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Threonine Deaminase from Salmonella typhimurium

EFFECT OF REGULATORY LIGANDS ON THE BINDING OF SUBSTRATES AND SUBSTRATE ANALOGUES TO THE ACTIVE SITES AND THE DIFFERENTIATION OF THE ACTIVATOR AND INHIBITOR SITES FROM THE ACTIVE SITES*

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Biosynthetic threonine deaminase, EC 4.2.1.16, displays a positive circular dichroism with a maximum rotation at 410 nm owing to the aldimine-bonded cofactor pyridoxal 5'-monophosphate. The substrates, L-threonine, L-allothreonine, and L-serine, and the competitive inhibitors of enzyme activity, D-threonine, D-allothreonine, and D-serine, cause a loss of circular dichroism presumably because of transfer of the aldimine bond from the protein to these ligands. The "allosteric" effector ligands, L-isoleucine and L-valine, have no effect on the optical activity of the enzyme. By employing these features of the enzyme, it is shown that L-isoleucine prevents the L-threonine- and L-allothreonine-imparted loss of circular dichroism but does not prevent the D-threonine-, D-allothreonine-, D-serine-, or L-serine-imparted loss of optical activity. These results imply that the inhibition of enzyme activity by L-isoleucine, depending upon the substrate, is explicable in terms of perturbation of either initial substrate binding or the catalytic mechanism per se, i.e., L-isoleucine exerts a K effect when either L-threonine or L-allothreonine serves as substrate but a V effect when L-serine is substrate. These results also definitively demonstrate the separation of the inhibitor and active sites of biosynthetic L-threonine deaminase.

The separation of the activator site and active sites of biosynthetic threonine deaminase is demonstrated by the inability of L-valine to compete with L-threonine in causing loss of circular dichroism. It is shown that L-serine mimics L-threonine as a substrate for L-threonine deaminase, i.e., the reaction velocity becomes a high order function of L-serine concentration in the presence of L-isoleucine. Loss of circular dichroism in the presence of L-isoleucine, however, is a first order function of L-serine concentration. These results together with the observation that L-valine overcomes isoleucine inhibition of the enzyme reaction with L-serine as substrate show that the modulation of the activity of L-threonine deaminase is wrought by the interaction of activator and inhibitor sites and also demonstrates that the activator site is separate from the active sites.

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conversion to product subsequent to binding. The classical characterization of biosynthetic L-threonine deaminase as a K system (4) could be based in the relationship between the activator and inhibitor sites described above, which leaves open the question regarding the manner in which l-isoleucine inhibits enzyme activity.

In order to explore this problem, circular dichroic measurements were employed to directly monitor the interaction of substrates and substrate analogues with the active sites of L-threonine deaminase. Circular dichroic measurements have been used to monitor the interaction of substrates and substrate analogues with a number of enzymes that contain pyridoxal 5'-phosphate as cofactor. For example, Nakazawa et al. (5) and Niederman et al. (6) have employed circular dichroism to study the interaction of L-threonine and substrate analogues with the degradative l-threonine deaminase from Escherichia coli. These measurements are based upon the following properties of enzymes which contain pyridoxal 5'-monophosphate as cofactor. The union of pyridoxal 5'-phosphate to a protein usually involves the formation of an aldime bond between the cofactor and an ε-amino group of a lysyl residue in the protein; this adduct is a chromophore which displays optical activity. If, in the course of ligand binding, the aldime bond is transferred to ligand (substrate), the chromophore is preserved, but optical activity is lost (Fig. 1).

Previous results have shown that biosynthetic L-threonine deaminase from S. typhimurium exhibits a positive extrinsic Cotton effect in its optical rotatory dispersion spectrum owing to the presence of the aldime-bound cofactor, pyridoxal 5'-monophosphate (2). We will show in this report that this optical activity is expressed as a positive circular dichroism with a maximum rotation at 410 nm which is lost when substrates or substrate analogues bind to the active site of threonine deaminase. We have employed this technique to further clarify the relationship among the inhibitor, activator, and active sites of biosynthetic L-threonine deaminase.

MATERIALS AND METHODS

Enzyme Purification-Biosynthetic L-threonine deaminase was prepared from Salmonella typhimurium, strain 11cE231, as previously described (3).

Enzyme Assay—Threonine deaminase was assayed by direct measurement of product formation at 235 nm using a Gilford 2400-S spectrophotometer (3); the reaction mixtures contained 0.1 mm potassium phosphate, pH 7.5, and substrates or substrate and inhibitors at the concentrations indicated in the text.

Circular Dichroism Measurements—A Cary model 60 spectrophotometer with a CD/ORD attachment was used. All measurements were made in 1.0-cm round cuvettes at an enzyme concentration of approximately 5 mg/ml. The enzyme had been dialyzed overnight against several changes of 0.1 m potassium phosphate, pH 7.5, containing 0.5 mm ethylenediaminetetraacetate and 0.5 mm dithiothreitol.

Preparation of D,L-Allothreonine—The D- and L-allothreonines were obtained from a racemic mixture by enzymatic means (7). The isolation of the D isomer was achieved with hog kidney D-amino acid oxidase (Worthington). The reaction mixture contained 10 mmol of D,L-allothreonine (ICN Pharmaceuticals, Inc.), 200 μg of D-amino acid oxidase, and 100 μg of catalase in 0.02 m sodium pyrophosphate adjusted to pH 8.3. The mixture was placed in a water bath at 37° and gently aerated by swirling. After 7 h incubation, an additional portion of D-amino acid oxidase and catalase was added and the reaction allowed to proceed overnight. The L-allothreonine was removed by a similar procedure using Ophiophagus hanahk toxin (Miami Serpentarium Laboratories). The reaction mixture contained 10 mmol of D,L-allothreonine, 5 mg of toxin, and 100 μg of catalase in 0.05 m Tris/HCl adjusted to pH 8.0. The reaction mixture was incubated as described above. After 7 h incubation, additional portions of toxin and catalase were added and the reaction allowed to proceed overnight.

In both cases, the formation of α-ketobutyrate was monitored as the 2,4-dinitrophenyl hydrazone (8). Each mixture was treated as follows. The mixtures were adjusted to pH 6.5 with dilute HCl and loaded onto a column of Dowex AG50W-8x (50 to 100 mesh). The column was then washed with water until the effluent contained no α-keto acid and the amino acid was eluted with 1 m ammonium hydroxide. The eluent was evaporated to dryness and the residue dissolved in a minimum volume of hot water; the amino acid was crystallized by addition of ethanol (7 volumes) and the crystals were dried with ether. The samples were recycled through the entire resolution procedure and finally checked for optical purity by the use of the D-amino acid oxidase and the venom. Thin layer chromatography of the resolved samples on microcrystalline cellulose plates, developed with a solvent system containing 1-butanol:methyl ethyl ketone:H2O:ammonium hydroxide (5:2:1:1) and which is known to resolve all-threonine and threonine (9), shows that each sample is approximately 5% contaminated with threonine.

Kinetic Parameters for L-Allothreonine—The Ks for L-allothreonine was estimated by measuring the tangential slope of a continuous reaction at the concentrations of L-allothreonine shown in Fig. 6. This procedure was made necessary by the apparent low affinity of the enzyme for L-allothreonine as well as by an appreciable level (~5%) of contamination with L-threonine. The reaction mixture contained 0.1 mm potassium phosphate, pH 7.5, and the reaction was initiated with 8 mm L-allothreonine. The reaction was allowed to proceed until the contaminating L-threonine was depleted as shown by thin layer chromatography (9) of a duplicate reaction mixture. The concentration of L-allothreonine at various times during the reaction was calculated by difference; the reaction was allowed to go to completion and the total concentration of L-allothreonine present following depletion of the L-threonine was determined by using the relationship that one absorbance unit at 235 nm equals 5.6 μmol of α-ketobutyrate.

RESULTS

Effect of l-Isoleucine and L-Valine on Circular Dichroic Spectrum of L-Threonine Deaminase—Fig. 2 depicts the circular dichroic spectrum of biosynthetic L-threonine deaminase in the 300 to 500 nm range and shows a maximum rotation at 600 nm which corresponds to the absorption maximum of the aldime-bound pyridoxal 5'-monophosphate chromophore at this wavelength; the peak value in the circular dichroic
spectrum corresponds to $+1.17 \times 10^5$ dm/dmol of pyridoxal 5'-monophosphate. The circular dichroic spectrum is unchanged by the addition of a concentration of l-isoleucine (1 mM) or l-valine (10 mM) which is saturating for kinetic parameters.

Effect of Threonine on Circular Dichroic Spectrum—
Owing to the fact that the high concentration of enzyme required for circular dichroic analysis rapidly converts L-threonine to $\alpha$-ketobutyrate and ammonia, it was not feasible to determine the effect of this substrate within the range of wavelengths where optical activity is observed in the unliganded enzyme. A rapid measurement of the effect of this substrate on the circular dichroism can be made by monitoring the optical activity at 410 nm. Fig. 3 shows that L-threonine causes a loss of optical activity which returns as this substrate is catalytically depleted by the enzyme. The results of a similar analysis, also presented in Fig. 3, show that the optical activity is preserved when l-isoleucine is present in the reaction mixture. The inability of the aldime bond to be transferred from the enzyme to the substrate when isoleucine is present suggests that this inhibitor prevents interaction of L-threonine with the active site of the enzyme. The possibility exists, however, that transfer of the aldime bond to the substrate occurs concomitantly with the formation of the transition state intermediate which is produced in the course of catalysis in which case inhibition of enzyme activity by L-isoleucine need not involve a perturbation of initial binding of the substrate but rather inhibition of the catalytic mechanism per se. Nakazawa et al. (5), studying the degradative L-threonine deaminase from Escherichia coli, have shown that the "dead-end" competitive inhibitor, d-threonine, causes a loss of optical activity which demonstrates, in the degradative enzyme, that transfer of the aldime bond and catalysis are separable events. The effect of d-threonine on the optical activity of biosynthetic L-threonine deaminase was determined by measuring the effect of this inhibitor on the circular dichroism in the range of 300 to 500 nm. The results of this analysis show that d-threonine causes a complete loss of optical activity which indicates that the transfer of the aldime bond from the enzyme is not necessarily followed by a catalytic event. As in the case of L-threonine, the transfer of the aldime bond from the enzyme to D-threonine is reversible. A 200-fold dilution of the enzyme-d-threonine mixture into reaction mixture containing L-threonine as substrate and without added pyridoxal 5'-monophosphate resulted in identical rates of catalysis; this also shows that the loss of optical activity is not caused by a d-threonine-promoted resolution of cofactor from the enzyme. Standard kinetic analysis indicates that d-threonine is a dead-end competitive inhibitor of biosynthetic L-threonine deaminase. The data depicted in Fig. 4A show that inhibition of activity is a first order function of d-threonine concentration; this observation is consistent with d-threonine acting at the active site rather than the inhibitor (L-isoleucine) site because inhibition of enzyme activity by l-isoleucine is a second order function of concentration (3). The $K_i$ computed from these data is 1.9 (±1.9) mM. Fig. 5 shows the effect of d-threonine on the optical activity of threonine deaminase expressed as loss of circular dichroism at 410 nm as a function of amino acid concentration; the change in circular dichroism was monitored at 410 nm. The enzyme (5 mg/ml) was dissolved in 0.05 M potassium phosphate, pH 7.5, in the presence of 2 mM L-threonine without (-- --) and with (---) 2 mM L-isoleucine.

Fig. 2. Circular dichroic spectrum of L-threonine deaminase. The circular dichroism as related to concentration of aldime bond present was measured as described under "Materials and Methods..." The enzyme and ligands were dissolved in 0.05 M potassium phosphate, pH 7.5, 0.5 mM ethylenediaminetetraacetate, and 0.5 mM dihydroferritin. No ligand; -- - - , 2 mM L-isoleucine; ---- , 5 mM L-valine.

Fig. 3. The circular dichroism of L-threonine deaminase as a function of time in the presence of L-threonine with and without L-isoleucine. The change in circular dichroism was monitored at 410 nm. The enzyme (5 mg/ml) was dissolved in 0.05 M potassium phosphate, pH 7.5, in the presence of 0.2 mM L-threonine without (---) and with (----) 2 mM L-isoleucine.

Fig. 4. Inhibition of threonine deaminase activity by d-threonine (A), d-allo-threonine (B), and d-serine (C). The reaction mixtures contained 0.1 M potassium phosphate, pH 7.5, 20 mM L-threonine, and the inhibitors at concentrations shown. The rates of product formation were measured spectrophotometrically at 233 nm. The inhibition constants were computed from these graphs employing the relationship $K_{i} = K \times [1 + ([s]/K_{m})]$. 

1 J. G. Hofler, unpublished observations.
Threonine Deaminase Regulatory Sites-Active Sites Interaction

Activity of Threonine Deaminase—It has been reported that similar measurements were made using other compounds extracted from this analysis, is presented as a single point in Fig. 5. These results indicate that whereas L-isoleucine prevents binding of L-threonine it does not prevent binding of D-threonine to the active site of L-threonine deaminase.

In order to explore the basis for the differential effect of L-isoleucine on the binding of L-threonine and D-threonine, similar measurements were made using other compounds which bind to the active sites of threonine deaminase.

Effect of D- and L-Allothreonine on Enzymatic and Optical Activity of Threonine Deaminase—It has been reported that D-allothreonine is a dead-end competitive inhibitor of biosynthetic L-threonine deaminase (10). The only commercially available allothreonine is the racemic mixture, therefore, this was enzymatically resolved into its D and L-components as described under "Materials and Methods." Preliminary examination of the D and L isomers for inhibitory properties revealed that the L isomer is, in fact, a substrate for biosynthetic L-threonine deaminase (Fig. 6). The D-allothreonine was shown, by conventional kinetic analysis, to be a competitive inhibitor. The data depicted in Fig. 4B show that inhibition of enzyme activity is a first order function of D-allothreonine concentration with a $K_i$ of 0.19 mM, and, as in the case of D-threonine, support the conclusion that this analogue inhibits by interaction with the active site rather than with the inhibitor site. The relative affinities of the allothreonines explain the previously observed inhibitor properties of the DL mixture; i.e. the affinity of D-allothreonine for the enzyme is 3 orders of magnitude greater than is that of L-allothreonine.

The ability of the D and L isomers of allothreonine to cause the loss of 300 to 500 nm circular dichroism of L-threonine deaminase was tested. These compounds exhibit the same properties as do the threonine isomers; L-allothreonine causes an immediate loss of optical activity which returns as the substrate is removed by the action of the enzyme and D-allothreonine causes a loss which is typical of that seen with D-threonine. The ability of L-isoleucine to prevent the allothreonine-imparted loss of optical activity was tested; L-isoleucine prevents the loss of optical activity caused by L-allothreonine, whereas it does not prevent the loss of optical activity caused by D-allothreonine. Presumably, L-isoleucine prevents the binding of L-allothreonine, but not the binding of D-allothreonine to the active site. Fig. 7 shows the loss of circular dichroism as a function of allothreonine concentration; the $K_i$ determined from these data is 0.61 (±0.31) mM. These results are identical in the presence or absence of 2 mM L-isoleucine, as is shown in Fig. 7 by the single point which was obtained as in the previous case with D-threonine.

Effect of D- and L-Serine on Enzymatic and Extrinsic Optical Activity of L-Threonine Deaminase—It has been known for some time that L-serine is a substrate for L-threonine deaminase (11). The kinetic analysis with L-serine as substrate, shown in Fig. 8, indicates that this substrate mimics L-threonine. The rate of catalysis is a first order function with respect to L-serine concentration in the absence of L-isoleucine, becomes higher order in the presence of the allosteric inhibitor, and L-valine restores the first order relationship. The maximal rate of conversion of L-serine to pyruvate is the same as that for conversion of L-threonine to a-ketobutyrate. The major difference is that L-serine does not

![Fig. 5. The circular dichroism of L-threonine deaminase as a function of D-threonine concentration and the effect of L-valine and L-isoleucine on the D-threonine-promoted loss of circular dichroism. The loss of circular dichroism at 410 nm was measured as a function of D-threonine concentration. The titration mixture contained 0.1 M potassium phosphate, pH 7.5, and enzyme (5 mg/ml). The inset scale represents the results of a titration of the enzyme in the presence of 3.7 mM D-threonine with L-valine. The D-threonine titration curve has one point (o) (the second from the left) in the presence of 2 mM L-isoleucine.](http://www.jbc.org/)

![Fig. 6. Reaction velocity of L-threonine deaminase as a function of L-allothreonine concentration. The reaction rate was measured as the tangential slope of a continuous reaction at the indicated concentration of L-allothreonine as described under "Materials and Methods." The reaction mixture contained 0.1 M potassium phosphate and the reaction was initiated with 8 mM L-allothreonine.](http://www.jbc.org/)

![Fig. 7. The circular dichroism of L-threonine deaminase as a function of D-allothreonine and the effect of L-isoleucine on the D-allothreonine-promoted loss of circular dichroism. The loss of circular dichroism at 410 nm was measured as a function of D-allothreonine concentration. The titration mixture contained 0.1 M potassium phosphate, pH 7.5, and enzyme (5 mg/ml). The titration curve contains one point (o) in the presence of 2 mM L-isoleucine.](http://www.jbc.org/)
The basis for the differential effect of L-isoleucine on the optical activity of the substrates and competitive inhibitors with the active sites. These results, which show that L-valine does not prevent the loss of circular dichroism caused by D-threonine, D-allotrhreonine, or D- and L-serine; these observations show that L-isoleucine does not compete with the binding of these compounds to the active site of threonine deaminase. The data presented in Fig. 8 which shows that L-isoleucine is an efficient inhibitor of enzyme activity when L-serine is the substrate, together with the observation that L-isoleucine does not prevent the binding of L-serine, provides convincing evidence for the complete separation of the inhibitor sites and active sites of L-threonine deaminase. The data depicted in Fig. 5 demonstrate that the activator site is also separate from the active sites. These results, which show that L-valine does not prevent the L-threonine-specific loss of circular dichroism, indicate that this activator ligand does not bind to the active sites of threonine deaminase.

**Differentiation of Activator and Inhibitor Sites from Active Sites of Biosynthetic L-Threonine Deaminase—**The results presented in Fig. 2 show that neither L-valine nor L-isoleucine causes the loss of the circular dichroism of threonine deaminase. Also, L-isoleucine does not prevent the loss of circular dichroism caused by D-threonine, D-allotrhreonine, or D- and L-serine; these observations show that L-isoleucine does not compete with the binding of these compounds to the active site of threonine deaminase. The data presented in Fig. 8 which shows that L-isoleucine is an efficient inhibitor of enzyme activity when L-serine is the substrate, together with the observation that L-isoleucine does not prevent the binding of L-serine, provides convincing evidence for the complete separation of the inhibitor sites and active sites of L-threonine deaminase. The data depicted in Fig. 5 demonstrate that the activator site is also separate from the active sites. These results, which show that L-valine does not prevent the L-threonine-specific loss of circular dichroism, indicate that this activator ligand does not bind to the active sites of threonine deaminase.

**DISCUSSION**

The basis for the differential effect of L-isoleucine on the ability of the substrates and competitive inhibitors to bind to the active sites is not entirely clear. The stereospecificity of the active sites of biosynthetic L-threonine deaminase is assumed to result from transfer of the aldime bond from the protein to the amino group of these compounds. The possibility exists, however, that the observed loss of optical activity is the consequence of a conformational change in the protein (12). The results of the present study show that only those compounds which are substrates or competitive inhibitors of enzyme activity cause loss of circular dichroism, whereas the allosteric effectors, L-valine and L-isoleucine, both of which most probably are effective by causing conformational changes, do not affect the circular dichroism of the enzyme. It seems reasonable to assume that the observed change in optical activity is because of the removal of the aldime bond from the protein. This view is supported by the work of Rabinowitz et al. (13) which shows that competitive inhibitors of degradative L-threonine deaminase from *Escherichia coli* which do not contain α-amino groups are unable to cause a loss in circular dichroism. Regardless of the basis for the effect, it is clear that the loss of circular dichroism is the consequence of interaction of the substrates and competitive inhibitors with the active sites.

The basis for the differential effect of L-isoleucine on the ability of the substrates and competitive inhibitors to bind to the active sites is not entirely clear. The stereospecificity of the active sites of biosynthetic L-threonine deaminase is relatively indiscriminate as shown by the ability of all four isomers of threonine and the two isomers of serine to cause loss of circular dichroism. Examination of the space filling models depicted in Fig. 11, although failing to reveal the
functions which may be related to the substrate nature of which lacks a methyl group. This line of reasoning appears to be inconsistent with the $K_m$ and $K_I$ values for $n$-serine and way to appear remarkably similar with the methyl group of nine. Fig. 11 shows $L$-serine and $L$-allothreonine oriented in a this compound. The substrates, $L$-serine and $L$-allothreonine, have $K_m$ values considerably higher than the one for $L$-threonine. Fig. 11 shows $L$-serine and $L$-allothreonine oriented in a way to appear remarkably similar with the methyl group of $L$-allothreonine partially eclipsed so as to appear like $L$-serine which lacks a methyl group. This line of reasoning appears to be inconsistent with the $K_m$ and $K_I$ values for $d$-serine and $n$-allothreonine which are nearly 2 orders of magnitude less than those for the respective $L$ isomers and where the sole similarity appears to be the orientation of the amino and hydroxyl groups. The basis for the relatively avid binding of the $d$ isomers is not clear. It is unlikely that the $K_m$ values for $L$-serine and $L$-threonine do not reflect the true $K_d$ values for these compounds. The direct measurement of the $K_d$ for $L$-serine in the presence of $L$-isoleucine (Fig. 10) as measured by loss of circular dichroism gives a value similar to the $K_m$. Also, the observation that threonine deaminase is inactive on the enantiomeric mixture of allothreonine is consistent with the $K_d$ of $L$-allothreonine being considerably higher than the $K_d$ for $d$-allothreonine which was measured directly. A possible basis for the avid binding of the $d$ isomers is the orientation of the carboxylate ion which may be capable of electrostatic interaction with the protonated amino group which is made available on the protein as a consequence of the transfer of the aldimine bond from the protein to the amino group of the inhibitor.

The differential $K$ and $V$ (4) effect must be based in the structural attributes of the threonines and serines as well as in the conformational change in the protein exerted by $L$-isoleucine. The conformational change which prevents the catalytic function of the active site could simultaneously result in steric hindrance to those compounds with $\beta$ substituents which are properly oriented relative to the functional binding groups on the molecules. Space filling models of the compounds under consideration show that the methyl group of $L$-threonine and $L$-allothreonine could provide hindrance to the intrusion of these molecules into the active sites when the enzyme is in the catalytically inactive conformation. Serine lacks the methyl group and $L$-threonine and $L$-allothreonine can be pictured as having methyl groups oriented away from the binding plane of these molecules. A separate line of evidence is available to suggest a role for $\beta$ substituents in binding to biosynthetic $L$-threonine deaminase. Two altered biosynthetic threonine deaminases have been purified from mutants of Salmonella typhimurium which were selected on the basis of containing feedback negative threonine deaminases. These enzymes, in addition to showing a decreased sensitivity to inhibition by $L$-isoleucine, also show a 10-fold increase in $K_m$ for $L$-threonine but a slight decrease in $K_m$ for $L$-serine. These results point either to a perturbation of a methyl group binding function or to a weaker version of the steric exclusion of the methyl group which, as pointed out above, may be linked functions.

The data presented in Table I present a subtle but interesting contrast between those values which were determined as the basis of enzymatic activity ($K_d$ and $K_m$) and those based upon loss of circular dichroism ($K_d$). It is of interest to note that the $K_d$ values which were measured by change in a physical property are approximately 2-fold higher than the $K_m$ values (as well as the one $K_m$ where the comparison is available). The $K_d$ values, $K_m$, and $K_I$ values were determined under the same conditions of ionic strength and temperature with the sole difference in conditions being the enzyme concentration employed; the concentration of enzyme in the $K_d$ measurements was 3 to 4 orders of magnitude greater than that used in typical enzyme activity measurements. Although the large difference in enzyme concentration may be the basis for the observed differences, other factors also must be considered. It is well established that Michaelis' constants do not necessarily correspond to dissociation constants and this or the fact that the $K_d$ for $L$-serine was determined in the presence of $L$-isoleucine could explain the difference between the $K_m$ and $K_d$ for $L$-serine. The situation with the competitive inhibitors, however, is different since $K_d$ and $K_m$ values should be equal. A possible explanation for the observed difference in the two sets of parameters is a type of "half of the site" activity (14). In the case of the competitive inhibitors, the liganding of one of the two active sites could be sufficient to

| Ligand        | $K_m$   | $K_I$   | $K_d$   |
|---------------|---------|---------|---------|
| $L$-Threonine | 4.5 (±0.5)* |         |         |
| $d$-Threonine | 88 (±6.0) |         |         |
| $L$-Serine    | 90 (±5.0) |         | 147 (±14.0) |
| $d$-Serine    | 7.4 (±1.9) | 25.8 (±9.3) |         |
| $L$-Allothreonine | 0.19 (±0.00) | 0.61 (±0.31) |         |
| $d$-Allothreonine | 2.4 (±1.0) | 3.7 (±0.7) |         |

* From Decedue et al. (3).
inhibit the enzyme and in the case of the substrate, although both active sites are capable of binding ligand, only one of the sites could be catalytically active. In contrast, if the loss of circular dichroism is the result of aldimine bond transfer, then both pyridoxal 5' monophosphates must be transferred in order to obtain total loss of optical activity. According to these speculations, the enzyme activity employed to determine the $K_m$ and $K_a$ would be the expression of only one of two equivalent sites each with the same probability of liganding the effector molecule, whereas in the case of the $K_d$ measurements, the loss of circular dichroism is a function of the liganding of both sites. Therefore, probability considerations dictate that since there are two ways to realize a single event when measuring $K$ or $K_m$ and only one way to decrease the circular dichroism, then the $K_a$ and $K_m$ values should be approximately one-half of the $K_d$ values. Efforts are currently under way to determine whether or not a type of half site activity is, in fact, operative in threonine deaminase.

The results presented in the present investigation provide additional insight into the relationship among the stereospecific sites on biosynthetic L-threonine deaminase. The inability of L-valine to compete with n-threonine in binding to the active site (Fig. 5) shows that the activator site and active sites are separate. This is further supported by the observation that D-threonine does not compete with L-valine in direct binding experiments. The results of the kinetic analysis and the circular dichroism measurements with L-serine provide proof of the allosteric nature of L-threonine deaminase as well as the existence of an activator site. The inability of L-isoleucine to prevent the binding of L-serine but yet prevent its enzymatic conversion to pyruvate and ammonia shows that the inhibitor sites are separate from the active sites. The observation that the loss of circular dichroism is a first order function of L-serine concentration in the presence of L-isoleucine is a high order function of L-serine concentration shows that L-serine must be binding to a site (i.e. the activator site) other than the active site in order to overcome L-isoleucine inhibition.

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REFERENCES
1. Burns, R. O., and Zarlenzo, M. H. (1965) J. Biol. Chem. 243, 178-185
2. Zarlenzo, M. H., Robinson, G. W., and Burns, R. O. (1965) J. Biol. Chem. 243, 186-191
3. Decedue, C. J., Hoffer, J. G., and Burns, R. O. (1967) J. Biol. Chem. 250, 1563-1570
4. Monod, J., Wyman, J., and Changeux, J.-P. (1965) J. Mol. Biol. 12, 98-118
5. Nakazawa, A., Tokushige, M., and Hayashi, O. (1965) Biochem. Biophys. Res. Commun. 29, 184-188
6. Niederman, R. A., Rabinowitz, K. W., and Wood, W. A. (1969) Biochem. Biophys. Res. Commun. 36, 951-956
7. Winitz, N., Bloch-Frankenthal, L., Izumiya, N., Birnbaum, S. M., Baker, C. G., and Greenstein, J. P. (1965) J. Am. Chem. Soc. 78, 2423-2429
8. Friedemann, T. E., and Haugen, G. E. (1943) J. Biol. Chem. 147, 415-422
9. Wheat, R. W., Rollins, E. L., and Leatherwood, J. M. (1962) Biochem. Biophys. Res. Commun. 9, 120-125
10. Maeha, P., and Sanwal, R. D. (1966) Biochemistry 5, 575-580
11. Wood, W. A., and Gunsalus, I. C. (1949) J. Biol. Chem. 181, 171-182
12. Tanabe, T., Shizuta, Y., Inoue, I., Kuratawa, A., and Hayashi, O. (1964) J. Biol. Chem. 241, 873-876
13. Rabinowitiz, K. W., Niederman, R. A., and Wood, W. A. (1973) J. Biol. Chem. 248, 8297-8215
14. Levitsky, A., Stullcup, W. B., and Koshland, D. E., Jr. (1971) Biochemistry 10, 3371-3377

R. O. Burns, unpublished observations.
Threonine deaminase from Salmonella typhimurium. Effect of regulatory ligands on the binding of substrates and substrate analogues to the active sites and the differentiation of the activator and inhibitor sites from the active sites.

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