

## Threonine Deaminase from *Salmonella typhimurium*

SUBSTRATE-SPECIFIC PATTERNS OF INHIBITION IN AN ACTIVATOR SITE-DEFICIENT FORM OF THE ENZYME\*

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Richard O. Burns, John G. Hofler,‡ and Geraldine H. Luginbuhl§

From the Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

A mutant strain of *Salmonella typhimurium*, selected on the basis of its ability to grow on L-threonine as principle source of nitrogen, produces a biosynthetic L-threonine deaminase (EC 4.2.1.16) with altered kinetic properties. The  $K_i$  for L-isoleucine (1 mM) and the  $K_a$  for valine (19 mM) are increased 380- and 330-fold, respectively, as compared with the wild type enzyme. The  $K_m$  for L-threonine is increased 4-fold as compared with the wild type enzyme; the  $K_m$  for L-serine and the  $K_i$  values for D-serine, D-threonine, and D-allothreonine are essentially unchanged. The  $K_a$  values for L-threonine and L-serine, in the altered enzyme extrapolated from the increase in  $K_a$  for L-valine, suggested that these ligands which function as activators in the wild type enzyme would be ineffective for this function in the mutated enzyme. The altered enzyme was used to demonstrate the relationship between the inhibitor sites and active sites of the enzyme in the absence of the intervening effects of the activator site. Standard kinetic analysis of the altered enzyme reveal that L-isoleucine functions as a competitive inhibitor when L-threonine serves as substrate and a noncompetitive inhibitor when L-serine serves as substrate. Unlike in the wild type enzyme the activity is a first order function with either substrate in the presence of L-isoleucine. Inhibition of the activity of the altered enzyme by L-isoleucine is a first order function of inhibitor concentration when L-serine serves as substrate but is a high order function of inhibitor concentration when L-threonine serves as substrate; these results are explained in terms of a "half-of-the-sites" reactivity.

Circular dichroism measurements show that inhibition of the altered enzyme occurs by the interaction of L-isoleucine with the residual inhibitor sites on the enzyme and that interaction of L-isoleucine with these sites does not prevent the binding of the competitive inhibitor D-serine to the active sites.

The properties of this activator site-deficient mutant are predictable as the basis of the previous demonstration with the wild type enzyme that L-isoleucine prevents the binding of L-threonine to the active site but does not prevent the binding of L-serine to the active site.

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‡ United States Public Health Service Trainee. Present address, Institute Pasteur, Neurobiologie Moleculaire, Paris, France.

§ Recipient of a postdoctoral research fellowship from the National Institute of General Medical Sciences. Present address, Department of Microbiology, North Carolina State University, Raleigh, N. C.

The activity of biosynthetic L-threonine deaminase from *Salmonella typhimurium* is modulated by the interaction of inhibitor sites which bind L-isoleucine and an activator site which binds L-valine and the substrate L-threonine or L-serine (1). The results of recent experiments which used circular dichroism measurements to monitor the effect of regulatory ligands on the binding of substrates and substrate analogues to the active sites of threonine deaminase revealed that L-isoleucine prevents the binding of L-threonine, but not the binding of L-serine to the active sites (2). L-isoleucine, however, inhibits enzyme activity with either substrate. These observations underscore the role of the activator site in restoring activity to the isoleucine-inhibited enzyme and provide a basis for the dependence of enzyme activity upon a high order substrate concentration when L-isoleucine is present; that is, the L-isoleucine-inhibited enzyme requires two qualitatively different binding events in order to be active, one occurring at the activator site and another at the active site. The rate of the threonine deaminase reaction in the presence of L-isoleucine, therefore, is a function of the product of the fractional saturation of the activator site and the catalytic site when either L-threonine or L-serine serves as substrate (1). The circular dichroism measurements, mentioned above, are based upon the observation that the extrinsic optical activity of L-threonine deaminase caused by the aldimine-bonded cofactor pyridoxal 5'-monophosphate is lost when substrates or substrate analogues bind to the active site. The loss in optical activity is thought to occur because of transfer of the aldimine bond from the protein to the amino group of the ligand. It is clear that loss of optical activity reflects binding at the catalytic site but the question remains whether the failure of a ligand to cause the loss can be taken as an indication that the ligand is not bound to the catalytic site. In order to clarify this point an independent manifestation of substrate-active site interaction was sought.

Owing to the differential effect which L-isoleucine exerts on the binding of L-threonine and L-serine to the active sites of threonine deaminase, it was assumed that a derivative of the enzyme which lacks a functional activator site might be useful in verifying the relationship between the inhibitor sites and catalytic sites of the enzyme. Based upon previous results it would be predicted that in the absence of an activator site, L-isoleucine would be a competitive inhibitor of enzyme activity when L-threonine serves as substrate and a noncompetitive inhibitor when L-serine is the substrate. These expectations are based upon the conclusion that L-isoleucine prevents the binding of L-threonine but does not prevent the binding of L-serine to the catalytic sites of threonine deaminase. Also, such behavior of an activator site-deficient enzyme would be consistent with the previous interpretation that the inability of active site ligands to cause a loss of extrinsic optical activity represents a perturbation of binding.

## MATERIALS AND METHODS

## RESULTS

**Isolation of *S. typhimurium* Strain DU-21**—The isolation of strain DU-21 was based on its increased growth advantage on medium containing L-threonine as the principal source of nitrogen. Approximately 50% of the mutant strains selected in this manner contain biosynthetic L-threonine deaminase which is less sensitive to inhibition by L-isoleucine. *S. typhimurium ilvD18* lacking dihydroxy acid dehydratase (EC 4.2.1.9) activity, is spread ( $2 \times 10^8$  cells/ml) onto agar plates containing 16.8 mM L-threonine, 0.043 M  $K_2HPO_4$ , 0.024 M  $KH_2PO_4$ , 1.2 mM  $MgSO_4$ , 0.38 mM L-isoleucine, 0.76 mM L-leucine, 0.85 mM L-valine, and 1.5% Difco Bacto-agar. The plates are incubated at 37°C and colonies appear against a lawn of moderate growth after 3 to 5 days. These colonies are picked and carried through three single colony isolation procedures. Crude extracts are prepared from the purified mutant strains and the sensitivity to L-isoleucine of biosynthetic L-threonine determined. The genetic lesion responsible for the "feedback-negative" phenotype in each of the isolates was shown to be within the *ilvA* gene by transductional analysis with the phage PL22 Ht105(3). Further details of the isolation procedure and the genetic analysis will be presented elsewhere.

**Growth of Cells and Preparation of Crude Extract**—Strain DU-21 was grown in a minimal medium which contained 0.086 M  $K_2HPO_4$ , 0.048 M  $KH_2PO_4$ , 0.016 M  $(NH_4)SO_4$ , 0.2 mM  $MgSO_4$ , 0.038 mM L-isoleucine, 0.21 mM L-valine, and 0.23 mM L-methionine. The L-isoleucine and L-valine are required to satisfy the auxotrophy (because of the *ilvD* mutation) and the L-methionine is required as a conditional growth requirement brought about by a combination of the drain on the common biosynthetic intermediate L-homoserine caused by the feedback-negative character of threonine deaminase and its derepression (see below) and the accumulation of  $\alpha$ -aminobutyrate which inhibits methionine biosynthesis.<sup>1</sup> Two hundred liters of minimal medium in a New Brunswick Fermentor F-250 was inoculated with 1 liter of an overnight nutrient broth culture of strain DU-21. The culture, at 37°C, was aerated with 2 cu ft/min at 1.33 atm. After 12 h of growth, 2 g of L-isoleucine in 4 liters of water was pumped at constant rate into the growth vessel for an additional 6 h. The cells were harvested by means of an industrial Sharples centrifuge. The cell paste was suspended in 2 liters of 1 M potassium phosphate, pH 7.5, containing 0.5 mM EDTA and 0.5 mM dithiothreitol (standard buffer). The cells were disrupted with a Branson J-17A sonifier at full power; an acetone/ice bath was used to maintain the temperature near 4°C. The cellular debris was removed by centrifugation at  $16,700 \times g$  for 1 h. The resulting crude extract was used in the purification procedure described under "Results." All steps were performed at 4°C.

**Preparation of Phenylalanine-substituted Sepharose**—Sepharose 4B (Pharmacia) was substituted with DL-phenylalanine according to the procedure of March *et al.* (4).

**Preparation of L-Isoleucine-substituted Sepharose**—L-Isoleucine was coupled to Sepharose as described by Sundberg and Porath (5). Sepharose 4B was activated by mixing 1 volume of washed, packed gel with 1 volume 1,4-butanediol diglycidyl ether (Aldrich Chemical Co.) and 1 volume of 0.6 M sodium hydroxide containing 2 mg/ml of sodium borohydride. The suspension was mixed by rotation for 2 h at 25°C after which the gel was washed on a sintered glass funnel with large volumes of water. Two volumes of a 50 mg/ml of suspension of L-isoleucine in 0.3 M sodium bicarbonate, pH 10, was added to the activated Sepharose and mixed by rotation for 24 h at 25°C at which time the suspension was made 0.1 M with ethanolamine. After standing overnight at 23°C the gel was washed with water and equilibrated with appropriate buffer.

**Preparation of Controlled Pore Glass Beads**—Controlled pore glass beads of 170A pore size (Electro-Nucleonics, Inc.) were prepared by washing with several changes of a 1% Carbowax 20M (Union Carbide Corp.) solution which had previously been degassed. A column (225  $\times$  1.5 cm) was gravity-packed with the beads. The column was flushed with degassed water and equilibrated with 0.1 M standard buffer immediately before use.

**Enzyme Assay**—Threonine deaminase was assayed spectrophotometrically as previously described (1).

**Circular Dichroism Measurements**—A Dichrographe III (Jobin-Yvon) was used. All measurements were made in 1.0-cm round cuvettes at an enzyme concentration of approximately 3 mg/ml. The enzyme had been dialyzed 12 h against several changes of 0.1 M potassium phosphate, pH 7.5, containing 0.5 mM ethylenediaminetetraacetate and 0.5 mM dithiothreitol.

**Isolation of Mutant Strains**—Mutant strains of *S. typhimurium* that contain biosynthetic L-threonine deaminase with altered kinetic properties can be selected by plating the wild type strain (LT2) on a medium which contains L-threonine as sole source of nitrogen (see "Materials and Methods"). Mutated cells which contain biosynthetic L-threonine deaminase with a decreased sensitivity to L-isoleucine have a growth advantage on this medium and form discrete colonies against a background of moderate growth. In the course of examining the properties of the threonine deaminase in crude extracts prepared from a series of these spontaneously arising mutant strains one was found not only to have an increased  $K_i$  for L-isoleucine but also an increased  $K_m$  for L-threonine and an increased  $K_a$  for L-valine. This altered enzyme, although less sensitive to inhibition by L-isoleucine, is still sensitive to this inhibitor in the millimolar range. An interesting feature of this altered enzyme is the differential change in the apparent affinity of L-valine for the activator site and the  $K_m$  for L-threonine. Preliminary results using crude extracts containing the mutant enzyme showed that whereas the  $K_a$  for valine is increased 200-fold, the  $K_m$  for L-threonine is only moderately affected. The affinity of L-threonine for the activator site and its  $K_m$  in the wild type enzyme are 3.0 mM and 4.5 mM, respectively (1). If the affinity of L-threonine (and L-serine) for the activator site in the mutant enzyme is affected in the same manner as is the affinity of L-valine for this site, then saturation of the catalytic site would require much lower concentrations of L-threonine than would saturation of the activator site. In other words, the activator site would be inoperative at concentrations of substrate necessary for typical measurements of enzymatic activity. This mutationally altered enzyme is useful for examining the relationship between the inhibitor and catalytic sites in the absence of the intervening effects of the activator site.

**Purification of Mutant Enzyme**—Preliminary analyses indicated that most of the mutationally altered enzymes are much less stable in crude extracts than is the wild type enzyme. The previously described procedure (6) employed for purification of the wild type enzyme proved inadequate for purification of the mutant enzyme, therefore a more rapid purification procedure based upon hydrophobic chromatography, ion exchange chromatography, and inverse filtration was developed. An outline of this procedure is as follows.

The cells were grown, harvested and disrupted as described under "Materials and Methods." The resulting crude extract was mixed with approximately one-half volume of phenylalanine-substituted Sepharose, which had previously been equilibrated with standard 1 M potassium phosphate, pH 7.5. The mixture was stirred for 1 h at 4°C. The viscous mixture was filtered on a sintered glass funnel and washed with standard 1 mM potassium phosphate buffer until the filtrate became clear.

After washing, the Sepharose with adsorbed protein was poured into a column (5.5  $\times$  42 cm) and washed with 2 column volumes of standard 1 mM potassium phosphate buffer. The L-threonine deaminase was step-eluted with standard 0.05 M potassium phosphate buffer. The active fractions were pooled and the phosphate concentration was adjusted to 1 mM with 5 M potassium phosphate, pH 7.5.

As with the first column resin, the isoleucine-substituted Sepharose, prepared as described under "Materials and Methods" and equilibrated with standard 1 M potassium phosphate buffer, is poured in a slurry with the enzyme solution for 1 h at 4°C and filtered on a sintered glass funnel. Again, the resin is washed with 1 M buffer and then poured into a column (4.0  $\times$  4.0 cm). After washing with 200 ml of 1 M buffer, the column

<sup>1</sup> R. O. Burns, unpublished observations.

is further washed with 250 ml of standard 0.85 M potassium phosphate, pH 7.5. As the L-threonine deaminase begins to elute at a potassium phosphate concentration of 0.80 M, this concentration is critical. The enzyme is then eluted with standard 0.2 M potassium phosphate, pH 7.5.

The active fractions are pooled and concentrated by vacuum dialysis against a solution of a 0.5 mM EDTA and 0.5 mM dithiothreitol to lower the phosphate concentration to less than 0.1 M. The concentrated enzyme is loaded on a Sephadex DEAE A-50 column (1 × 20 cm) and eluted with 0.05 to 0.5 M standard buffer. The enzyme elutes at a potassium phosphate concentration of approximately 0.25 M. The fractions with a specific activity greater than 600 are pooled and vacuum-concentrated. The concentrated enzyme solution was dialyzed overnight against 0.05 M standard buffer and stored frozen at -20°C. The enzyme was usually used without further purification.

If the enzyme is not pure after Sephadex A-50, the protein can be purified by filtration through a uniform pore 170A glass bead column ("Materials and Methods"). The column (225 × 1.5 cm) is equilibrated with standard 0.1 M potassium phosphate buffer by pumping at 180 ml/h. After applying the enzyme, the column is developed by pumping the same buffer at the same rate. Fractions are collected and the fractions of uniform specific activity are pooled and concentrated as previously described. Table I presents a summary of the purification procedure.

This purification procedure can also be used with extracts containing the wild type enzyme and has the advantage that large volumes of extract can be handled with ease. Many of the mutant enzymes, including that from strain DU-21, became stable following the initial hydrophobic step. It should be pointed out that the efficacy of the isoleucine-substituted Sepharose is not based, to any great degree, as a stereospecific affinity property of the matrix and primarily functions on a hydrophobic principle.

**Physical Properties of Mutant Enzyme**—Sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis was used to

estimate the subunit molecular weight for the enzyme purified from the mutant strain. The polypeptide chains from the mutant enzyme co-migrate in 5% gels with the wild type enzyme which has a molecular weight equivalent to 48,500.

The absorption spectrum of the mutant enzyme is indistinguishable from that of the wild type enzyme. The protein has a broad peak with a maximum at 415 nm resulting from the aldimine-bonded chromophore (pyridoxyl 5'-phosphate) and an absorption maximum at 280 nm. The enzyme's circular dichroism spectrum between 300 and 500 nm is indistinguishable from that of the wild type enzyme. The  $E_{280}^{1\%}$  is 6.4, the same as for the wild type enzyme. The  $A_{280}:A_{415}$  ratio is 8.8 which indicates that, like the wild type enzyme, the mutant enzyme possesses 2 pyridoxal 5'-phosphate residues per tetramer (6).

**Kinetic Parameters of the Mutant Enzymes**—The kinetic parameters of the mutant enzyme were measured as previously described for the wild type enzyme (1, 2). Table II compares the standard kinetic parameters of the wild type and the mutant enzyme. Included in this table are the  $K_a$  values for L-threonine and L-serine; these numbers were computed assuming that the observed change in  $K_a$  for L-valine represents the magnitude of change in the  $K_a$  values for the activators L-threonine and L-serine in the altered enzyme. It is of interest to note that the  $K_m$  for L-threonine is elevated in the mutant enzyme but the  $K_m$  for L-serine is unchanged. Previous results have shown that the substrate L-serine and the competitive inhibitors D-serine, D-threonine, and D-allo-threonine exhibit the common property of binding to the catalytic site of L-threonine deaminase in the presence of L-isoleucine (2). The similarity in the binding of L-serine and the competitive inhibitors also pertains to the mutant enzyme. Table II shows that the  $K_i$  values for D-serine, D-threonine, and D-allo-threonine are unaltered in the mutant enzyme.

**Patterns of Inhibition of Enzyme Activity by L-Isoleucine**—Fig. 1 depicts the results of a kinetic analysis of the altered enzyme with L-threonine as substrate in the presence of various concentrations of L-isoleucine. The pattern of inhibition by L-isoleucine appears to be strictly competitive. A striking feature of these results is the lack of apparent cooperativity which is normally seen with the wild type enzyme when the reaction velocity is measured as a function of substrate concentration in the presence of L-isoleucine (1). These results are consistent with the lack of a functional activator site in the mutant enzyme and the requirement for a single type of binding event to overcome L-isoleucine inhibition of enzyme activity. The inset in Fig. 1 shows a secondary plot of the data in which the apparent  $K_m$  values are plotted as a function of the concentration of L-isoleucine. This analysis indicates that inhibition of enzyme activity is a high order function of isoleucine concentration. The mutant and wild type enzymes are similar in the dependence upon a high order

TABLE I  
Purification of L-threonine deaminase from *S. typhimurium* strain DU-21

Step	Total protein mg	Units <sup>a</sup> retained	Specific activity	Units discarded
Crude extract	30,000	51,700	1.7	
Sepharose (phenyl-alanine)	2,080	41,300	19.8	2,800
Sepharose (isoleucine)	126	32,800	260	7,390
Sephadex A-50	37 <sup>b</sup>	25,300	683	

<sup>a</sup> Amount of enzyme producing 1 μmol of α-ketobutyrate/min.

<sup>b</sup> Protein was measured by  $E_{280}^{1\%} = 6.4$ .

TABLE II  
Comparison of kinetic parameters of L-threonine deaminase from wild type and strain DU-21 *S. typhimurium*

Ligand	Wild type <sup>a</sup>			Strain DU-21		
	$K_m$	$K_a$	$K_i$	$K_m$	$K_a$	$K_i$
	mM			mM		
L-Threonine	4.5 (± 0.5)	3.0		19 (± 4)	1,140 <sup>b</sup>	
L-Serine	90 (± 5.0)	55		80 (± 5)	20,900 <sup>b</sup>	
L-Valine		0.05 (± 0.00)			19.0 (± 4.0)	
L-Isoleucine			0.003 (± 0.000)			1.0 (± 0.3)
D-Serine			2.4 (± 0.3)			3.3 (± 0.4)
D-Threonine			7.4 (± 1.9)			7.5 (± 1.5)
D-Allo-threonine			0.19 (± 0.00)			0.21 (± 0.00)

<sup>a</sup> From Hoffer and Burns (2).

<sup>b</sup> Calculated from the ratio 19.0:0.05, the -fold change in  $K_a$  for L-valine in enzyme from strain DU-21 and wild type.

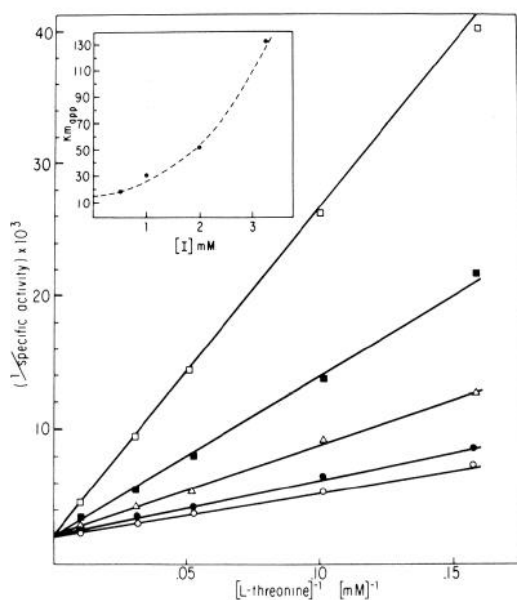


FIG. 1 (left). The effect of L-isoleucine on the activity of L-threonine deaminase from strain DU-21 with L-threonine as substrate. The initial velocity of the L-threonine deaminase reaction was measured spectrophotometrically (1) as a function of L-threonine concentration in the presence of (○) no addition, (●) 0.5 mM, (△) 1.0 mM, (□) 2.0 mM, and (■) 3 mM L-isoleucine. The reaction mixture, at 24°C, contained 0.1 M potassium phosphate, pH 7.5, and enzyme which had been dialyzed against 0.1 M standard buffer.

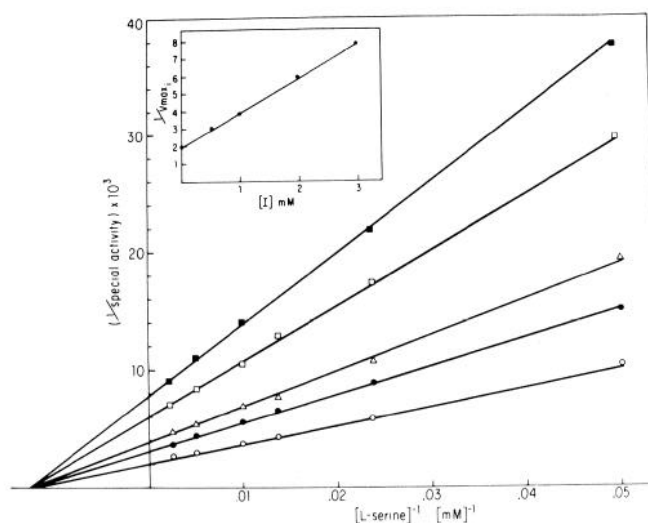


FIG. 2 (right). The effect of L-isoleucine on the activity of L-threonine deaminase from strain DU-21 with L-serine as substrate. The initial velocity of the L-threonine deaminase reaction was measured spectrophotometrically (1) as a function of L-serine concentration in the presence of (○) no addition, (●) 0.5 mM, (△) 1.0 mM, (□) 2.0 mM, and (■) 3.0 mM L-isoleucine. The reaction mixture, at 24°C, contained 0.1 M potassium phosphate, pH 7.5, and enzyme which had been dialyzed against 0.1 M standard buffer.

concentration of L-isoleucine for inhibition of enzyme activity when L-threonine serves as substrate. The basis for this requirement, however, may differ in the two forms of the enzyme (see "Discussion").

Fig. 2 depicts the results of a kinetic analysis with L-serine as substrate and the pattern of inhibition exerted by L-isoleucine; inhibition of enzyme activity appears to be strictly non-competitive. A secondary plot of the data depicted in the inset to Fig. 2 shows that inhibition of enzyme activity with L-serine as substrate is a first order function of L-isoleucine concentration.

*The Allosteric Nature of the Inhibitor Site and the Effect of L-Isoleucine on Ligand Interaction with the Active Sites*—The substrate-specific patterns of inhibition of enzyme activity exerted by L-isoleucine are consistent with the previously demonstrated relationship between this inhibitor and the binding of L-threonine and L-serine to the catalytic sites of the wild type enzyme, *i.e.* isoleucine prevents the binding of L-threonine but does not prevent the binding of L-serine to the catalytic sites. Unfortunately, a circular dichroic analysis of the differential effect of L-isoleucine on the binding of L-threonine and L-serine to the active sites, as performed with the wild type enzyme (2), is not possible with the altered enzyme owing to the elevated  $K_i$  for L-isoleucine. The high concentration of enzyme required for circular dichroic measurements and the inability to obtain concentrations of L-isoleucine sufficient to inhibit completely the mutationally altered enzyme prevented a direct analysis of the effect of L-isoleucine on the binding of L-threonine and L-serine to the catalytic sites. In order to approach this question, circular dichroic analysis of the altered enzyme was performed using the isosteric competitive inhibitor D-serine (2). The loss of extrinsic optical activity of the altered enzyme was measured as a function of D-serine concentration in the presence and absence of 100 mM L-isoleucine. The results of this analysis, depicted in double reciprocal form in Fig. 3, show that L-isoleucine has a moderate effect on D-serine binding; the  $K_d$

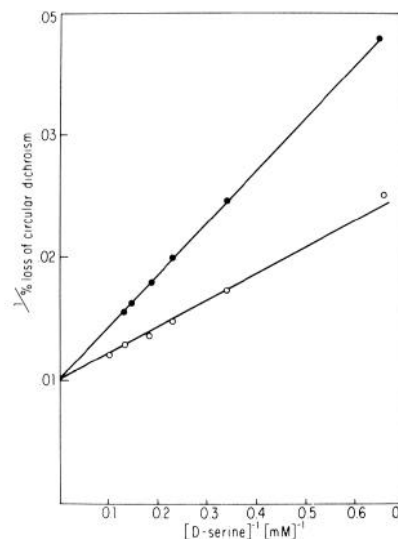


FIG. 3. The effect of L-isoleucine on the D-serine-promoted loss of circular dichroism of threonine deaminase from strain DU-21. Approximately 3 mg/ml of L-threonine deaminase from strain DU-21 in 0.1 M standard buffer was titrated with D-serine in the presence (●) and absence (○) of 100 mM L-isoleucine. The loss of circular dichroism was monitored at 415 nm.

for D-serine computed from these data is 2.1 mM and the  $K_{dapp}$  is 3.9 mM. The inhibitor constant for L-isoleucine computed from the data in Fig. 3 using the relationship  $K_i = K_d(I)/K_{dapp} - K_d$  is 116 mM. This inhibitory effect is apparently unrelated to the inhibition of enzyme activity by L-isoleucine and represents a weak affinity of this inhibitor for the catalytic site. This conclusion is based on the observation that the  $K_i$  for L-isoleucine computed from kinetic analysis (Table II) is 1 mM, approximately 2 orders of magnitude less than the  $K_d$  computed from the data in Fig. 3. It is concluded, therefore, that inhibition of activity of the altered enzyme by

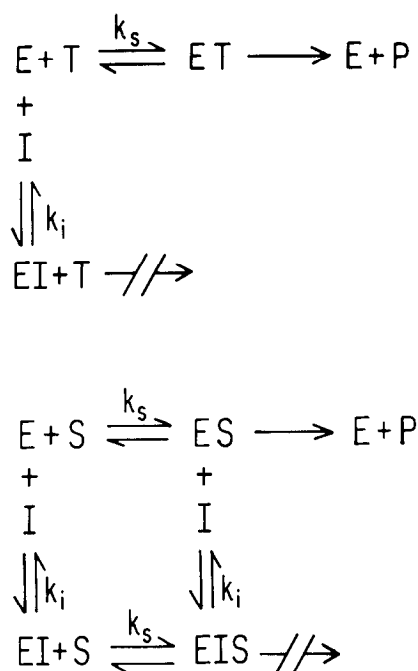


FIG. 4. Reaction schemes for threonine deaminase from strain DU-21 with L-threonine (*T*) and L-serine (*S*) as substrates.

L-isoleucine, although less efficient than in the wild type enzyme is "allosteric." It also is concluded that occupancy of this residual allosteric site by L-isoleucine does not prevent the binding of D-serine to the catalytic site, and by inference does not prevent the binding of L-serine to the catalytic site.

#### DISCUSSION

The altered enzyme described in this report was derived from a spontaneously arising mutant strain of *S. typhimurium*; the properties of the enzyme, therefore, are most probably the consequence of a single mutational event. The results of genetic analysis of this mutant strain show that the mutation responsible for these properties lies in the *ilvA* gene which is the structural gene encoding biosynthetic L-threonine deaminase (3). The observation that the apparent affinity of the three types of stereospecific sites present in this enzyme are affected by a single mutation, although not surprising, demonstrates the functional linkage among these sites. It is noteworthy that although the apparent affinity of the altered enzyme for threonine is decreased that the apparent affinity for serine is unaffected. This property of the mutant enzyme may be related to the previously proposed role of the methyl growth of L-threonine in binding to the catalytic site and in exclusion of this substrate from the L-isoleucine-inhibited enzyme (2). It has been suggested that the methyl group of L-threonine not only aids in binding to the uninhibited enzyme but provides steric hindrance which prevents binding to the inhibited enzyme (2). The change in the altered enzyme which leads to a decreased affinity for L-threonine without affecting L-serine binding may also be related to the orientation of the methyl group. The results in Table II, which show that the  $K_i$  values for the competitive inhibitors D-serine, D-threonine, and D-allothreonine are unaffected in the mutant enzyme support this notion. It was previously suggested that the methyl groups in D-threonine and D-allothreonine are eclipsed relative to the enzyme-substrate binding plane and therefore should mimic serine (which lacks the methyl group) in binding properties (2). This feature was invoked to explain the ability

of these inhibitors to bind to the isoleucine-inhibited, wild type enzyme (2).

The substrate-specific patterns of inhibition of the activity of the altered enzyme are consistent with previously demonstrated or conjectured features of the enzyme. The apparent lack of a functional activator site in the enzyme permits a discernment of the relationship between the inhibitor and catalytic sites by traditional kinetic analysis. The results of these analyses are fully consistent with previous conclusions reached from the circular dichroism measurements with the wild type enzyme (2); *i.e.* L-isoleucine prevents the binding of L-threonine but does not prevent the binding of L-serine to the catalytic site. The reaction schemes for biosynthetic L-threonine deaminase which lacks a functional activator site can be reduced to the reaction schemes depicted in Fig. 4 (7). In the scheme depicted for threonine, the inhibitor and substrate compete for the same form of the enzyme resulting in a strictly competitive relationship. The scheme does not specify an isosteric or allosteric relationship but simply mutually exclusive binding of the two ligands. The reaction scheme shown for serine is that for a noncompetitive situation. In this case the energy of binding of serine to the free enzyme and the isoleucine-liganded enzyme is the same and the distribution of active and inactive enzyme is strictly a function of the concentration of L-isoleucine.

The kinetic analysis of the mutant enzyme with L-serine as substrate supports previous conclusions that the cooperativity seen in the wild type enzyme is the consequence of a functional activator site. The case with threonine as substrate is different however because a definite cooperativity is seen in inhibition by L-isoleucine. Inhibition of the wild type enzyme is a second order function of isoleucine concentration with either L-threonine or L-serine as substrate (2). This observation has been explained by the requirement for the binding of 2 molecules of L-isoleucine to prevent the binding of the single activating ligand (1). The basis for the apparent cooperative nature of isoleucine inhibition in the mutant and wild type enzymes is probably not the same; the mutant enzyme lacks the activator site and the cooperativity is only seen when the substrate is threonine. A possible basis for the apparent cooperativity with respect to inhibition of the activity of the altered enzyme by L-isoleucine is a half-of-the-sites reactivity which was previously invoked for the wild type enzyme (2). According to this principle, only one of the two active sites of threonine deaminase is capable of catalysis at any time, *i.e.* the catalytic potential of the enzyme is the same if one or both of the active sites are occupied by the substrate L-threonine. In the present case, an enzyme liganded with a single L-isoleucine molecule is not totally inactive and becomes so only when both isoleucine sites are occupied; this would result in the cooperativity observed in isoleucine inhibition. The lack of cooperativity in isoleucine inhibition when L-serine serves as substrate indicates that either half-of-the-sites reactivity does not occur with this substrate or that it is obscured by the noncompetitive nature of the inhibition.

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