ is varied. These plots demonstrate that at typical substrate concentrations, apparent first order substrate saturation curves are observed only when the $K_a$ is at least two orders of magnitude less than $K_m$. Curve A in Fig. 9 which is obtained for Case I using $K_a = 4.5$ mM, $K_m = 0.05$ mM is identical with one obtained for Case II, in the absence of isoleucine using $K_a = 4.5$ mM. Curves B, C, and D (Fig. 9) were generated employing the $K_{ob}$ determined by the curve fitting analysis of Curves B, C, and D in Fig. 1; the points in Fig. 9 were transposed from the experimental data in Fig. 1. It is clear that the $K_a$ determined from the experimental data is consistent with Case II, and suggests that the activator site is used to “reactivate” the isoleucine-inhibited enzyme. This model, which specifies ambivalent recognition of threonine and valine by the activator site, and which adequately explains high order substrate saturation curves in the presence of L-isoleucine as well as the ability of valine to restore first order substrate saturation curves, is supported by the observation described below.

**Ligand-binding to Borohydride-reduced Holoenzyme**—The models described above are based on the supposition that threonine binds the site on threonine deaminase originally

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**Fig. 8.** Inhibition of L-valine binding to L-threonine deaminase as a function of L-isoleucine concentration. Binding was measured by ultrafiltration, each well containing 0.525 nmol of L-threonine deaminase and 60 µM L-valine (9.75 × 10^6 cpm per µmol). Counts per min bound at each L-isoleucine concentration was subtracted from counts per min bound in the absence of L-isoleucine to determine counts per min released. a, release of L-valine from L-threonine deaminase as a function of L-isoleucine concentration. b, release of L-valine from L-threonine deaminase as a function of [l-ile]^p.

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\[
N_v = \frac{2K_v'(s) + 2K_v'(s)^2}{1 + 2K_v'(s) + K_v'(s)^2}
\]
recognized as a valine-binding site. Unfortunately, it is not possible to study threonine binding to native threonine deaminase owing to the rapid catalysis of this substrate. However, an enzymatically inactive derivative of native threonine deaminase can easily be prepared. Treatment of threonine deaminase with borohydride specifically reduces the aldimine bond through which the cofactor pyridoxal 5'-monophosphate is linked to the enzyme (Fig. 10). This treatment causes total inactivation of the catalytic site and appears to be highly specific since similar reduction of apoenzyme followed by removal of excess borohydride and addition of pyridoxal 5'-monophosphate results in complete restoration of activity. Borohydride-treated enzyme was prepared as described under "Materials and Methods" and used in binding studies. Figs. 11 and 12 show the results of isoleucine and valine binding to the borohydride-reduced derivative of the enzyme; the results are qualitatively and quantitatively identical with those obtained with the native enzyme. The availability of an inactive derivative of threonine deaminase in which the native properties of the isoleucine and valine sites are preserved presents the opportunity to determine whether or not threonine is capable of binding at the "valine site." Initial attempts to measure threonine binding directly yielded unsatisfactory results which suggested that the affinity of this ligand for the borohydride-reduced derivative of the enzyme is less than predicted by Case I. It was therefore determined whether or not threonine competitively inhibits valine binding. Fig. 13 shows the effect of L-threonine on the binding of valine. These results show that the binding of these ligands is competitive and suggest they bind to a common site. The $K_s$ of threonine computed from these data is 3 mM and is in close agreement with the value obtained from the kinetic analysis as applied to Case II cited above.

**DISCUSSION**

The foregoing results suggest that the catalytic activity of threonine deaminase is modulated by the interaction of the enzyme with 2 molecules of negative effector and a single molecule of positive effector and that the binding of these

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**Fig. 9.** Theoretical substrate saturation curves for L-threonine deaminase. Curves were generated from the equations described in the text. The points on these graphs are data points transposed from Fig. 1. A, $K_a = 0.05$ mM, $K_s = 4.53$ mM; B, $K_a = 1.61$ mM, $K_s = 4.53$ mM; C, $K_a = 2.57$ mM, $K_s = 4.53$ mM; D, $K_a = 5.01$ mM, $K_s = 4.53$ mM.

**Fig. 10.** Absorption spectrum of (A) holothreonine deaminase, pH 7.5, and (B) NaBH₄-reduced L-threonine deaminase, pH 7.5.

**Fig. 11.** L-Isoleucine binding to NaBH₄-reduced L-threonine deaminase. Binding of L-[¹⁴C]isoleucine was measured by ultrafiltration analysis. Wells contained 0.24 nmol of reduced enzyme, 296,000 cpm of L-[¹⁴C]isoleucine, and varying concentrations of L-[¹⁴C]isoleucine.

**Fig. 12.** L-Valine binding to NaBH₄-reduced L-threonine deaminase. Binding of L-[¹⁴C]valine was measured by ultrafiltration analysis. Each well contained 0.24 nmol of reduced enzyme, 296,000 cpm of L-[¹⁴C]valine, and varying concentrations of L-[¹⁴C]valine.
ligands is competitive. As a result of ligand-binding experiments the possibility is raised that reversal of L-isoleucine-induced inhibition by L-valine and L-threonine is wrought through the mediation of an activator site, and that association of either of these ligands with the enzyme prevents isoleucine binding. It seems reasonable, on the basis of the results presented here, that the active sites play no role in reversal of inhibition, but rather, that the activator site is primarily involved.

Our results suggest that the activator site of threonine deaminase is required solely for release from inhibition and in the absence of inhibitor the active sites are operational without liganding the activator site. The question arises as to why this enzyme requires an activator site to relieve isoleucine inhibition. One plausible explanation has its basis in the observation that threonine deaminase is a hysteretic enzyme (16) and the time required for the isomerization of the inactive form of the enzyme to an active form is relatively long. This fact means, as pointed out by Frieden (17), that even in the presence of low levels of inhibitor, the enzyme remains poised (buffered) in the inactive conformation. In such a case little if any enzyme would be in a form where the active sites are accessible for liganding substrate therefore permitting disruption of an equilibrium between active and inactive enzyme. It appears as if the most efficient manner for reversing inhibition under these circumstances is to prevent inhibitor binding by an event which concomitantly induces a conformational change to produce an active form of the enzyme. Although this paradigm is subject to verification, it is consistent with what is presently known about threonine deaminase.

It is clear from the results presented here that a concerted transition mechanism ought to appear as cooperative does not. Although the apparent reciprocal relationship between isoleucine inhibition and restoration of enzyme activity by threonine appears consistent with a concerted transition the inconsistencies cited above demand an alternate explanation. We have shown that threonine and valine bind to a common site on the enzyme which appears to be distinct from the active site. This site is most reasonably viewed as an activator site. According to Case II described under "Results," enzyme activity in the presence of inhibitor depends upon two events one of which is contingent upon the other but both depending upon a first order concentration of ligand; with appropriate respective dissociation constants the second binding event, which is recognized as catalysis, demonstrably depends upon an order of concentration of ligand greater than one, but less than two. On the other hand we have shown that the exclusion of activator requires the binding of 2 molecules of isoleucine and therefore bears a dependence on [L-isoleucine]². These two high order concentration-dependent phenomena have different bases and therefore are not reciprocal.

The question arises as to the precise relationship between the inhibitor sites and the activator site and specifically whether or not they are composed of the same or different structural elements within the protein. The observations that 2 isoleucine molecules must bind to the enzyme in order to exclude a single valine molecule and that neither L-valine nor L-threonine induce cooperative binding of L-isoleucine are most reasonably interpreted as involving induced conformational changes. The question remains, however, whether this mutual exclusion involves two different (allosteric) or the same (isosteric) sites. The stoichiometry of isoleucine and valine binding and what appears in both kinetic and ligand-binding analyses to be a strictly competitive relationship must be considered as related properties, furthermore, these properties are probably related to the fact that tetrameric threonine deaminase possesses two cofactor residues. Many enzymes are now known in which the number of stereospecific sites is less than the number of constituent polypeptide chains, and the mechanisms involved in explanation of this condition have been discussed (19). The observation that threonine deaminase is composed of four identical polypeptide chains but possesses only 2 residues of cofactor has been reconciled with the identification of dimeric protomers and evidence has been presented which suggest that these dimers constitute the interacting protomers within the holotetramer (20). The tentative identification of the protomers gave rise to the possibility that the subunits composing the dimers are asymmetrically arranged but that the dimers are symmetrically arranged within the tetramer so as to provide two rather than four cofactor sites. Although this reasoning also can be applied as an explanation for the two isoleucine sites other possibilities also must be considered. It is possible that association of pyridoxal 5'-monophosphate with the enzyme is strongly anticooperative (21) and that conformational or other changes which obscure the remaining potential cofactor sites also obscure two of the isoleucine sites, or alternatively, that the obscured cofactor sites are the isoleucine sites. These latter views are supported by the results of preliminary binding experiments which suggest that apo-threonine deaminase is capable of binding 4 rather than 2 isoleucine molecules.² The observation that threonine deaminase binds a

²C. Decedue, unpublished observation.
single activator ligand could be explained by an extension of the notion of anticooperativity, i.e. the holoenzyme possesses two potential activator sites which display extreme anticooperativity. Owing to the apparent strict competitive relationship between activator-ligand and isoleucine binding it is tempting to speculate that this hypothetical anticooperativity is exerted by binding of activator ligand to one of the isoleucine sites. This notion is supported by the observation that the enzyme must bind 2 molecules of isoleucine in order to prevent binding of activator ligand. In other words, liganding of a single isoleucine molecule leaves another isoleucine site which also is available for valine or threonine binding. The presence of activator ligand at the isoleucine site then triggers a conformation change which expels the single isoleucine molecule by the same mechanism which obscures the other potential activator-binding site. Continued binding studies using apoenzyme hopefully will clarify this relationship.

The physiological significance of the specificity of the activator site for L-threonine lies in the apparent fact that it endows threonine deaminase with those properties which are thought to be required for the proper integration of an enzyme into the total metabolism of the organism; i.e. in the presence of low levels of isoleucine, the enzyme responds to a high order concentration of substrate, thus allowing maintenance of a level of threonine sufficient for protein synthesis. The second order concentration-dependence of isoleucine inhibition prevents autointoxication of the pathway while maintaining a pool level of the end product sufficient for protein synthesis. The observation that L-valine exhibits a greater affinity for the activator site than does L-threonine suggests that it also must play a significant physiological role. It is of interest in this regard that valine causes threonine deaminase to be less sensitive to isoleucine inhibition but nonetheless, under conditions of appropriate ligand concentration, permits a high order dependence on substrate. The significance of the valine effect may be related to the fact that isoleucine and valine are synthesized via parallel pathways (22) and that acetohydroxy acid synthetase, isomeromutase, dihydroxyacid dehydrase, and transaminase B use the product of the threonine deaminate reaction, a-ketobutyrate, as well as pyruvate as substrates or as source of substrates. This pattern of biosynthesis means that isoleucine and valine biosynthetic intermediates are competitive substrates for these four enzymes. The activator role of valine would provide a mechanism for insuring a balanced flow of the valine and isoleucine intermediates through this common pathway.

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