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Amino Acid Substitutions in the C-terminal Regulatory Domain Disrupt Allosteric Effector Binding to Biosynthetic Threonine Deaminase from *Escherichia coli**

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Shifts in the sigmoidal kinetics of allosteric threonine deaminase promoted by isoleucine and valine binding control branched chain amino acid biosynthesis in *Escherichia coli*. A highly conserved α-helix in the C-terminal regulatory domain of the tetrameric enzyme was previously implicated in effector binding and feedback inhibition. Double (447, 451) and triple (447, 451, 454) alanine replacements for the conserved amino acids leucine 447, leucine 451, and leucine 454 in this region yield enzyme variants that show increased sigmoidality in steady-state kinetics, and which are less sensitive to the allosteric modifiers isoleucine and valine. Equilibrium binding studies using fluorescence, enzyme kinetic, and calorimetric approaches indicate that the enzyme variants possess reduced affinity for isoleucine and valine, and suggest that heterotropic ligands can bind to the same site to promote their different effects. The increase in sigmoidal kinetics for the mutants relative to wild-type threonine deaminase may be attributable to the elimination of L-threonine binding to the effector sites, which activates the wild-type enzyme. Enzyme kinetic data and isotherms for active site ligand binding to the mutants can be analyzed in terms of a simple two-state model to yield values for allosteric parameters that are consistent with previous estimates based on an expanded two-state model for homotropic cooperativity for threonine deaminase.

Control of branched-chain amino acid biosynthesis in plants and microorganisms is achieved in part by biosynthetic threonine deaminase. Threonine deaminase (TD[1]; threonine dehydratase; L-threonine hydrolyase (deaminating), EC 4.2.1.16) (1) from *Escherichia coli* catalyzes the committed step in the biosynthesis of isoleucine, the pyridoxal phosphate-dependent conversion of L-threonine to α-ketobutyrate, in a controlled fashion (1). The initial velocity of threonine deaminase follows a sigmoidal dependence on L-threonine concentration and is shifted in the presence of the end products isoleucine and valine. Isoleucine, which binds preferentially to the low activity T state of TD, is an allosteric inhibitor, resulting in an increase in the sigmoidality and the mid-point of saturation curves. Valine activates the enzyme by preferentially binding to the high activity R state, thereby giving rise to virtually hyperbolic kinetics, although it only slightly affects the apparent $K_{0.5}$ (2). Despite the structural similarity of these ligands, neither isoleucine nor valine show appreciable affinity for the active site of threonine deaminase. However, the affinity of L-threonine and its analogs for the regulatory sites is greater than that seen for the active site, which results in a synergistic effect on the allosteric transition. This effect can be treated by incorporating a provision into the classic two-state model of Monod *et al.* (3) to account for the shift in the ratio of the T state to the R state upon substrate binding to effector sites (4).

A key issue in the regulation of allosteric enzymes concerns the manner whereby different feedback modifiers bind to the same regulatory site but yield dramatically different effects on enzyme kinetics and ligand binding (5). For example, activation by ATP and inhibition by CTP and UTP occurs by binding to the same sites on the regulatory chains of aspartate transcarbamoylase (6–10). This is an especially significant problem for the regulation of threonine deaminase since the allosteric effectors isoleucine and valine are so structurally similar yet promote such dramatically different effects on the kinetic properties of the enzyme. Previous studies identified an interesting amino acid substitution, $Leu^{447} \rightarrow Phe$, in a conserved region of the regulatory domain of the enzyme that resulted in a loss of feedback regulation through a defect in the allosteric transition (4). In an effort to assess the role of this highly conserved region of the regulatory domain, multiple amino acid substitutions were introduced into this region in TD, and their effects on quaternary structure, effector binding, and the regulation of catalysis were investigated. Results for a double and triple mutant with reduced affinity for isoleucine and valine suggest that effectors bind to the same site in the regulatory domain of TD. Interestingly, the mutant enzymes show more sigmoidal saturation curves in steady-state kinetics and inhibitor binding to the active site, which suggests that L-threonine and its analogs also have reduced affinity for the regulatory site. These results provide support for a complex mechanism for regulation of wild-type threonine deaminase.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, polymerases, and ligases were from New England BioLabs or Life Technologies, Inc. Isoleucine and valine were from Sigma, and L-α-aminobutyrate was from ICN Biomedicals. Oligonucleotides were synthesized on an Applied Biosystems model 380 B synthesizer and purified by high performance liquid chromatography prior to use.

Construction and Purification of TD Variants—Site-directed mutagenesis of *E. coli ilvA* was used to introduce multiple mutations into a highly conserved region of the regulatory domain of threonine deami-

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[1] The abbreviations used are: TD, threonine deaminase; TD_{DBL}, enzyme variant with the two amino acid substitutions $Leu^{447} \rightarrow Ala$ and $Leu^{451} \rightarrow Ala$; TD_{TPL}, enzyme variant with the three amino acid substitutions $Leu^{447} \rightarrow Ala$, $Leu^{451} \rightarrow Ala$, and $Leu^{454} \rightarrow Ala$.

nase. A double mutation in *ilvA* containing alanine substitutions for Leu^{447} and Leu^{451} was constructed and designated TD_{DBL}. A variant with three substitutions including $Leu^{447} \rightarrow$ Ala, $Leu^{451} \rightarrow$ Ala, and $Leu^{454} \rightarrow$ Ala was also constructed, and designated TD_{TPL}. Mutagenesis was performed with pEE27 as a single-stranded template (11) using the Sculptor IVM kit (Amersham Pharmacia Biotech) for mutagenesis reactions. The oligonucleotides that were synthesized to introduce the mutations into *ilvA* also introduced convenient sites for restriction endonucleases to facilitate screening. The mutants were verified by DNA sequencing using Sequenase version 2.0 (Amersham Pharmacia Biotech). Double and triple mutants of TD were purified essentially as described previously for wild-type threonine deaminase (2, 12).

Enzyme Kinetics—The effect of the mutations on the activity of threonine deaminase was assessed from measurements of the initial velocity *versus* L-threonine concentration using a continuous spectrophotometric assay for α-ketobutyrate (13). Initial rates were determined in the absence and presence of 0.5 mM valine and 50 μM isoleucine. Additionally, the effect of increasing isoleucine and valine on the activity of the mutants was measured at low, fixed concentrations of threonine to obtain a preliminary estimate for the relative affinity of the mutants for isoleucine and valine. All the experiments were performed at 25 °C.

Fluorescence Measurements—Fluorescence spectra to detect ligand binding to TD variants were collected using a FluoroMax 2000 spectrofluorimeter from Spex Industries. An excitation wavelength of 295 nm was used to measure changes in the tryptophan emission spectrum upon effector site binding (14), and an excitation wavelength of 400 nm was used to measure changes in the emission of pyridoxal phosphate upon active site ligation (12). Aliquots of concentrated stock solutions of isoleucine, valine, or α-aminobutyrate were added to 2.5 ml of a 100–200 μg/ml enzyme solution, which was stirred continuously at a constant 25 °C. Corrections to the spectra were made to account for background emission and enzyme dilution.

Titration Calorimetry—The exchange of heat upon addition of isoleucine to wild-type TD and TD_{DBL} was measured in a Microcal Omega titration calorimeter. The measurements were performed in 0.05 M potassium phosphate, 0.1 mM dithiothreitol, and 0.1 mM EDTA, pH 7.5. Microliter aliquots of a 10 mM isoleucine solution were added by a rotating stirrer-syringe to an enzyme solution containing 266 μM effector sites. Estimates for the average and stepwise enthalpies of isoleucine binding to TD_{DBL} were made as described previously for the wild-type enzyme (14).

Sedimentation Equilibrium—Sedimentation equilibrium was performed with a Beckman Optima XL-A analytical ultracentrifuge. Data in the form of absorbance *versus* radius were collected at 280 nm and 412 nm after reaching equilibrium, usually between 16 and 22 h. Data were analyzed using nonlinear least-squares analysis to assess for the effect of mutations on the quaternary structure of the tetramer. Analyses in terms of a single, homogeneous species were performed using equilibrium data usually obtained at three different protein concentrations, and also in the presence of isoleucine, valine, or α-aminobutyrate.

Data Analysis—All data were analyzed using nonlinear least-squares methods (15). Sigmoidal ligand binding and enzyme kinetic data for the mutants were analyzed first by the Hill (16) equation to obtain empirical parameters. Because the apparent average affinity of a protein for a cooperatively binding ligand is dependent on the number of binding states and the energetic barriers between their interconversion, estimates for average apparent binding constants were obtained by analysis of equilibrium binding data in terms of an Adair (17) equation. This analysis was used as described previously in order to obtain estimates for stepwise and average binding energies (12, 14). Stepwise binding energies were also used to extract the stepwise and average enthalpies of isoleucine binding to TD_{DBL} from titration calorimetry data as described previously (14). Finally, enzyme kinetic data and active site ligand binding data were analyzed in terms of the two-state model (3) to obtain the allosteric parameters L, K_R, and c.

RESULTS

Effect of Amino Acid Substitutions in the Regulatory Domain Attenuate the Effects of Isoleucine and Valine in Steady-state Kinetics—The effects of the amino acid substitutions in the region of Leu^{447} in the regulatory domain were first assessed by examining the steady-state kinetics of TD_{DBL} and TD_{TPL} in the absence and presence of isoleucine and valine. As can be seen in Fig. 1, the values estimated for the maximal activities of TD_{DBL} and TD_{TPL} were similar to that for wild-type threonine deaminase. However, the velocities of the mutant enzymes displayed a markedly more sigmoidal dependence on the L-threonine concentration than does wild-type TD. Additionally, the mid-points of these curves are elevated compared with those of the wild type. Analysis of the kinetics data presented in Fig. 1 in terms of the Hill equation yields values for the n_H of 2.2 and a $K_{0.5}$ of 18.7 mM for TD_{DBL}, and an n_H of 2.3 and a $K_{0.5}$ of 25.6 mM for TD_{TPL}.

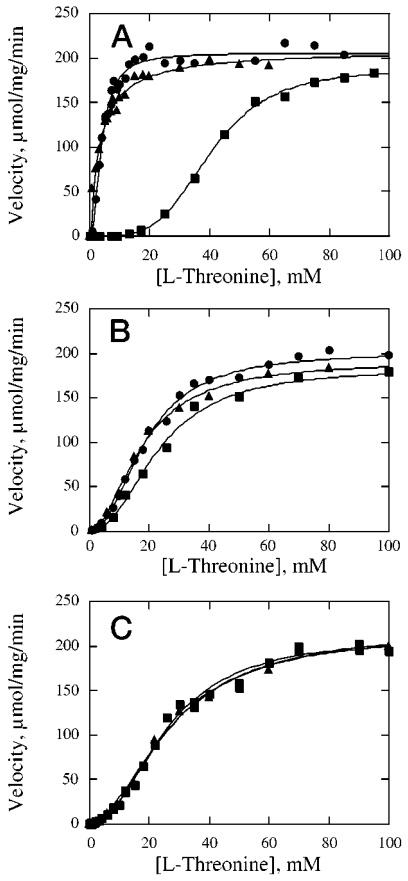

FIG. 1. **Effect of isoleucine and valine on the steady-state kinetics of wild-type TD, TD_{DBL}, and TD_{TPL}.** The kinetics of formation of α-ketobutyrate for wild-type TD and the two mutational variants was followed continuously at 230 nm in 0.05 M potassium phosphate buffer, pH 7.5, at 25 °C, using enzyme concentrations in the range of 0.87 to 1.51 μg/ml. The effects of isoleucine (■) and valine (▲) were examined at 50 μM and 0.5 mM, respectively, and are compared with results in the absence of effectors (●). *A*, wild-type threonine deaminase; *B*, TD_{DBL}; *C*, TD_{TPL}.

Addition of 50 μM isoleucine or 0.5 mM valine, saturating effector concentrations for wild-type threonine deaminase, resulted in almost no effect on the steady-state kinetics seen for TD_{DBL} and TD_{TPL}. As can be seen in Fig. 1, *B* and *C*, the sigmoidality of the saturation curves and their mid-points are roughly the same even in the presence of the heterotropic effectors. Although enzyme activation was undetectable even at millimolar concentrations of valine for either variant, feedback inhibition of TD_{DBL} could be achieved by the addition of elevated concentrations of isoleucine. Steady-state kinetics with TD_{DBL} performed in the presence of 100 μM isoleucine yielded an increased $K_{0.5}$ of 30 mM, and the addition of 350 μM isoleucine resulted in nearly complete inhibition of the enzyme. Similar concentrations of isoleucine had little effect on TD_{TPL}, however. Thus, the double and triple mutations in the vicinity of Leu^{447} result in enzyme variants that are significantly reduced in their sensitivity to feedback modifiers. A summary of the kinetic data for TD_{DBL} and TD_{TPL}, as well as the parame-

TABLE I

Effects of isoleucine and valine on the steady-state kinetic parameters for regulatory domain mutants of threonine deaminase

The kinetic parameters are reported for experiments conducted in 0.05 M potassium phosphate buffer, pH 7.5, at 25 °C.

Enzyme	Effector	n_H[a]	$K_{0.5}$	V_{max}
			mM	
TD_{DBL}	None	2.2	18.7	202
	Isoleucine	2.2	23.2	184
	Valine	1.9	17.6	191
TD_{TPL}	None	2.1	25.6	212
	Isoleucine	1.8	27.3	220
	Valine	2.0	26.6	213
Wild type TD	None	2.0	3.9	206
	Isoleucine	4.0	40.4	188
	Valine	1.0	3.4	210

[a] The Hill parameters were determined as described under "Experimental Procedures," with 65% confidence intervals ranging from about 5% for the Hill coefficients, and between 10 and 20% for the values for V_{max} and for $K_{0.5}$.

ters for wild-type TD obtained under similar conditions, is presented in Table I.

Feedback Resistance of TD_{DBL} and TD_{TPL} Result from Defects in Isoleucine and Valine Binding—Because the previously isolated mutant TD_{L447F} was found to bind heterotropic effectors strongly but was feedback-resistant because of defective allosteric communication (4), it was of interest to examine the interaction of isoleucine and valine with TD_{DBL} and TD_{TPL}. Additionally, since the binding of feedback modifiers promotes significant changes in tryptophan fluorescence in wild-type threonine deaminase, this approach was initially employed to assess the interaction of isoleucine and valine with the variant enzymes. As can be seen in Fig. 2*A*, the addition of millimolar levels of isoleucine and valine to TD_{DBL} produced only a small, yet measurable, change in tryptophan fluorescence. By contrast, neither the addition of isoleucine nor of valine promoted any change in the tryptophan fluorescence of TD_{TPL}. As can be seen in Fig. 2*B*, the fluorescence spectra for TD_{TPL} are virtually identical in the presence of either of the heterotropic effectors.[2] There was no significant effect of either isoleucine or valine on the fluorescence properties of the pyridoxal phosphate cofactor for either variant. Thus, one explanation for the reduced sensitivity of the two variants to feedback regulation may be attributable to defects in isoleucine and valine binding.

The small fluorescence change in TD_{DBL} at reasonable concentrations of isoleucine permitted an evaluation of its binding isotherm. As can be seen in Fig. 3, isoleucine binds cooperatively to TD_{DBL}, with an average dissociation constant of 70 μM. Not only is the binding of isoleucine to TD_{DBL} weaker than the wild type, but the isotherm is more sigmoidal. Thus, one effect of the mutations may be to slightly destabilize the T state of the enzyme, thereby reducing the value for the allosteric equilibrium constant and resulting in more cooperative isotherms for ligands that bind preferentially to the T state. No accurate information could be obtained for the affinity of valine to TD_{DBL}, nor for the association of either isoleucine or valine for TD_{TPL}, since any fluorescence changes were immeasurably small.

Isothermal titration calorimetry was employed to corroborate the interpretation from the fluorescence binding experiments. As can be seen in Fig. 4, there was a significant release

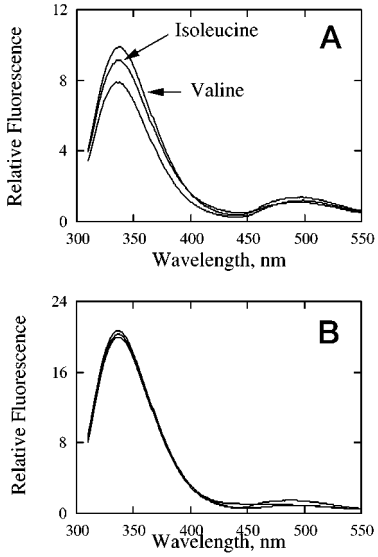

FIG. 2. **Effect of isoleucine and valine on the fluorescence properties of TD_{DBL} and TD_{TPL} mutants.** The relative fluorescence emission spectrum for each mutant was measured in 0.05 M potassium phosphate buffer, pH 7.5, upon excitation at 295 nm. The effect of isoleucine and valine is noted relative to the mutant enzymes in the absence of ligands. *A*, TD_{DBL}; only the addition of millimolar concentrations of isoleucine or valine resulted in a change in the fluorescence. *B*, TD_{TPL}; neither isoleucine nor valine, even at relatively high concentrations, promote a significant change in the fluorescence of the mutant enzyme.

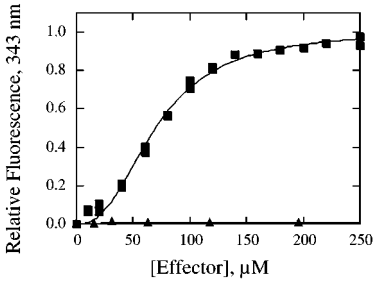

FIG. 3. **Heterotropic effector binding to TD_{DBL}.** The change in tryptophan fluorescence for TD_{DBL} upon addition of either isoleucine (■) or valine (▲) is plotted *versus* ligand concentration at 25 °C. The data for isoleucine binding to TD_{DBL} were analyzed in terms of the Hill equation to yield a $K_{0.5}$ of 69 ± 3 μM and a Hill coefficient of 2.4 ± 0.2. There was no measurable change in fluorescence upon the addition of up to millimolar concentrations of valine to the enzyme variant. Analysis of the isoleucine binding isotherm in terms of a four term Adair model yields the following overall binding constants: $\beta_1 = 4780\ M^{-1}$, $\beta_2 = 1.425 \times 10^8\ M^{-2}$, $\beta_3 = 1.207 \times 10^{12}\ M^{-3}$, and $\beta_4 = 4.219 \times 10^{16}\ M^{-4}$, which gives a value for K_{av}, the average dissociation constant for isoleucine binding, of 69.8 μM. Errors on the first three Adair constants range about 25–50% of the parameter value, whereas the error on β_4 is only about 10% of the parameter value.

of heat from TD_{DBL} upon addition of microliter aliquots of isoleucine. Analysis of the data indicates that 4 mol of isoleucine are bound per tetramer, and that the average enthalpy for isoleucine binding to TD_{DBL} is −20.98 kcal/mol, about twice the value (−10.7 kcal/mol) seen for isoleucine binding to wild-type TD. Neither the addition of valine to TD_{DBL}, nor isoleucine or valine to TD_{TPL}, yielded sufficient heat exchange to estimate energetic parameters.

Enzyme kinetic titrations were also used to estimate the affinity of isoleucine and valine to the TD variants. This approach, which relies on the effect of feedback modifiers to shift the activity of threonine deaminase at concentrations of substrate which are low enough that they do not significantly perturb the allosteric equilibrium, has yielded binding curves for wild-type enzyme that agree surprisingly well with other,

[2] The relative fluorescence of TD_{TPL} is almost 2-fold greater that TD_{DBL} at equivalent concentrations. This may be due to a quenching of the intrinsic fluorescence of Trp^{458} by Leu^{454}, which is reduced by the $Leu^{454} \rightarrow Ala$ substitution. These two residues are in close proximity to one another at the end of helix 17 of the x-ray crystal structure of TD (23).

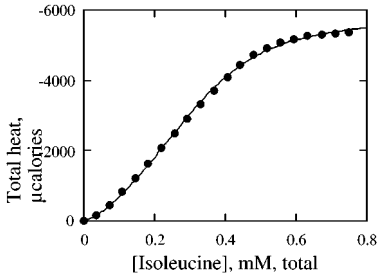

FIG. 4. **Effect of isoleucine on the release of heat from TD_{DBL}.** The total heat released upon addition of 10 μL aliquots of 10 mM isoleucine to a 65.5 μM solution of TD_{DBL} is plotted against the total isoleucine concentration. The theoretical curve was generated using the values of the Adair coefficients from the analysis of the fluorescence binding data (shown in Fig. 2) and the following values for the stepwise enthalpies of binding: $\Delta H_1 = 3.36 \times 10^2$ kcal/mol ($\pm$32%), $\Delta H_2 = -4.75 \times 10^2$ kcal/mol ($\pm$ 23%), $\Delta H_3 = 6.40 \times 10^2$ kcal/mol ($\pm$14%), and $\Delta H_4 = -83.91$ kcal/mol ($\pm$4%). The last value is the total enthalpy change for the binding 4 mol of isoleucine, which yields a value of -20.98 kcal/mol for the average enthalpy of isoleucine binding to TD_{DBL}.

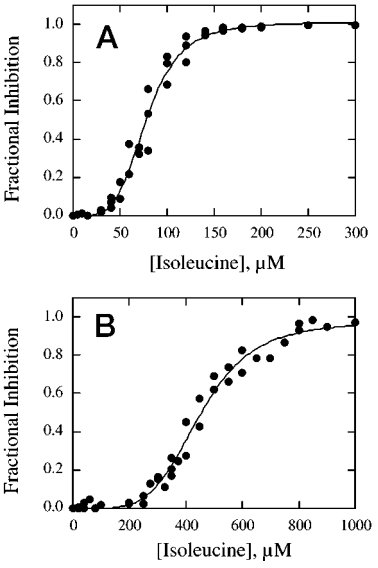

FIG. 5. **Effect of isoleucine on the activity of TD_{DBL} and TD_{TPL} mutants.** *A*, The initial velocity of TD_{DBL} was measured in the presence of 20 mM L-threonine, and the fractional inhibition of the enzyme was analyzed by the Hill equation. The theoretical curve corresponds to the parameters $K_{0.5} = 78 \pm 2$ μM and $n_H = 4.1 \pm 0.3$. This value for the $K_{0.5}$ is similar to that obtained from the fluorescence data shown in Fig. 2. *B*, the initial velocity of TD_{TPL} was measured at 25 mM L-threonine. The fractional inhibition of this mutant was also analyzed by the Hill equation. The theoretical curve corresponds to the parameters $K_{0.5} = 450 \pm 9$ μM and $n_H = 4.0 \pm 0.4$.

more direct approaches (14). As can be seen in Fig. 5*A*, the isoleucine-promoted inhibition of TD_{DBL} seen in enzyme assays performed at 20 mM L-threonine exhibit a sigmoidal dependence on isoleucine concentration. Analysis of the data in terms of the Hill equation yields a $K_{0.5}$ of 78 μM, which in reasonable agreement with that determined by fluorescence and calorimetry. No increase in the activity of TD_{DBL} was observed by the addition of valine. The fractional inhibition of TD_{TPL} can be seen in Fig. 5*B*. These assays were performed at a concentration of 25 mM L-threonine, and the dependence of the inhibition on isoleucine concentration is markedly sigmoidal. Analysis of this isotherm in terms of the Hill equation yields a $K_{0.5}$ of 450 μM. As with TD_{DBL}, there was no effect of valine on the activity of TD_{TPL} at these L-threonine concentrations. Assuming that changes in enzyme activity are directly proportional to the fractional saturation of TD by allosteric effectors, these results indicate that the binding of isoleucine is decreased by roughly 20- and 100-fold in TD_{DBL} and TD_{TPL}, respectively, and that the affinity of valine for the mutants is negligible.

Cooperative Binding of α-Aminobutyrate to the Active Sites of TD_{DBL} and TD_{TPL}—Because the initial velocity curves seen for TD_{DBL} and TD_{TPL} appear more sigmoidal than typical curves for wild type, it was of interest to assess the effect of the amino acid substitutions on the cooperativity of active site ligand binding. A convenient approach to measure ligand binding to the active sites arises from the formation of an external aldimine Schiff base with amino acid substrates and analogs such as α-aminobutyrate, which results in a substantial increase in the fluorescence of the pyridoxal phosphate cofactor in threonine deaminase (12, 18). As can be seen in Fig. 6, TD_{DBL} and TD_{TPL} bind α-aminobutyrate cooperatively, with average dissociation constants of 25.7 and 33.6 mM, respectively. Analysis of cooperative ligand binding to the active sites of wild-type threonine deaminase is complicated by the higher affinity that substrates and analogs possess for the regulatory site. This complication can be circumvented by including a term in an expanded two-state allosteric model for substrate binding to both active sites and effector sites (4). However, for TD_{DBL} and TD_{TPL}, which possess virtually no affinity for valine, a simple two-state model can be used to analyze α-aminobutyrate binding data and, by assuming that initial velocity is proportional to fractional saturation, to the steady-state kinetics data with L-threonine as substrate. Analysis of the data in Figs. 1 and 6 in terms of the simple two-state model yields a range of values for the allosteric equilibrium constant, L, of 55–80 for TD_{DBL}, and 45–90 for TD_{TPL}, as well as a range of values for K_R of

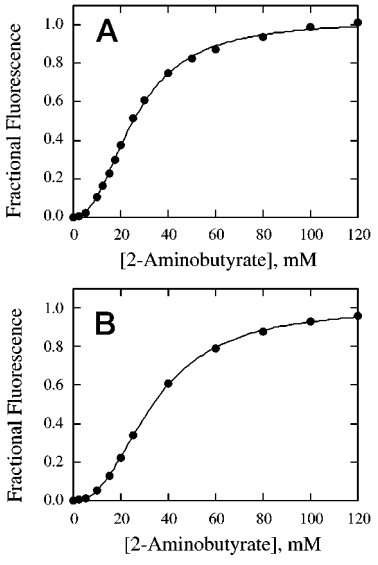

FIG. 6. **Cooperative binding of α-aminobutyrate to TD_{DBL} and TD_{TPL}.** The change in pyridoxal phosphate fluorescence associated with α-aminobutyrate binding to TD_{DBL} and TD_{TPL} was measured in 0.05 M potassium phosphate buffer, pH 7.5, at 25 °C, using enzyme concentrations in the range of 100–200 μg/ml. Theoretical curves were generated using the Hill equation. *A*, analysis of the isotherm for α-aminobutyrate binding to TD_{DBL} yields values for $K_{0.5}$ of 25.7 $\pm$ 0.4 mM and $n_H = 2.25 \pm 0.06$. *B*, Hill analysis of the isotherm for α-aminobutyrate binding to TD_{TPL} yields a value for $K_{0.5}$ of 33.62 $\pm$ 0.41 mM and $n_H = 2.35 \pm 0.05$.

11–16 for α-aminobutyrate and 7–9 for L-threonine. A summary of the allosteric parameters for the two mutant enzymes is presented in Table II.

DISCUSSION

Amino acid substitutions in a conserved region of the regulatory domain of threonine deaminase yielded mutant enzymes with dramatic alterations of heterotropic effector binding, and

TABLE II
Allosteric parameters for cooperative active site ligand binding to regulatory domain mutants of threonine deaminase evaluated from a simple two-state model

The allosteric parameters are reported for experiments conducted in 0.05 M potassium phosphate buffer, pH 7.5, at 25 °C.

Enzyme	Ligand	L^a	K_R
			mM
TD_{DBL}	L-Threonine	78	7
	α-Aminobutyrate	54	11
TD_{TPL}	L-Threonine	92	9
	α-Aminobutyrate	46	16

[a] The allosteric parameters were determined as described under "Experimental Procedures," with 65% confidence intervals ranging from about 40% of the parameter value for L, and about 20% of the parameter value for K_R. The value of c, the ratio of K_R/K_T, was kept at 0.0001 for these analyses (12).

a smaller, but significant effect on the allosteric equilibrium between the T and the R states of the enzyme. These properties of the mutants have revealed some interesting clues about the allosteric regulation of threonine deaminase.

The apparent affinity of TD_{DBL} and TD_{TPL} for isoleucine and valine, and the effect of these ligands on the enzymes, is markedly reduced. Spectroscopic (Fig. 3), calorimetric (Fig. 4), and activity titrations (Fig. 5) all yield apparent dissociation constants for isoleucine binding to TD_{DBL} of 70–80 μM, about 15-fold weaker than is isoleucine binding to wild-type TD. The affinity of TD_{TPL} for isoleucine appears much weaker and could only be estimated by the effect of the allosteric inhibitor on activity titrations (Fig. 5). Although these latter data rely on the assumption that the allosteric equilibrium is not shifted significantly in the presence of low concentrations of threonine, and that changes in TD activity are directly proportional to the fractional saturation of the enzyme with isoleucine, they enable an estimate for an apparent binding constant of about 450 μM, roughly 100-fold weaker than the wild type (14). On the other hand, valine binding is simply too weak to measure accurately by any of the methods for either TD_{DBL} or TD_{TPL}. Taken together, these data suggest that the feedback resistance of the mutants is primarily attributable to defects in effector binding. The small decreases seen for the allosteric equilibrium constants for the mutants also make a contribution to their feedback-resistant properties, however. Additionally, the reduction in the apparent affinity for heterotropic ligands resulting from the small cluster of mutations suggests that both isoleucine and valine bind to a similar region, if not the same site, in the regulatory domain of TD. Although the substituted amino acids fall within the same, conserved helix in the regulatory domain, it is unclear at the current resolution of the unligated structure of TD how they all contribute to effector binding or how isoleucine and valine binding can promote such dramatic differences in the activity of the enzyme.[3]

Inspection of the isoleucine binding isotherms for TD_{DBL} and TD_{TPL} suggests that in addition to the weaker binding of heterotropic effectors, the T state has been destabilized relative to the R state for these variants. This is reflected in the greater sigmoidality of the isotherms, which suggests a greater population of R state molecules relative to wild-type TD. Additionally, isoleucine binding to TD_{DBL} occurs with a large negative average enthalpy of about −21 kcal/mol (Fig. 4). This value is almost double that of −10.7 kcal/mol seen for isoleucine binding to wild-type TD, which is predominantly in the T state (14), as well as that of −11.3 kcal/mol for the enthalpy of isoleucine binding to TD_{L447F}, a mutational variant that is stabilized completely in the R state (4). Thus, if the binding enthalpies of isoleucine to the T and the R states are roughly equivalent, then the large negative enthalpy change seen for isoleucine binding to TD_{DBL} reflects not only ligand binding, but also a favorable enthalpic component for the transition from the R state to the T-stabilized form of the enzyme, and predicts that the T → R transition occurs with a positive enthalpy change.

In addition to the measurable effects of the amino acid substitutions on the apparent affinity for isoleucine and valine, the variants also manifest changes in active site ligand binding isotherms. However, this would not be unexpected. If L-threonine was unable to interact with the activator sites, it could not perturb the allosteric equilibrium, and a more sigmoidal saturation curve than wild type might be expected since the R state would be less populated at low substrate concentrations. This is, in fact, seen for both TD_{DBL} and TD_{TPL} in both enzyme kinetics using L-threonine (Fig. 1), and in the binding of the inhibitor α-aminobutyrate (Fig. 6). Moreover, this interpretation is qualitatively consistent with the fact that these ligands do not possess measurable affinity for the effector sites as seen in fluorescence assays. This is in sharp contrast to wild-type TD, which displays an increase in fluorescence at 343 nm (attributable to Trp^{458})[4] when substrates or analogs bind to the effector sites (14). Thus, the isotherms for L-threonine and α-aminobutyrate only reveal cooperativity parameters for active site ligand binding, and there is no need to invoke the expanded two-state model previously developed for wild-type threonine deaminase to analyze cooperative active site ligand binding.

It is necessary to analyze with cooperative ligand binding to the active sites of wild-type threonine deaminase by an expanded two-state model in order to obtain accurate values for L, the allosteric equilibrium constant (4). This model, which is based on the tenets of the simple two-state model of Monod *et al.* (3), can account for the relatively high affinity binding of substrates and analogs to the effector sites of TD, which results in an additional shift in the allosteric equilibrium in the direction of the R state as active site saturation is measured. However, because TD_{DBL} and TD_{TPL} possess significantly weaker affinity for ligands to their effector sites, it is unnecessary to invoke the expanded two-state model for analyses of cooperative active site ligand binding to the variants. Analysis of active site ligand binding to TD_{DBL} and TD_{TPL} in terms of a simple two-state model (3) yields values for L of between 80 and 90 from enzyme kinetics, and between 45 and 55 from α-aminobutyrate binding (Table II). These values are somewhat lower than those for wild-type TD, which fall around 1500 as evaluated by the expanded model (4). However, they are significantly greater than the estimates of 1–10 for wild-type TD that are obtained when substrate and inhibitor binding to the effector sites is not taken into account (12). Additionally, analysis of the binding and kinetic data for TD_{DBL} and TD_{TPL} in terms of a simple two-state model yield excellent fits, which display no signs of the systematic errors that are seen for wild-type data when analyzed in this way. Finally, since the TD_{L447F} mutant is completely stabilized in the R state, and the calorimetric data on TD_{DBL} suggests that another effect of these amino acid substitutions is to slightly destabilize the T state of the enzyme, the lower estimates of L for TD_{DBL} and TD_{TPL} seem reasonable.

Early studies on threonine deaminase suggested several possible complex mechanisms for allosteric control of substrate binding and catalysis based on ligand competition studies (19–

[3] D. T. Gallagher and D. Chinchilla, personal communication.

[4] H.-D. Yu, D. Porter, J. Knutson, and E. Eisenstein, unpublished observations.

22). More recently, direct binding measurements on mutant enzymes with altered regulatory properties have indicated a pattern of regulation in which substrates (and inhibitors) shift the allosteric equilibrium by binding to the effector site and simulating the action of valine-activated enzyme (4). A common feature of these proposals is that substrates bind to the effector sites on TD, which necessarily complicate any analysis of active site ligand binding. Because the two variants described here possess altered effector binding properties, they should be quite useful in studies aimed at probing the role of amino acid residues in the affinity for substrates, and also in promoting the allosteric transition of the enzyme solely through homotropic interactions.

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