An Expanded Two-State Model Accounts for Homotropic Cooperativity in Biosynthetic Threonine Deaminase from *Escherichia coli*†

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ABSTRACT: The linkage between substrate and regulatory effector binding to separate sites on allosteric enzymes results in shifts in their sigmoidal kinetics to regulate metabolism. Control of branched chain amino acid biosynthesis in *Escherichia coli* occurs in part through shifts in the sigmoidal dependence of α-ketobutyrate production promoted by isoleucine and valine binding to biosynthetic threonine deaminase. The structural similarity of threonine, valine, and isoleucine have given rise to suggestions that there may be competition among different ligands for the same sites on this tetrameric enzyme, resulting in a complex pattern of regulation. In an effort to provide a coherent interpretation of the cooperative association of ligands to the active sites and to the effector sites of threonine deaminase, binding studies using single amino acid variants were undertaken. A previously-isolated, feedback-resistant mutant identified in *Salmonella typhimurium*, *ilvA219*, has been cloned and sequenced. The phenotype is attributable to a single amino acid substitution in the regulatory domain of the enzyme in which leucine at position 447 is substituted with phenylalanine. The mutant exhibits hyperbolic saturation curves in both ligand binding and steady-state kinetics. These results, in addition to calorimetric and spectroscopic measurements of isoleucine and valine binding, indicate that the low affinity (T) state is destabilized in the mutant and that it exists predominantly in the high affinity (R) conformation in the absence of ligands, providing an explanation for its resistance to isoleucine. Chemical and spectroscopic analyses of another mutant, in which alanine has replaced an essential lysine at position 62 that forms a Schiff base with pyridoxal phosphate, indicate that the cofactor is complexed to exogenous threonine and is therefore unable to bind additional amino acids at the active sites. Isoleucine and valine binding to this inactive, active site-saturated enzyme revealed that it too was stabilized in the R state, yielding binding constants in excellent agreement with the leucine to phenylalanine mutant. The lysine to alanine mutant was further utilized to demonstrate that both threonine and 2-aminobutyrate bind with stronger affinity to the regulatory sites than to the active sites. A direct consequence of these results is that substrates and analogs have a synergistic effect on the allosteric transition since, in effect, they act as both homotropic and heterotropic effectors. When these coupled equilibria are considered in an expanded two-state model, good agreement was obtained for the allosteric parameters determined from homotropic and heterotropic ligand binding, and furthermore, they provide an estimate of 4.3 kcal/mol for the average energetic difference between the T and R conformations.

Cooperative ligand binding by allosteric proteins provides an important means for achieving metabolic regulation. Shifts in the sigmoidal kinetics of cooperative enzymes that are promoted by heterotropic effector binding to regulatory sites result in the control of metabolism by increasing or decreasing turnover at intermediate substrate concentrations. The regulation of branched chain amino acid biosynthesis in *Escherichia coli* is achieved in part by the sigmoidal dehydration/deamination of threonine by biosynthetic threonine deaminase (threonine dehydratase; L-threonine hydrolyase (deaminating); EC 4.2.1.16). Feedback inhibition of the enzyme by isoleucine, the end product of the pathway, leads to a decrease in activity at threonine concentrations near the midpoint in saturation curves, and to an increase in sigmoidality. Alternatively, valine activates the enzyme by increasing activity at low to moderate threonine concentrations, yielding isotherms that are less cooperative (Eisenstein, 1991).

Recent studies of the cooperative binding of substrate analogs and regulatory effectors to threonine deaminase, that were analyzed in terms of a simple two-state model (Monod *et al.*, 1965) in which the enzyme converts from a low affinity (T) conformation to a higher affinity (R) state, presented an apparent paradox. Low values for *L*, the allosteric equilibrium constant, were estimated from the cooperative binding of the substrate analogs 2-aminobutyrate

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and alanine, suggesting that if the enzyme actually did exist in two functional forms, the energetic difference between them was small (Eisenstein, 1994). Alternatively, analyses of cooperative isoleucine and valine binding to the effector sites on the enzyme, as well as of the cooperative binding of D-threonine to the active sites, suggested that L was higher, arguing that there was a considerable energetic difference between the putative T and R states (Eisenstein *et al.*, 1994; Eisenstein, 1995). These apparently conflicting data suggested an intricate mechanism for the enzyme's regulation.

The structural similarity among isoleucine, valine, threonine, and their analogs has long been recognized as a possible source of complexity in the analysis of binding isotherms for threonine deaminase (Changeux, 1962; Freundlich & Umbarger, 1963; Harding *et al.*, 1970; Hatfield, 1971; Umbarger, 1973; Decedue *et al.*, 1975; Hofler & Burns, 1978). Competition among similar ligands for either the active sites, the effector sites, or both would complicate any simple analysis of binding isotherms, thereby impeding attempts to reach a consensus for the regulation of the enzyme (Changeux, 1964; Hatfield, 1971; Hofler & Burns, 1978). In this study, single amino acid variants of threonine deaminase have been used to ask which, if any, ligands possess measurable affinity for both the regulatory and catalytic sites on the tetrameric enzyme. The results show that substrates and L-amino acid analogs not only bind to the active sites of threonine deaminase but, surprisingly, bind with even greater affinity to the regulatory sites. A consequence of binding to both the regulatory and catalytic sites is that substrates and their analogs synergistically promote the allosteric transition. Considering these coupled equilibria within the framework of the two-state model of Monod, Wyman, and Changeux (Monod *et al.*, 1965) results in an expanded model that has been used to analyze sigmoidal active-site ligand binding and steady-state kinetics. The expanded model adequately describes homotropic cooperativity in threonine deaminase and yields parameters for the allosteric transition that are in good agreement with previous values obtained from cooperative isoleucine and valine binding to the effector sites on the enzyme (Eisenstein *et al.*, 1994) and cooperative D-threonine binding to the active sites (Eisenstein, 1995). While this analysis provides a potentially important step in studies of the homotropic cooperativity seen in wild-type and mutationally altered variants of threonine deamianse, the results presented also raise puzzling questions concerning the regulation of enzyme activity by isoleucine and valine.

EXPERIMENTAL PROCEDURES

Materials and Analytical Methods. Restriction enzymes, polymerases, and ligases were from New England BioLabs. Isoleucine, valine, threonine, and 2-aminobutyrate were from either Sigma, Serva, or ICN Biochemicals, with no detectable differences between suppliers. Trifluoroacetic acid was from Pierce. TPCK-trypsin[1] was from Worthington Biochemicals. HPLC solvents and all other chemicals were of reagent grade purity. The stoichiometry of pyridoxal phosphate binding to the mutant enzymes was determined using phenylhydrazine as previously described (Eisenstein, 1991).

Cloning ilvA219 and Site-Directed Mutagenesis. An isoleucine-resistant strain of *Salmonella typhimurium* generously provided by R. LaRossa was used as a source of the *ilvA219* gene (LaRossa *et al.*, 1987). Chromosomal copies of *ilvA* from TV088, harboring the *ilvA219* allele, and TV087, containing the wild-type *ilvA* gene, were amplified using PCR techniques in two overlapping segments. The first segment was amplified using an oligonucleotide that was complementary to the noncoding sequence of the first 23 nucleotides, and an oligonucleotide that was complementary to a 27-nucleotide stretch of the coding strand beginning at 841 bp of *Salmonella ilvA* (Taillon *et al.*, 1988). The second segment was amplified using an oligonucleotide that was complementary to the noncoding sequence beginning at 804 bp and an oligonucleotide that was complementary to the coding sequence of the last 21 nucleotides of the *ilvA* sequence of *Salmonella*. The amplified fragments were cloned into M13mp18 and M13mp19 for DNA sequence determinations with Sequenase (US Biochemicals). Three isolates from TV087 and TV088 were sequenced to compare nucleotide substitutions between the two strains, or any other changes arising from amplification with *Taq* polymerase. Several differences were found in all six clones relative to the published nucleotide sequence for *S. typhimurium ilvA*. These differences include A211G, C370T, G278C, A927C, A996G, G1016C, C1017G, C1018T, A1024G, A1029G, A1047G, A1047G, A1052G, C1056T, C1056T, C1058T, and C1449T. None of these nucleotide differences yield a change in the deduced amino acid sequence, however. In addition, the three TV088 isolates contained an additional single mutation, G1341T, which results in a coding change of leucine at position 447 to phenylalanine. Since this was the only coding change detected in the *ilvA219* sequence, it was constructed in *E. coli ilvA* by site-directed mutagenesis (Kunkel *et al.*, 1987) using pEE27 as previously described (Fisher & Eisenstein, 1993). The mutant TD_{L447F} enzyme was expressed using pEE28 in an *ilvA* deletion strain (Fisher & Eisenstein, 1993) and was purified as previously described for wild-type threonine deaminase (Eisenstein, 1991, 1994).

Site-directed mutagenesis of *E. coli ilvA* was also performed to construct the substitution of alanine for lysine at position 62, the highly conserved residue which forms a Schiff base with the pyridoxal phosphate in threonine deaminase. The TD_{K62A} mutant was expressed as described above, but was purified in the presence of threonine rather than isoleucine as in the case for wild-type TD.[2]

Purification of a Borohydride-Reduced, Pyridoxamine Phosphate-Containing Proteolytic Fragment of Threonine Deaminase. Reduction of the pyridoxal phosphate Schiff base in threonine deaminase was achieved with $NaBH_4$ in the dark at 4 °C in 0.05 M potassium phosphate, pH 8.0, containing 0.1 mM EDTA, at 3 mg mL^{-1} enzyme. The 10

[1] Abbreviations: TPCK-treated trypsin, trypsin treated with L-1-(tosylamino-2-phenylethyl chloromethyl ketone to inhibit chymotryptic activity; HPLC, high-pressure liquid chromatography; PCR, polymerase chain reaction; bp, base pairs; GdmCl, guanidinium chloride; TD, threonine deaminase; TD_{L447F}, nomenclature for a mutant in threonine deaminase in which leucine 447 has been replaced with phenylalanine; TD_{K62A}, lysine 62 to alanine mutant of threonine deaminase.

[2] TD_{K62A} was purified in the presence of threonine since it was found that when the usual purification protocol was used with isoleucine present, a significant amount of isoleucine as well as threonine was found covalently attached to the pyridoxal phosphate cofactor. Although this situation had no effect on the binding isotherms measured for the mutant, the inclusion of threonine in purification buffers resulted in a vast increase in the homogeneity of the ligand bound at the active sites.

mL reaction was initiated by the addition of a 100-fold molar excess of a fresh 1 M $NaBH_4$ solution, and a pH of 8.0 was maintained with microliter additions of 1 N HCl. The bright yellow color of the solution was immediately bleached upon addition of $NaBH_4$, and after 15 min, another 100-fold molar excess of $NaBH_4$ was added to ensure complete reduction. The reduced enzyme was dialyzed in the dark at 4 °C against two changes of 0.05 M potassium phosphate, pH 7.5, containing 0.1 mM EDTA, for acquisition of an absorbance spectrum and for enzyme kinetics assays.

Prior to proteolysis, the cysteines of $NaBH_4$-reduced threonine deaminase were reduced and alkylated with iodoacetamide. First, 20 mg of the reduced enzyme was made 6 M in GdmCl, and then 100 μM dithiothreitol was added to reduce any disulfide bonds. Then, the solution was made 10 mM in iodoacetamide to alkylate the free sulfhydryl groups. The denatured, alkylated, $NaBH_4$-reduced enzyme was dialyzed against 0.01 M ammonium carbonate, pH 8.0, and digestion was performed with 2% (w/w) TPCK-treated trypsin at 37 °C in ammonium carbonate buffer containing 0.1 mM $CaCl_2$ and a final concentration of 2 M urea. After 1 h, another equivalent of trypsin was added, the reaction was left at 37 °C for 24 h, and the resulting mixture containing the pyridoxamine peptide was lyophilized to dryness.

The lyophilized powder was resuspended in a minimal volume of water for separation on a Waters Maxima HPLC System using a Vydak C-18 column (218TP54/4.6 mm × 25 cm). The purification of the pyridoxamine peptide was achieved in two steps. First, a sample was applied to the column equilibrated in 0.1% trifluoroacetic acid (solvent A), washed with equilibration buffer for 5 min, and then eluted with a linear gradient of increasing solvent B (95% acetonitrile in 0.1% trifluoroacetic acid) at a rate of 0.25% solvent B/min. A peak that absorbed at 325 nm eluted at 55 min and was collected for further purification. This fraction was reapplied to the column equilibrated in solvent A, washed for 2 min, and then eluted using a linear gradient of increasing solvent B at a rate of 0.125% solvent B/min for 102 min. A single peak that absorbed at 325 nm eluted at 93.5 min and was collected and lyophilized. The compositional and sequence analysis of the peptide was performed at the Protein−Nucleic Acid Laboratory at the University of Maryland at College Park.

Fluorescence Measurements. Fluorescence experiments were performed as previously described for either active site (Eisenstein, 1994) or effector site (Eisenstein *et al.*, 1994) ligand binding. Binding isotherms were normalized to the fractional fluorescence change and analyzed as discussed below.

Titration Calorimetry. The exchange of heat promoted by isoleucine and valine binding to variants of threonine deaminase was measured as previously described for wild-type enzyme (Eisenstein *et al.*, 1994). Data were analyzed as discussed previously, utilizing the Adair coefficients of the binding polynomials for stepwise enthalpy estimation (Parody-Morreale *et al.*, 1987; Eisenstein *et al.*, 1994), thereby avoiding the high correlation among errors in the parameters. Ligand binding stoichiometries were determined directly in calorimetric titrations from the total heat exchanged and the total threonine deaminase concentrations as previously described (Lin *et al.*, 1991) using instrumental software from MicroCal.

Enzyme Kinetics. Measurements of initial velocity versus substrate concentration were determined with a continuous spectrophotometric assay (Davis, 1965), using an extinction coefficient of 540 M^{-1} cm^{-1} for 2-ketobutyrate at 230 nm (Davis, 1965; Eisenstein, 1991) and of 1090 M^{-1} cm^{-1} for pyruvate at 220 nm (Davis, 1965; Schonbeck *et al.*, 1975).

Data Analysis. Cooperative ligand binding to the mutants of threonine deaminase was generally analyzed as described for wild-type enzyme (Eisenstein, 1994; Eisenstein *et al.*, 1994). Briefly, the binding isotherms, in the form of fractional saturation versus free (~total) ligand concentration, were analyzed in terms of the Adair equation (Adair, 1925) to obtain stoichiometric binding parameters, a Hill equation (Hill, 1910) to obtain empirical cooperative binding parameters, and then in terms of either a simple two-state model (Monod *et al.*, 1965) or the alternative form of the two-state model presented below. An expanded form of the two-state model was used to describe the cooperative binding data of substrate analogs not only to the active sites but also to the effector sites, to synergistically promote the allosteric transition. This model accounts for the effects of regulatory site saturation in a manner analogous to that proposed by Rubin and Changeux for nonexclusive ligand binding to either the T or the R states in allosteric systems (Rubin & Changeux, 1966). This expanded form of the two-state model for homotropic cooperativity has the following functional form

$$\Delta F/F_{max} = \frac{\alpha(1+\alpha)^3}{(1+\alpha)^4 + L[(1 + c_{act,X}[X]/K_{Ract,X})/(1 + [X]/K_{Ract,X})]^4} \quad (1)$$

where L is the allosteric equilibrium constant ([T]/[R]) in the absence of ligands, $c_{act,X}$ is the ratio of dissociation constants, $K_{Ract,X}/K_{Tact,X}$, for the association of ligand X with the regulatory sites when the enzyme is in either the R or the T conformation, $K_{Ract,X}$ is the dissociation constant for ligand X binding to the regulatory sites when the enzyme is in the R conformation, and $\alpha = [X]/K_R$, where [X] is the particular ligand concentration and K_R is the dissociation constant for the ligand for the active sites in the R conformation. The parameter c, or the ratio K_R/K_T, was fixed at zero as previously discussed (Eisenstein, 1994). The fractional fluorescence change, $\Delta F/F_{max}$, was determined as previously described (Eisenstein, 1994). Analysis of the data by any model was performed by nonlinear least-squares methods (Johnson & Fraser, 1985), with the error on the parameters corresponding to 65% confidence intervals. This usually resulted in confidence intervals of about 10−20% of the parameter value for binding constants, and of about 20−40% of the value for the allosteric equilibrium constants (Eisenstein, 1994; Eisenstein *et al.*, 1994).

RESULTS

Identification of the Mutation in the ilvA219 Allele. The *ilvA219* allele was initially identified as a *S. typhimurium* strain that was capable of using threonine as a sole nitrogen source (Burns *et al.*, 1979), and which was subsequently shown to be isoleucine resistant (LaRossa *et al.*, 1987). It was of interest to identify this genetic defect since it seemed a reasonable candidate for encoding an isoleucine binding

variant of threonine deaminase. The mutation in the *ilvA219* allele was identified by cloning the *ilvA* gene from *Salmonella* strain TV088 and comparing its nucleotide sequence to the isogenic *Salmonella* strain TV087 containing a wild-type chromosomal copy of *ilvA*. A comparison of three isolates of *ilvA* from TV087 with three from TV088 revealed 15 nucleotide differences with a published sequence of *ilvA* from *Salmonella* (Taillon *et al.*, 1988), but only a single nucleotide change between wild-type and *ilvA219*, in which the wild-type G at nucleotide 1431 was a T in the mutant. This single substitution results in a change in the TTG codon for an invariant leucine at position 447 to TTG, encoding a phenylalanine. This change was subsequently made in *E. coli ilvA*, and the mutant threonine deaminase was expressed, purified, and characterized as to the basis for its feedback resistance to isoleucine.

TD_{L447F} Is Isoleucine Resistant Because It Exhibits R State Behavior in Binding and Kinetics. Both steady-state kinetics and active-site ligand binding isotherms revealed that the L447F mutation in TD results in isoleucine resistance because the enzyme behaves as though it were stabilized in the so-called R state. As can be seen in Figure 1, the TD_{L447F} mutant displays Michaelian steady-state kinetics with either threonine or serine, in marked contrast to that exhibited for wild-type threonine deaminase (Eisenstein, 1991). The K_m value for threonine is 5.7 mM for the mutant, whereas the K_m for serine is 30 mM. The maximal velocity for TD_{L447F} is approximately 215 μmol/(mg·min), which is virtually identical for either serine or threonine saturation curves of wild-type enzyme. 2-Aminobutyrate binding to threonine deaminase through Schiff base formation with the 4 mol of pyridoxal phosphate in the tetramer also shows no evidence of sigmoidality, in sharp contrast to wild-type TD (Eisenstein, 1994), yielding an average binding constant of 13 mM. The addition of up to 1 mM isoleucine or valine had no effect on the saturation curves seen in Figure 1 (data not shown), consistent with the interpretation that the tetrameric mutant enzyme is stabilized in the high activity R conformation.

Since TD_{L447F} showed no effect of isoleucine or valine in active-site ligand binding isotherms or in steady-state kinetics, it was of interest to assess whether these ligands possessed any measurable affinity for the regulatory sites on the enzyme. As can be seen in Figure 2, isoleucine and valine both bind strongly to the mutant enzyme, in a nearly hyperbolic manner. The isoleucine binding isotherm shows a slight sigmoidality, characterized by a Hill coefficient, n_H, of 1.5 and a $K_{0.5}$ of 4.4 μM, and a stoichiometric analysis of binding yields an average dissociation constant of 4.5 μM. Valine binding is noncooperative, characterized by a simple binding constant of 19 μM. This value is identical with the predicted value of 19 μM for valine binding to the R state of the enzyme based on an analysis of effector binding to wild-type threonine deaminase (Eisenstein *et al.*, 1994). Thus, it does not appear that strains harboring the *ilvA219* allele are feedback resistant because of a simple binding defect, but rather because TD_{L447F} exhibits the functional properties of an "R state" enzyme, even in the presence of isoleucine.

In spite of the strong affinity of TD_{L447F} for isoleucine and valine as evidenced in the spectroscopic binding studies, titration calorimetry revealed the mutation had an effect on the stoichiometry of effector site binding. As can be seen in Figure 3, the association of ligands to the regulatory sites occurs with a release of heat, similar to that seen for wild-type enzyme (Eisenstein *et al.*, 1994). However, an analysis of these data reveal that only 2 mol of isoleucine or valine bind to the tetrameric enzyme, as compared with 4 mol of effector for wild-type TD and for TD_{K62A} (see below). Analysis of isoleucine binding to two sites yields an average enthalpy change of −12.3 kcal/mol for the L447F mutant, which is close to the value of −10.7 kcal/mol seen for wild-type enzyme. Alternatively, an analysis of valine binding to two sites on TD_{L447F} yields an enthalpy change of −18.4

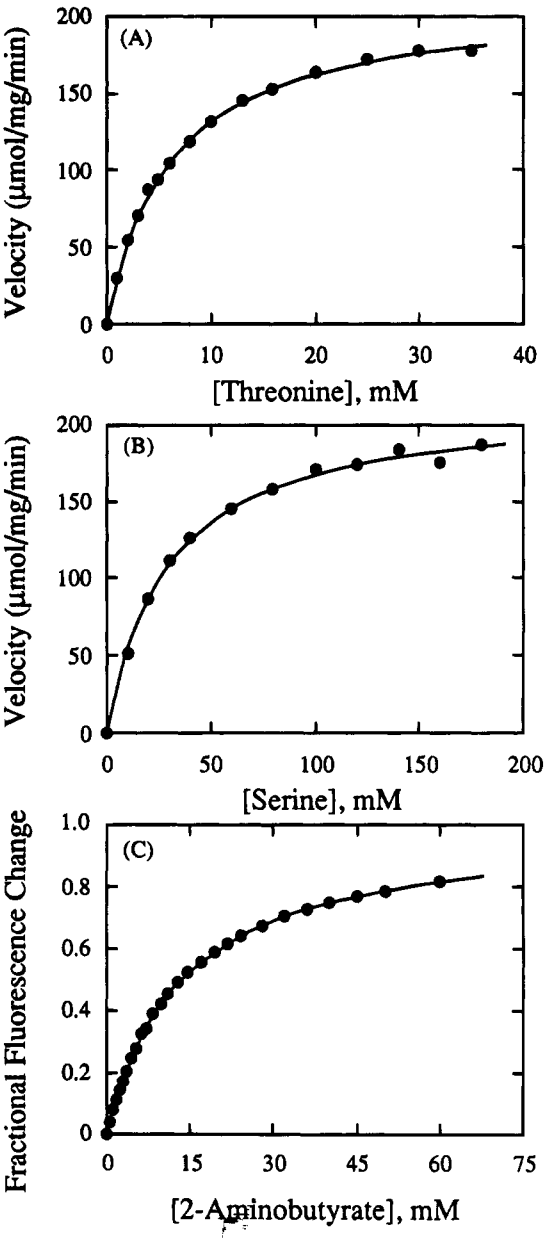

FIGURE 1: Noncooperative ligand binding to the active sites of TD_{L447F}. Hyperbolic steady-state kinetics of TD_{L447F} using either (A) threonine or (B) serine in assays. Product formation was followed continuously at either 230 nm for 2-ketobutyrate or 220 nm for pyruvate in 0.05 M potassium phosphate, pH 7.5, at 25 °C. The theoretical curves were constructed from an analysis in terms of the Michaelis−Menten equation, yielding values for the K_m of 5.7 mM for threonine and 30 mM for serine, with equivalent V_{max} values of 215 μmol/(mg·min). (C) Noncooperative binding of the substrate analog 2-aminobutyrate assessed by the increase in pyridoxal phosphate fluorescence at 480 nm. The theoretical curve was constructed from an analysis to a simple binding isotherm, yielding an average dissociation constant of 13 mM. Binding experiments were conducted in 0.05 M potassium phosphate buffer, pH 7.5 at 25 °C.

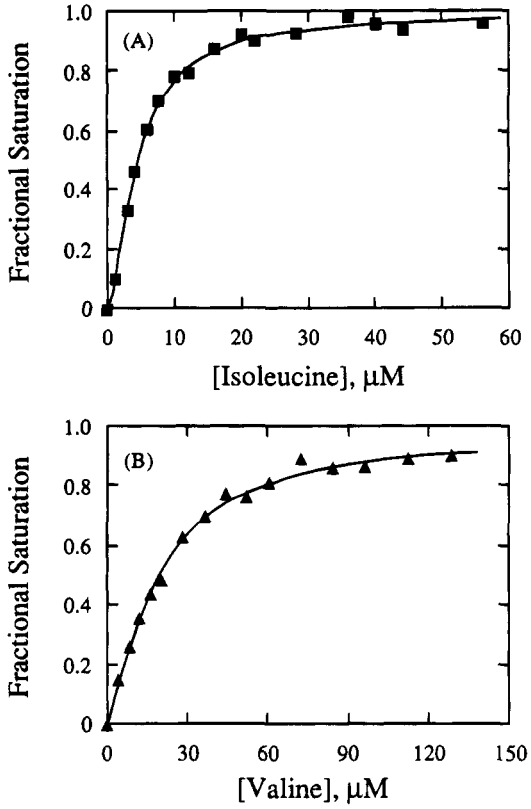

FIGURE 2: Isoleucine and valine binding to the regulatory sites in TD_{L447F}. The fractional fluorescence change was determined by the increase in tryptophan emission at 343 nm and is plotted versus free (~total) ligand concentration in 0.05 M potassium phosphate buffer, pH 7.50, at 25 °C. (A) Isoleucine saturation plot. The data were analyzed in terms of a two-term Adair equation, and the theoretical curve was generated using the parameters (and errors) $\beta_1 = 1.39 \times 10^5\ M^{-1}$ (±21%) and $\beta_2 = 4.95 \times 10^{10}\ M^{-2}$ (±17%), yielding an average dissociation constant of 4.5 μM. (B) Valine saturation plot. The data were analyzed in terms of a two-term Adair equation, and the theoretical curve was generated using the parameters (and errors) $\beta_1 = 5.88 \times 10^4\ M^{-1}$ (±31%) and $\beta_2 = 2.77 \times 10^9\ M^{-2}$ (±24%), yielding an average dissociation constant of 19 μM.

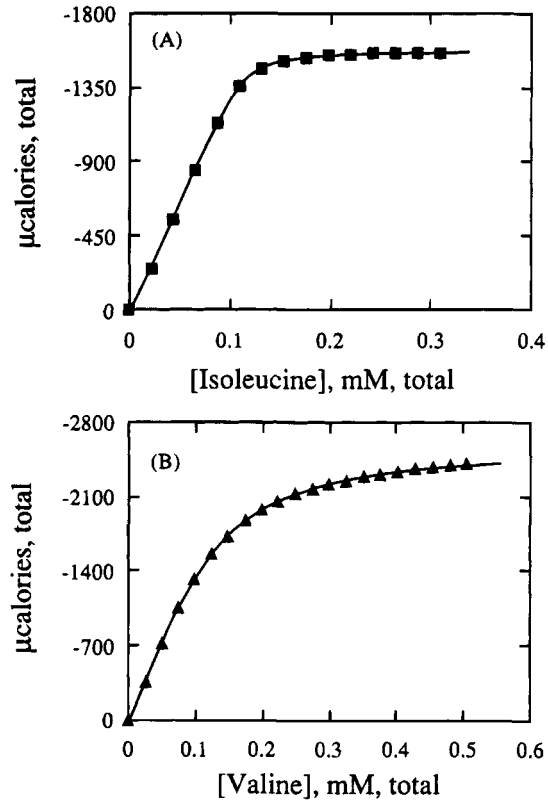

FIGURE 3: Effect of isoleucine and valine on the release of heat from TD_{L447F}. (A) The total heat released upon the addition of 5 μL aliquots of 6 mM isoleucine to a 48 μM TD_{L447F} solution is plotted versus the total isoleucine concentration. The data were analyzed as described under Experimental Procedures, and the theoretical curve was generated using the Adair coefficients given in the legend to Figure 2 and the following values for the stepwise enthalpies (and errors): $\Delta H_1 = -0.9$ kcal/mol (±26%) and ΔH_2, the total enthalpy for the reaction, of −24.5 kcal/mol (±1.8%), yielding an average enthalpy of −12.3 kcal/mol for isoleucine binding to TD_{L447F}. (B) The total heat released upon addition of 5 μL aliquots of 6.7 mM valine to a 48 μM TD_{L447F} solution versus the total valine concentration. The theoretical curve was generated using the values of the Adair coefficients given in Figure 2 and the following values of the stepwise enthalpies (and errors): $\Delta H_1 = -23.4$ kcal/mol (±42%) and ΔH_2, the total enthalpy for the reaction, of −36.7 kcal/mol (±8.5%), yielding an average enthalpy of −18.4 kcal/mol for valine binding to TD_{L447F}.

kcal/mol, more than twice that measured for wild-type TD. A summary of the average thermodynamic parameters for isoleucine and valine binding to TD_{L447F} is presented in Table 1.

Lysine 62 Forms a Schiff Base with the Pyridoxal Phosphate Cofactor in Threonine Deaminase. Because the stoichiometry of regulatory effector binding to TD_{L447F} was only half that seen for wild-type enzyme, additional evidence was needed to confirm that the binding parameters for the mutant were an accurate reflection of the putative R state. Another mutant variant was therefore sought which displayed R state-like binding properties, but for which the stoichiometry of ligand binding was the same as that for wild-type threonine deaminase. In a parallel effort toward identifying potential functional residues in the active site of threonine deaminase, lysine 62 was identified as the residue that forms a Schiff base with pyridoxal phosphate in the active site of the enzyme. Sodium borohydride reduction of threonine deaminase resulted in a loss of activity, which was proportional to the decrease in pyridoxal phosphate absorbance at 412−413 nm. Digestion of $NaBH_4$-reduced enzyme with trypsin yielded a mixture of small fragments, and the pyridoxamine phosphate-containing peptide was purified using acetonitrile gradients on reverse phase HPLC. Because

Table 1: Average Thermodynamic Parameters for Isoleucine and Valine Binding to the Regulatory Sites of TD_{L447F}[a]

parameter[b]	isoleucine	valine
K_{av}	4.5 μM	19 μM
$\Delta G°$	−7.3 kcal/mol	−6.4 kcal/mol
$\Delta H°$	−12.3 kcal/mol	−18.4 kcal/mol
$-T\Delta S°$	+5.0 kcal/mol	+12.0 kcal/mol

[a] The thermodynamic parameters are reported for binding experiments that were conducted in 0.05 M potassium phosphate, pH 7.50, at 25 °C. [b] Average free energies were determined from fluorescence changes as described under Experimental Procedures. 65% confidence intervals for the average Gibbs free energies are approximately 5% of the parameter value. The calorimetrically determined average enthalpies show 65% confidence intervals that are less than 10% of the parameter values.

the N-terminal residue was blocked to sequencing, the location of the peptide in the amino acid sequence of threonine deaminase was ascertained by determining the amino acid composition of the fragment. The amino acids found from this analysis were arginine, histidine, leucine, phenylalanine, proline, serine, and valine, in addition to two

other unidentified peaks. This composition was in good agreement with a potential trypsin-generated sequence that possesses strong primary sequence similarity between several pyridoxal phosphate requiring dehydratases for which the Schiff base has been identified (Datta *et al.*, 1987; Ogawa *et al.*, 1989) and pointed to lysine 62 as the residue that forms the Schiff base with pyridoxal phosphate. This residue, previously implicated from genetic studies to be essential for catalysis in threonine deaminase (Fisher & Eisenstein, 1993), was therefore targeted by site-directed mutagenesis for replacement with alanine.

The lysine 62 to alanine mutation in threonine deaminase yields a completely inactive enzyme as evidenced by the isoleucine auxotrophy of an *ilvA* deletion strain carrying a plasmid encoding the variant. On the other hand, the purified enzyme retained 4 mol of strongly bound pyridoxal phosphate, although its spectral properties differed significantly from that seen for the wild-type (Eisenstein *et al.*, 1994). Absorption, fluorescence, and circular dichroism spectra indicated not only that the internal aldimine Schiff base with the active site lysine had been broken, but that the enzyme contained exogenous amino acid bound to the cofactor (data not shown). Hence, an aliquot of purified enzyme was acidified to precipitate polypeptide chains and to liberate the cofactor–amino acid complex. The composition of the mixture identified threonine as the most abundant amino acid, comprising about 85% of the bound material.[3] Additional amino acids included trace amounts of aspartate (4%), alanine (<2%), valine (<2%), methionine (<3%), isoleucine (<2%), leucine (<3%), and tryptophan (<2%). This is similar to what is seen for some other pyridoxal phosphate-dependent enzymes (Lu *et al.*, 1993; Schirch *et al.*, 1993) in which removal of the internal aldimine Schiff base by mutagenesis results in a purified variant enzyme with a ligand bound covalently to the cofactor.

The TD_{K62A}–Threonine Complex Binds Regulatory Ligands as an R State Enzyme. Since the K62A mutant of threonine deaminase was purified with threonine bound to the active site, it was expected that the enzyme might be stabilized sufficiently in the R state to display noncooperative binding isotherms for isoleucine and valine. As can be seen in Figure 4, isoleucine binding to the TD_{K62A}–threonine complex exhibits a nearly hyperbolic isotherm, characterized by a Hill coefficient of 1.4 and a $K_{0.5}$ of 6.3 μM, yielding an average dissociation constant of 5.3 μM. On the other hand, valine binds with no evidence of sigmoidality, reflected in the simple binding constant of 26 μM, in good agreement with that seen for TD_{L447F}, and also with the value of 19 μM for valine binding to the R state of wild-type enzyme (Eisenstein *et al.*, 1994). Titration calorimetry was used to assess the energetics and stoichiometry of regulatory effector binding to TD_{K62A}, and the results are summarized in Table 2. Calorimetric titrations revealed simple binding of 4.1 mol of valine per TD_{K62A}–threonine complex, with a dissociation constant of 26 μM, and an enthalpy change of −12.4 kcal/mol. Similarly, 4 mol of isoleucine were bound per tetrameric mutant, with an average enthalpy change of −11.3 kcal/mol, assuming an average binding constant of 5.3 μM.

[3] Quantitative amino acid analysis kindly performed by Zafar I. Randawa at Otsuka America Pharmaceutical, Inc., Rockville, MD, revealed that approximately 90–95% of the pyridoxal phosphate contained bound amino acid.

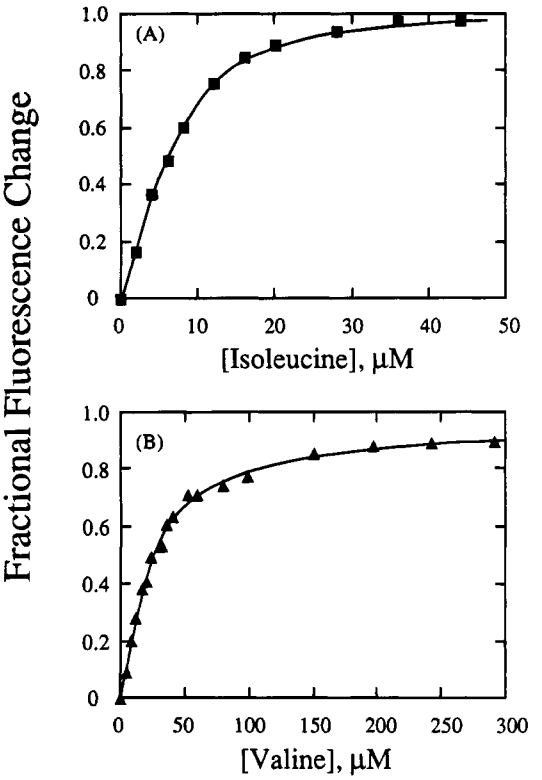

FIGURE 4: Isoleucine and valine binding to the regulatory sites of the TD_{K62A}–threonine complex. Ligand binding was monitored by the increase in tryptophan fluorescence at 343 nm. (A) Isoleucine binding isotherm. The data were analyzed in terms of a four-term Adair equation, and the theoretical curve was generated using the parameters (and errors) $\beta_1 = 3.58 \times 10^5$ M^{-1} (±24%), $\beta_2 = 9.43 \times 10^{10}$ M^{-2} (±18%), $\beta_3 = 2.62 \times 10^{15}$ M^{-3} (±6.2%), and $\beta_4 = 1.31 \times 10^{21}$ M^{-4} (±8.8%), yielding an average dissociation constant of 5.3 μM. (B) Valine saturation plot. The data were analyzed in terms of a four-term Adair equation, and the theoretical curve was generated using the parameters (and errors) $\beta_1 = 8.85 \times 10^5$ M^{-1} (±17%), $\beta_2 = 4.77 \times 10^9$ M^{-2} (±19%), $\beta_3 = 1.98 \times 10^{14}$ M^{-3} (±46%), and $\beta_4 = 2.16 \times 10^{18}$ M^{-4} (±4.3%), yielding an average dissociation constant of 26 μM.

Table 2: Average Thermodynamic Parameters for Isoleucine and Valine Binding to the Regulatory Sites of the TD_{K62A}–Threonine Complex[a]

parameter[b]	isoleucine	valine
K_{av}	5.3 μM	26 μM
$\Delta G°$	−7.2 kcal/mol	−6.3 kcal/mol
$\Delta H°$	−11.3 kcal/mol	−12.4 kcal/mol
$-T\Delta S°$	+4.1 kcal/mol	+6.1 kcal/mol

[a] The thermodynamic parameters are reported for binding experiments that were conducted in 0.05 M potassium phosphate, pH 7.50, at 25 °C. [b] Average free energies were determined from fluorescence changes as described under Experimental Procedures. 65% confidence intervals for the average Gibbs free energies are approximately 5% of the parameter value. The calorimetrically determined average enthalpies show 65% confidence intervals that are less than 10% of the parameter values.

Thus, the TD_{K62A}–threonine complex behaves as though it was stabilized significantly toward the R state, yielding parameters for isoleucine and valine binding that are consistent with analyses of wild-type isotherms, as well as with ligand binding to the R state-like TD_{L447F} mutant.

Substrates and Analogs Bind More Strongly to the Regulatory Sites Than to the Active Sites of Threonine Deaminase. Because TD_{K62A} was completely inactive, yet contained near-stoichiometric levels of threonine covalently bound to

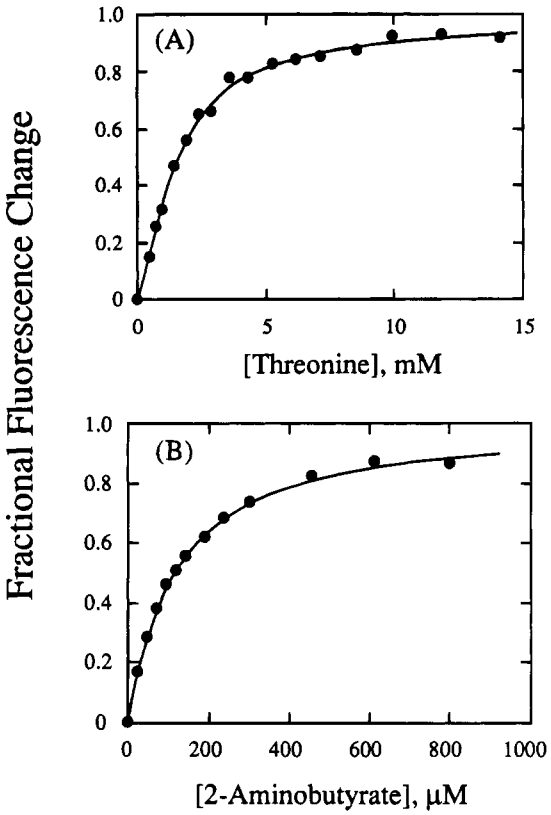

FIGURE 5: Active site ligands bind to the effector sites of the TD_{K62A}–threonine complex. Ligand binding was monitored by the increase in tryptophan fluorescence at 343 nm. (A) Threonine saturation curve. The data were analyzed in terms of a four-term Adair equation, and the theoretical curve was generated using the parameters (and errors) $\beta_1 = 3.97 \times 10^3\ M^{-1}$ (±17%), $\beta_2 = 3.54 \times 10^5\ M^{-2}$ (±17%), $\beta_3 = 9.86 \times 10^8\ M^{-3}$ (±15%), and $\beta_4 = 3.38 \times 10^{11}\ M^{-4}$ (±18%), yielding an average dissociation constant of 1.3 mM. (B) 2-Aminobutyrate saturation curve. The data were analyzed in terms of a four-term Adair equation, and the theoretical curve was generated using the parameters (and errors) $\beta_1 = 3.12 \times 10^4\ M^{-1}$ (±23%), $\beta_2 = 4.17 \times 10^8\ M^{-2}$ (±27%), $\beta_3 = 2.26 \times 10^{12}\ M^{-3}$ (±23%), and $\beta_4 = 5.72 \times 10^{15}\ M^{-4}$ (±12%), yielding an average dissociation constant of 115 μM.

pyridoxal phosphate in the active sites, it was possible to ask whether substrates or analogs possessed measurable affinity for the effector sites on the enzyme. As can be seen in Figure 5, both threonine and 2-aminobutyrate bind to the regulatory sites of threonine deaminase, *with greater affinity than for the active sites*! Threonine binding is characterized by a nearly hyperbolic isotherm, yielding a Hill coefficient of 1.3, a $K_{0.5}$ of 1.5 mM, and an average dissociation constant of 1.3 mM. 2-Aminobutyrate also binds strongly to the effector sites, yielding hyperbolic isotherms that were characterized by an average binding constant of 115 μM. Thus, threonine binds about 5-fold, and 2-aminobutyrate binds about 100-fold more tightly to the regulatory sites than to the active sites.

In an effort to combine the observation that substrates and analogs bind strongly to the effector sites of threonine deaminase with an explanation for the discrepancy between estimates for the allosteric equilibrium constant evaluated by different methods, an expanded, two-state model was developed. The expanded model as represented by eq 1 was used to analyze the binding data of 2-aminobutyrate to threonine deaminase (Eisenstein, 1994) and, by assuming that initial velocity was proportional to fractional saturation, to

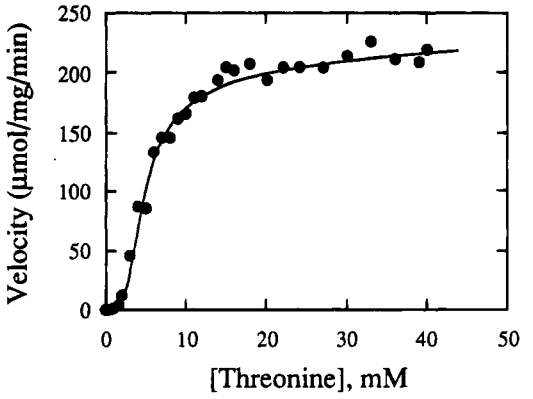

FIGURE 6: Steady-state kinetics of wild-type threonine deaminase. The formation of 2-ketobutyrate was followed continuously at 230 nm in 0.05 M potassium phosphate buffer, pH 7.5, at 25 °C. A Hill analysis yielded a value for n_H of 2.2, a $K_{0.5}$ of 5.4, and a V_{max} of 215 μmol/(mg·min). The data were also analyzed to the expanded two-state model described by eq 1 in Experimental Procedures, yielding the parameters (and errors) used for constructing the theoretical curve: $L = 1450$ (±46%), $K_{R,Thr} = 3.4$ mM (±11%), $K_{Ract,Thr} = 1.0$ mM (±10%), $c_{act,Thr} = 0.15$ (±22%), $V_{max} = 234$ μmol/(mg·min) (±12%), with c for threonine binding to the active site fixed at 0 as discussed previously (Eisenstein, 1994).

steady-state kinetics with threonine as substrate. As can be seen in Figure 6, the model fits well to the kinetic data using wild-type threonine deaminase. A value of 1450 was obtained for L, the allosteric equilibrium constant, which is in reasonable agreement with the value of 1870 seen for isoleucine and valine binding to wild-type enzyme (Eisenstein *et al.*, 1994), as well with the value of 1350 seen for D-threonine binding to the active sites (Eisenstein, 1995). In addition, the model returns an estimate for the binding constant for threonine to the regulatory sites in the R state of 1.0 mM, which is in good agreement with the value of 1.3 mM determined experimentally with TD_{K62A}. Analysis of the 2-aminobutyrate binding data presented previously (Eisenstein, 1994) yields an allosteric equilibrium constant of 1125, in sharp contrast to the value of 1.4 that was previously determined from a simple, two-state model, but which is in reasonable agreement with the new estimates. Moreover, the value of 105 μM for the binding constant for 2-aminobutyrate to the R state regulatory sites evaluated from the expanded model is also in excellent agreement with the value of 115 μM determined experimentally with TD_{K62A}. A summary of the allosteric parameters determined for wild-type threonine deaminase using the expanded, two-state model is presented in Table 3.

DISCUSSION

The cooperativity seen in substrate and analog binding to threonine deaminase can be accounted for by an expanded two-state model in both a qualitative and quantitative manner. The key provision in the expanded model relative to the two-state model originally formulated by Monod, Wyman, and Changeux (1965) concerns the effect of substrates and analogs in promoting the allosteric transition by binding to effector sites, as well as to the active sites. The simple, two-state model proposes that the preferential affinity of ligands for specific sites on allosteric enzymes affects cooperativity solely by displacing a putative equilibrium between the T and R states, but the functional form of the model used for most analyses of sigmoidal binding isotherms accounts only

Table 3: Allosteric Parameters for Homotropic Cooperativity in Threonine Deaminase[a]

parameter[b]	threonine[c]	2-aminobutyrate[d]
L	1450	1125
K_R	3.4 mM	11 mM
c	0	0
$K_{Ract,X}$	1.0 mM	105 μM
$c_{act,X}$	0.15	0.15

[a] The allosteric parameters are reported for binding experiments and steady-state kinetics that were conducted in 0.05 M potassium phosphate, pH 7.50, at 25 °C. [b] The allosteric parameters were determined as described under Experimental Procedures. 65% confidence intervals for the equilibrium constants range from approximately 20% of the parameter value for binding constants, and about 40% of the parameter values for L, the allosteric equlibrium constant. When binding constants were fixed to the independently determined values, the error on the allosteric equilibrium constants improved substantially to about 10% of the parameter value. [c] The parameters for threonine are derived from analysis of steady-state kinetics experiments in which it is assumed that fractional saturation is directly proportional to maximal velocity. [d] The allosteric parameters for 2-aminobutyrate binding are taken from saturation curves published previously (Eisenstein, 1994).

for the specific association of ligands with either the active sites or the effector sites (Monod *et al.*, 1965). Of course, substrate binding to the active sites will readily promote the T → R transition through preferential binding to the R state, an effect which is particularly significant for threonine deaminase because of the apparently low affinity of T state active sites for ligands (Eisenstein, 1994). In also binding to the effector sites, however, substrates and their analogs will shift the allosteric equilibrium by preferentially stabilizing the R state, in an analogous manner to heterotropic effectors proposed by Rubin and Changeux (1966), but in a progressive manner throughout the saturation curve. Thus, the 100-fold increase in the affinity of 2-aminobutyrate for the effector sites relative to the active sites should substantially shift the T → R equilibrium, resulting in an increase in the average concentration of R molecules in the range of analog concentration where it binds to the active sites. This interpretation is supported by the nearly hyperbolic isotherms seen for this ligand, yielding erroneously low values for L when analyzed with the simple two-state model. By contrast, threonine binds only 5-fold more strongly to the effector sites than to the active sites. This small difference results in a correspondingly smaller shift in the average population of R state molecules in the concentration range where active-site binding occurs, consistent with the more cooperative isotherms seen in steady-state kinetics. To a first approximation, therefore, the strong association demonstrated for substrates and analogs with effector sites is sufficient to explain the degree of homotropic cooperativity seen for these ligands in binding to the active sties of threonine deaminase and qualitatively suggests that active site ligands act synergistically to promote the allosteric transition.

In an effort to obtain a coherent, quantitative description of these equilibria, the expanded model presented in eq 1 was used to analyze both the 2-aminobutyrate binding isotherms and the threonine saturation curves obtained from steady-state kinetics with wild-type TD. As can be seen in Table 3, not only was there good agreement between the values for the allosteric equilibrium constant from these two saturation curves, but the estimates for L were in reasonable agreement with the values determined for isoleucine and valine binding isotherms, which associate solely with the regulatory sites (Eisenstein *et al.*, 1994), and also for D-threonine isotherms, which binds solely to the active sites (Eisenstein, 1995).[4] Considered together, these data yield an average value for L of 1525, well within the error of the various experiments, corresponding to an energetic difference between the T and the R states of 4.3 kcal/mol. Furthermore, the values for the R state dissociation constants for threonine and 2-aminobutyrate binding to the effector sites ($K_{Ract,X}$) were in excellent agreement with the estimates determined experimentally with the mutants (Figure 6), providing additional, quantitative justification for the use of this model.

Early studies on the regulation of threonine deaminase also suggested an intricate mechanism of control, despite complications from questionable preparations of wild-type enzyme that exhibited hyperbolic steady-state kinetics, substoichiometric cofactor levels, and noncooperative binding of isoleucine and valine (Burns & Zarlengo, 1968; Harding, 1969; Hatfield & Umbarger, 1970; Calhoun *et al.*, 1973; Decedue *et al.*, 1975; Koerner *et al.*, 1975). Of particular relevance are the findings by Decedue *et al.* (1975) that threonine was an effective competitor of valine for binding to a modified enzyme that was inactivated by borohydride reduction of the internal aldimine Schiff base. Their results, which were considered incompatible with the tenets of the two-state model, suggested that threonine could bind to a single regulatory site to activate the enzyme. Our efforts using mutant enzymes support these early observations by measuring directly the relatively strong binding of threonine and 2-aminobutyrate to the regulatory sites, as well as by the relative inability of isoleucine and valine to bind to the active sites (see below). Moreover, by providing an energetic framework for the allosteric transition that is consistent with the data for threonine, 2-aminobutyrate, isoleucine, valine, and D-threonine binding to threonine deaminase, we can readily extend these results by testing the expanded model with other mutants.

A key attribute of the two mutants that were used in this study was that they both exhibited the functional characteristics of an enzyme distribution that was stabilized in the R state. This was important not only for verifying the value of K_R for valine binding to the R state and to link it with an estimate for L, but also to assess the affinities of regulatory ligands for the active sites, as well as active site ligands for the regulatory sites. For example, the simple, hyperbolic saturation curves that TD_{L447F} displayed for substrates and analogs seen in Figure 1, which were unperturbed by the presence of millimolar levels of isoleucine and valine, enabled the measurement of binding constants for isoleucine and valine to an R state active site from competitive inhibition studies.[5] The K_i for isoleucine in 0.05 M potassium phosphate, pH 7.5 at 25 °C, was found to be 66 mM, whereas the K_i for valine binding to the R state was 120 mM (data not shown). Thus, under the conditions normally used to measure regulatory site binding by isoleucine and valine, there is negligible association of these effectors with

[4] Spectroscopic binding experiments have thus far failed to reveal the association of D-threonine with the regulatory sites of threonine deaminase.

[5] The hyperbolic nature of both steady-state kinetics and 2-aminobutyrate binding by TD_{L447F} is maintained over the pH range of 6.0–9.5, and from an ionic strength of 0.1 to 0.5 (H. D. Yu and E. Eisenstein, unpublished observations).

the active sites. On the other hand, because the allosteric equilibrium of TD_{K62A} appears shifted in the direction of the R conformation because threonine was bound covalently to pyridoxal phosphate in the active sites, this mutant was useful in establishing that both threonine and 2-aminobutyrate possess strong affinity for the unoccupied regulatory sites of the mutant−threonine complex (Figure 5). An additional attribute of the K62A mutant of threonine deaminase was that it eliminated any uncertainty attendant to the half-of-the-sites binding of effector ligands to TD_{L447F}. The isotherms in Figure 4 yielded binding constants for isoleucine and valine to TD_{K62A} that were in good agreement with those determined with TD_{L447F}, and also with the analyses of ligand binding to wild-type threonine deaminase. Moreover, calorimetric titrations (data not shown) yielded 4.1 sites for isoleucine and valine binding to the TD_{K62A}−threonine complex, in contrast to TD_{L447F}, but in agreement with wild-type TD (Eisenstein *et al.*, 1994).[6]

It is interesting to point out that the molecular basis for isoleucine resistance of the *ilvA219* allele does not appear to result from a binding defect for isoleucine *per se*. The strong affinity of the L447F mutant enzyme for isoleucine can be seen readily in the fluorescence binding measurements presented in Figure 2, as well as the calorimetric titrations in Figure 3. Alternatively, the hyperbolic steady-state kinetics and ligand binding properties displayed by TD_{L447F} argue that the variant is resistant to isoleucine because its allosteric equilibrium lies well in the direction of the R state. Further support for the interpretation that TD_{L447F} and the TD_{K62A}−threonine complex are in the so-called R conformation comes from three other lines of evidence. First, there was excellent agreement between the average dissociation constant for valine binding to either TD_{L447F} or the TD_{K62A}−threonine complex with the calculated value for K_R of 19 μM obtained from an analysis of cooperative valine binding to wild-type enzyme in terms of the two-state model (Eisenstein *et al.*, 1994). Second, the average enthalpy of valine binding to the two mutant variants was greater than that seen for wild-type TD, possibly reflecting the increased complementarity of valine with an R state regulatory site, an enthalpic cost for the protein structural rearrangements to make an R state regulatory site from a T state site, or both. Finally, although there was little evidence of cooperative valine binding either to the TD_{K62A}−threonine complex or to TD_{L447F}, the binding isotherms for isoleucine clearly showed sigmoidality, reflecting its preferential affinity for the T state. These data are consistent with the notion that the allosteric equilibrium for the two mutants is shifted toward the R state and imply that whereas isoleucine may alter the allosteric equilibrium slightly toward the T state, the shift is so small that it has no effect on TD_{L447F} in kinetics or active-site ligand binding.

The low stoichiometry for isoleucine and valine binding to TD_{L447F} revealed by titration calorimetry may be the result of a local distortion in the symmetry of the mutant enzyme. If leucine 447 was on or near a molecular 2-fold axis, then two of the four effector sites might be disrupted by steric restrictions in the vicinity of the mutation that prevent the larger phenylalanine rings from maintaining 2-fold symmetry. This interpretation is consistent with diffraction studies of threonine deaminase crystals belonging to the space group *I*222, with a single monomer per asymmetric unit, indicating that the wild-type tetramer has 222 symmetry, containing three, 2-fold axes.[7] At this point in our studies, however, it may be unwarranted to suggest that the L447F substitution, located in the C-terminal regulatory domain of the enzyme (Taillon *et al.*, 1988; Fisher & Eisenstein, 1993), shifts the allosteric equilibrium through a conformational change at an "allosteric" interface. Amino acid substitutions in diverse structural domains of other allosteric enzymes have also yielded variants that exhibit R state-like properties (Eisenstein *et al.*, 1990; Newell & Schachman, 1990; Wente & Schachman, 1991). Clearly, high resolution structural information will be of great use in evaluating these possibilities.

The expanded model presented here, predicated on the binding of substrates and analogs to the effector sites to promote the T → R transition, implies that when isoleucine or valine fully saturate the effector sites, active-site ligand binding might be described by the simple two-state model. However, the strong affinity of 2-aminobutyrate for the effector sites suggests a significant competition with modifier binding. Another limitation to applying the simple, two-state model to steady-state kinetics in the presence of effectors stems from the compound effect of isoleucine and valine on ligand binding and enzymic catalysis in threonine deaminase. Not only does isoleucine inhibit ligand binding to the active sites of threonine deaminase through stabilization of the T state, but the negative allosteric effector also inhibits transimination, the first step in pyridoxal-mediated catalysis (Eisenstein, 1995). This may explain the relatively large effect of isoleucine seen in steady-state kinetics (Eisenstein, 1991) as compared to that observed in ligand binding (Eisenstein, 1994, 1995). In addition, the possibility that isoleucine affects the rapid equilibration of threonine with the active sites during steady-state kinetics (Hatfield, 1971) may need to be analyzed by considering aspects of the numerous proposals put forth to account for kinetic effects that may lead to cooperativity in enzyme kinetics (Frieden, 1964; Neet, 1980; Ricard & Noat, 1984; Symcox & Reinhart, 1992). Investigations on the kinetics of ligand binding, as well as the structural basis for control of threonine deaminase, should be helpful in resolving these issues. Overall, the expanded two-state model represents an important step toward explaining the energetics of cooperative binding of substrates and analogs to wild-type threonine deaminase and will doubtless be useful in deciphering the association of ligands to numerous mutants of this regulatory enzyme.

[6] Our initial strategy to corroborate the R state binding parameters relied on measuring valine binding in the presence of 2-aminobutyrate. It was expected that, in the presence of millimolar levels of 2-aminobutyrate, which would be sufficient to saturate the active sites and promote the allosteric transition, valine would bind hyperbolically and with high affinity to the effector sites. However, saturable valine binding was rarely observed under these conditions, with estimates for binding constants above 200 μM, even at as low as 2 mM 2-aminobutyrate. These results can now be reconciled by the explanation that 2-aminobutyrate binds to the effector sites, even in a lower concentration range than where the active sites would be fully saturated.

[7] D. T. Gallagher, K. R. Ducote, E. Eisenstein, and G. L. Gilliland, unpublished observations.

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