# Biochemical and Mass Spectrometric Evidence for Quaternary Structure Modifications of Plant Threonine Deaminase Induced by Isoleucine‡

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ABSTRACT: *Arabidopsis thaliana* threonine deaminase (TD) is a tetramer composed of identical ∼59600 Da subunits. TD activity has been shown to be inhibited by isoleucine. This effect is reversed by a large excess of valine. Nondenaturant gel filtration, polyacrylamide gel electrophoresis, and mass spectrometry experiments demonstrated that binding of isoleucine on TD induces dimerization of the enzyme, whereas tetramerization is restored by addition of a high valine concentration. Nondenaturant gel filtration and electrospray ionization mass spectrometry of the enzyme in the presence of increasing amounts of isoleucine suggest a fast equilibrium between the tetramer and the dimer. Finally, study of TD mutants allowed us to focus on the specific role of each isoleucine-binding site.

The bacterial biosynthetic threonine deaminase (TD,[1] EC 4.2.1.16) is a homotetramer with a molecular mass of 224 kDa (*1*). The resolution of the structure of the *Escherichia coli* enzyme demonstrated that each of the identical subunits was organized into two distinct domains, a catalytic N-terminal domain containing the pyridoxal phosphate (PLP) cofactor and a regulatory C-terminal domain (*1*). The N- and C-terminal domains between which no contact occurred constituted two globular structures connected by a helix (*1*). The tetramer of *E. coli* TD has been described as a "dimer of dimers" because only weak interactions occurred between both catalytic domains of the dimers (*1*). Thus, most of the interactions involved in the quaternary structure take place at the level of the regulatory domain within each dimer (*1*).

TD catalyzed the deamination of threonine into α-ketobutyrate. Isoleucine and valine, the end products of the biosynthetic pathways, behaved like allosteric effectors (*2*, *3*). Isoleucine was shown to be an allosteric inhibitor by decreasing the affinity for threonine, whereas valine reversed the inhibition by isoleucine (*4*−*17*). Recently, kinetic and binding experiments on the wild type and mutants of *Arabidopsis thaliana* TD demonstrated that the regulatory domain of each monomer possessed two effector-binding sites constituted in part by Y449 and Y543 (*18*). This previous work demonstrated that Y449 belonged to a high-affinity binding site whose interaction with a first isoleucine molecule induced conformational modifications corresponding to a conformer displaying a higher activity which makes the low-affinity binding site Y543 efficient in fixing a second isoleucine molecule (*18*). Isoleucine interaction with the latter binding site was responsible for conformational modifications leading to the final inhibition of the enzyme (*18*). Y449 interacted with both isoleucine and valine. However, interaction of valine with the high-affinity binding site induced different conformational modifications, leading to the dissociation of isoleucine from binding sites and the reversion of inhibition (*18*).

As described above, regulation of TD operated via conformational modifications induced by sequential ligand binding on Y449 and Y543 (*18*). To determine if these conformational changes also modified the interactions between monomers, we analyzed in this paper the effect of isoleucine and valine on the quaternary structure of *A. thaliana* TD. For this purpose, several methodologies, such as nondenaturant gel filtration (nd-GF), native polyacrylamide gel electrophoresis (nd-PAGE), and nondenaturant mass spectrometry (nd-MS), were used.

## EXPERIMENTAL PROCEDURES

*Expression and Purification*. Expression and purification of TD were carried out as described previously (*18*).

*Protein Determination.* Protein concentrations were measured either by the method of Bradford (*19*) (for crude extract only) with bovine γ-globulin as a standard or by measuring the UV absorbance at 205 nm as described by Scopes (*20*).

*In Vitro Assays of TD.* Threonine deaminase activity was assayed in 100 mM $KH_2PO_4/K_2HPO_4$ (pH 8.0) in a final volume of 1 mL. Reactions were initiated by adding threonine (0−40 mM) with or without isoleucine (0−20 mM) and valine (0−20 mM). All the stock solutions of amino acids were prepared in 100 mM $KH_2PO_4/K_2HPO_4$ (pH 8.0). The progress of the reaction was monitored by the increase in absorbance of α-ketobutyrate at 230 nm. Enzyme activity

‡ Dedicated to the memory of Peter M. Wessel.

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[1] Abbreviations: nd, nondenaturant; GF, gel filtration; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PLP, pyridoxal phosphate; TD, threonine deaminase; TIC, total ion current.

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was expressed as the number of micromoles of α-ketobutyrate produced per minute per milligram of protein using an $\epsilon$ value of 536 $mol^{-1}$ $cm^{-1}$.

*Gel Filtration Chromatography*. Injections of TD on a HiLoad (1.6/60 cm) Superdex S 200 (Pharmacia biotech) column were carried out with 1 mL of buffer containing different amounts of enzyme (25−2500 $\mu$g), with or without different effector concentrations in the presence of 10 mg of BSA as an internal reference. Elutions were performed at 1 mL/min at room temperature, with a buffer containing 100 mM $K_2HPO_4/KH_2PO_4$ (pH 8.0) in the presence or absence of the effector. Elution of TD was monitored by determination of enzyme activity.

*Nondenaturant Polyacrylamide Gel Electrophoresis*. Samples were prepared in 10% glycerol, 0.02% bromophenol blue, and 60 mM Tris-HCl (pH 6.8) and separated on a 3.5 to 27% (w/v) gradient polyacrylamide slab gel (*21*). Electrophoresis was carried out at 4 °C and 16 mA for 16 h with a Tris-glycine buffer with or without the effector (isoleucine or valine at 1 mM).

*Nondenaturant Mass Spectrometry*. For MS experiments, samples [15 mg/mL TD in 20 mM Hepes-KOH (pH 7.5)] were thawed out extemporaneously prior to buffer desalting. For desalting, a 12 mL volume of 20 mM $NH_4OAc$ (pH 7.5) was used. Buffer changes were obtained using a 30 kDa cutoff ultrafiltration membrane (YM 30, Amicon, Millipore) at 4000*g* and 15 °C. Under nondenaturant conditions, the protein was prepared at a concentration of 30 $\mu$M in 20 mM $NH_4OAc$ (pH 7.5). Protein−ligand complexes were formed by adding isoleucine or valine to the desired final concentration (10−200 $\mu$M) and directly analyzed by MS without further treatment. Mass spectra were obtained by using a Zabspec T tandem magnetic sector mass spectrometer (Micromass, Manchester, U.K.), fitted with an electrospray ionization source (*22*). Samples were continuously introduced into the ion source at a flow rate of 5 $\mu$L/min using a model 22 Harvard syringe pump (Harvard apparatus, Les Ulis, France). Spraying was carried out by applying a needle voltage of 8 kV and a skimmer voltage ($V_s$) of 4 kV. The voltage difference between the sampling cone and skimmer ($V_{sc} - V_s$) was maintained at a value of 160 V. The capillary and the nebulizer gas ($N_2$) were heated at 80 °C. The mass spectra acquisition scale was fixed between *m*/*z* 500 and 9000 with a scan rate of 10 s/decade. The instrument was calibrated using a solution of CsI.

*Denaturant Mass Spectrometry*. For mass spectrometry performed under denaturant solvent conditions, the protein was diluted in a $H_2O$/MeOH/AcOH mixture (50/50/1, v/v) to a final protein concentration of 10 $\mu$M. Mass spectra were acquired following the experimental procedure described for nondenaturant experiments.

## RESULTS

*Integrity of TD*. We first tested the homogeneity of the recombinant TD by recording its mass spectrum under denaturant solvent conditions. TD was represented on the mass spectra by multiply charged ion peaks corresponding to $[M + 23H]^{23+}$ to $[M + 52H]^{52+}$ (Figure 1). The measured mean molecular mass was 59 567 ± 6 Da, while the calculated molecular mass was 59 695 Da (theoretical). This mass difference ($\Delta m$ = 128 ± 6 Da) indicated that the

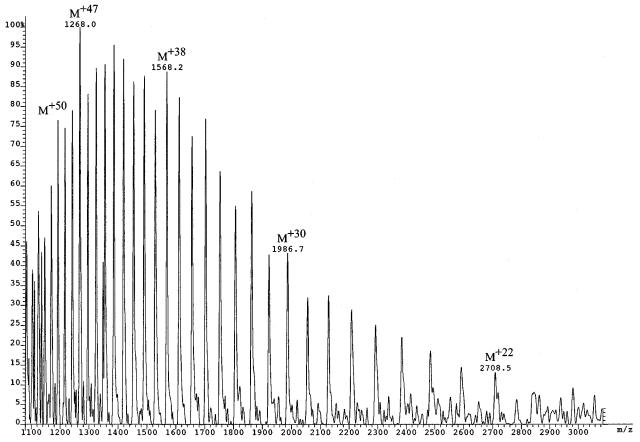


FIGURE 1: Mass spectrum of TD under denaturant conditions ($H_2O$/MeOH/AcOH). The calculated molecular mass was 56 567 ± 6 Da.

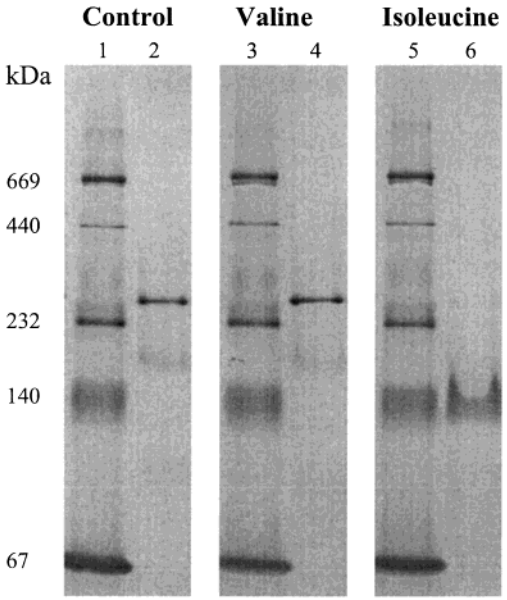


FIGURE 2: Polyacrylamide gel electrophoresis under nondenaturant conditions: lanes 1, 3 and 5, markers; lane 2, TD (20 $\mu$g) without effector; lane 4, TD (20 $\mu$g) with valine (1 mM); and lane 6, TD (20 $\mu$g) with isoleucine (1 mM). Electrophoreses were performed as described in Experimental Procedures.

N-terminal methionine of the recombinant enzyme had been cleaved in the overproduced enzyme.

*Effects of Isoleucine and Valine on the Oligomerization State of TD*. The apparent molecular mass of *A. thaliana* TD under various conditions was first determined by nondenaturant gel electrophoresis (Figure 2). TD behaved like a tetramer in the absence of the effector with an apparent molecular mass of 268 kDa (calculated molecular mass of the tetramer was 238 kDa). Addition of valine in the migration buffer and in the polyacrylamide gel had no effect on the quaternary structure of the enzyme. In contrast, addition of isoleucine induced dimerization of the protein molecule. Indeed, in the presence of isoleucine, TD migrated as a dimer with an apparent molecular mass of 140 kDa (calculated molecular mass of the dimer was 119 kDa). To determine if the TD behavior observed on nd-PAGE was linked to the effect of isoleucine on the quaternary structure or to artifact effects, the apparent molecular mass of the enzyme was also determined by gel permeation chromatog-

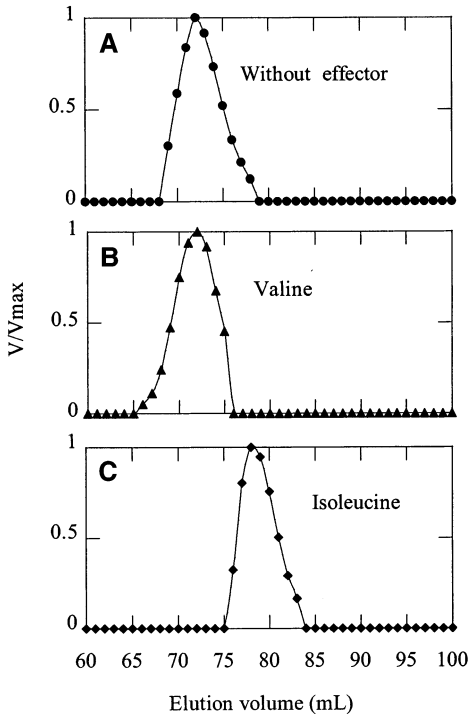


FIGURE 3: Gel filtration on Superdex S 200 in the absence of effector (A) or with 1 mM valine (B) or isoleucine (C). The enzyme (350 $\mu$g in 1 mL) was loaded on the column equilibrated in 100 mM $K_2HPO_4/KH_2PO_4$ buffer (pH 8.0) with or without effector. Elution was performed at 1 mL/min as described in Experimental Procedures.

raphy on a Superdex S 200 column. As shown in Figure 3, when elution was carried out without effector, the enzyme behaved as a tetramer with an apparent molecular mass of 250 kDa. Addition of valine did not modify the elution volume of the enzyme, whereas isoleucine led to a shift of the TD elution volume corresponding to the dimer form (Figure 3). In this case, the apparent molecular mass of the enzyme (150 kDa) determined with the addition of isoleucine (1 mM) did not match the theoretical mass of the dimer. To clarify this point, the effect of increasing concentrations of isoleucine was followed by using gel permeation chromatography.

*Effect of Isoleucine Concentration on TD Quaternary Structure Determined by nd-GF.* To better characterize the effect of isoleucine on the dimerization process, gel filtration experiments were carried out with injection of the protein at a constant concentration (350 $\mu$g of TD corresponding to 5.9 $\mu$M in 1 mL) and with a stepwise increase in the isoleucine concentration (up to 5 mM). In the presence of isoleucine, TD was always eluted as a single peak (Figure 4). The maximum effect on dimerization was reached when the isoleucine concentration reached 30 $\mu$M. Higher concentrations of isoleucine did not lead to a complete dimerization of the enzyme, which is evident from the apparent molecular mass of the enzyme that did not go below 150 kDa under the experimental conditions (Figure 4). From the data shown in Figure 4A, the effect of isoleucine on the dimerization of TD can be fitted by a cooperative equation allowing determination of an apparent $K_d$ value of 9.6 $\mu$M for isoleucine (Figure 4B).

*Effect of Valine Concentration on TD Quaternary Structure Determined by nd-GF.* Similarly, the effect of different

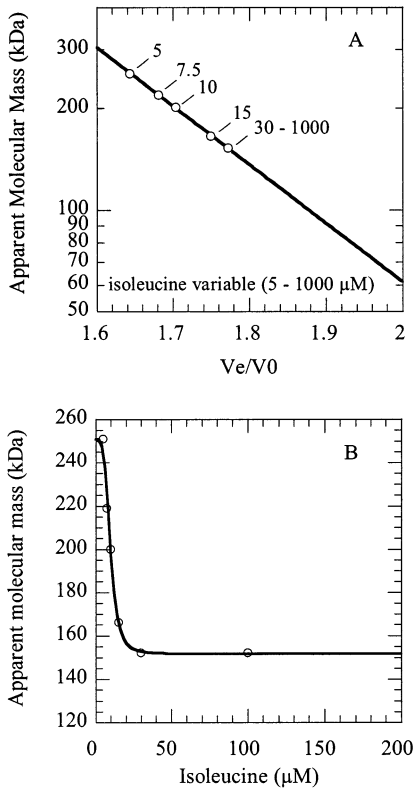


FIGURE 4: Effect of increasing isoleucine concentration on the dimerization of TD. (A) Effect of increasing isoleucine concentration on the elution of TD on Superdex S 200. (B) Plot of the apparent molecular mass as a function of isoleucine concentration. The data were fitted with a Hill equation ($K_d = 9.65 \pm 3.47$ $\mu$M; $n_H = 4.02 \pm 0.56$). Loading and elution were performed as described in Experimental Procedures.

valine concentrations was determined, and as noted above, valine by itself in the absence of isoleucine had little effect on the quaternary structure of TD. However, as previously demonstrated (*18*), valine reversed isoleucine inhibition. To determine if this reversion corresponded to a modification of the oligomerization state, gel filtrations were carried out with a constant concentration of protein (5.9 $\mu$M TD) and isoleucine (30 $\mu$M) and with increasing concentrations of valine (up to 2000 mM). As previously observed, TD was always eluted as a single peak. As shown in Figure 5, addition of valine led to a total recovery of the tetrameric form. From the data presented in Figure 5A, the effect of valine on the tetramerization of the enzyme can be fitted by a hyperbolic curve leading to the determination of an apparent $K_d$ value of 297 $\mu$M for valine (Figure 5B).

*Determination of the Quaternary Structure of TD by MS under Nondenaturant Conditions.* The effect of both allosteric effectors upon the TD quaternary structure was examined. The representative charge states of each species were ions $[D + 23H]^{23+}$ to $[D + 25H]^{25+}$ for the dimer and ions $[T + 32H]^{32+}$ to $[T + 39H]^{39+}$ for the tetramer. Mass spectra of TD were recorded in the absence of ligand and also in the presence of an excess of isoleucine or valine, namely, 100 $\mu$M each (Figure 6). Results showed that, in the absence of ligand, TD was represented mainly by the tetramer form with a small amount of dimer, with ion intensities corresponding to 90.3 and 9.7%, respectively, of the total ion current (TIC) (Figure 6A). As shown in Figure 6B, addition of an excess of isoleucine shifted the TD equilibrium from the tetramer

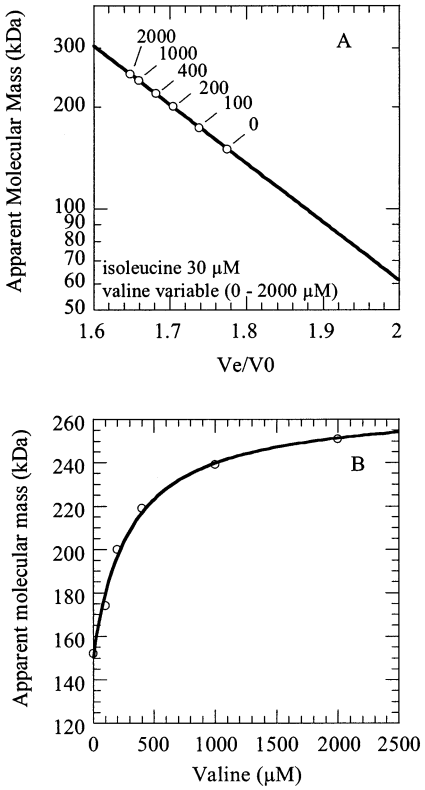


FIGURE 5: Reversion of the isoleucine effect by valine. (A) Effect of increasing concentrations of valine on the elution of TD on Superdex S 200 at a constant concentration of isoleucine (100 $\mu$M). (B) Plot of the apparent molecular mass as a function of valine concentration. The data were fitted with a hyperbolic equation ($K_d$ = 297.81 ± 53.74 $\mu$M). Loading and elution were performed as described in Experimental Procedures.

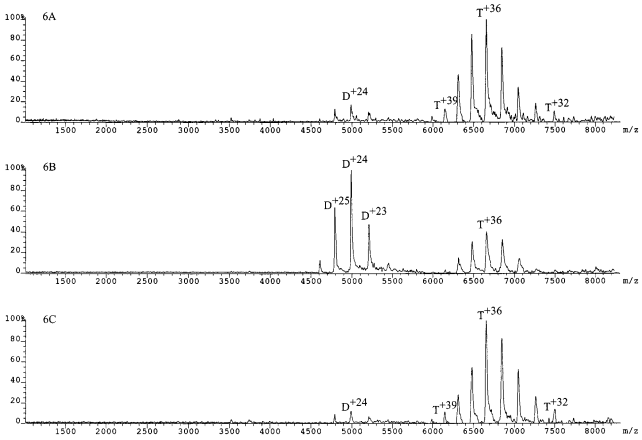


FIGURE 6: Mass spectra of TD under nondenaturant conditions in the absence of effector (A), in the presence of 100 $\mu$M isoleucine (B), and in the presence of 100 $\mu$M valine (C).

toward the dimer form. The latter represented 69% of the TIC at 100 $\mu$M isoleucine. Similarly, the valine effect was investigated on TD quaternary structure. Using a final valine concentration of 100 $\mu$M, no significant change in the tetramer ion intensity was noted. Under these conditions, the tetramer corresponded to 93% of the TIC instead of 90.3% which was observed in the absence of this ligand (Figure 6C). To further investigate the effect of isoleucine on this equilibrium, experiments were carried out at a constant concentration of TD (136 $\mu$g in 80 $\mu$L; 30 $\mu$M) and with increasing concentrations of isoleucine (up to 200 $\mu$M).

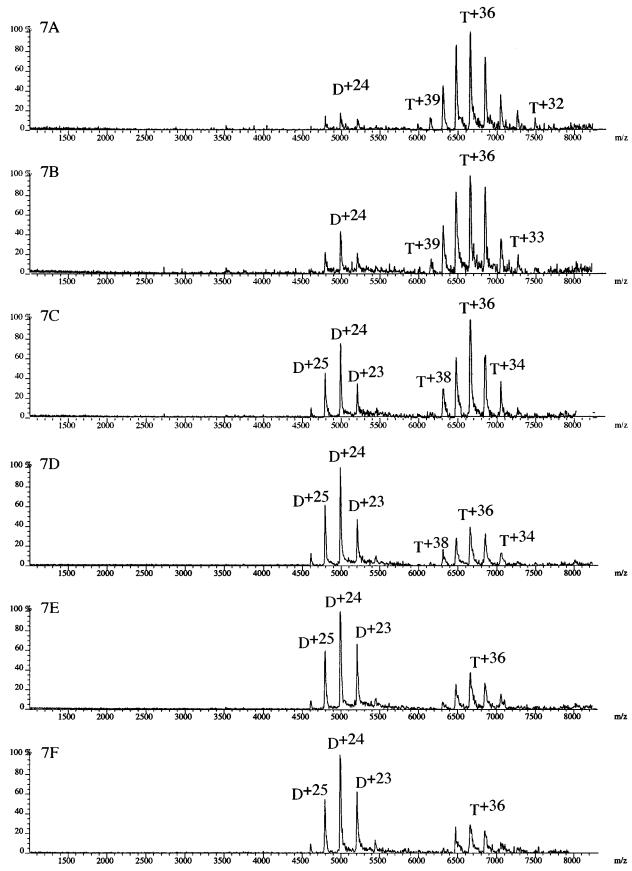


FIGURE 7: Mass spectra of TD under nondenaturant conditions in the presence of increasing isoleucine concentrations [0 (A), 10 (B), 30 (C), 60 (D), 100 (E), and 200 $\mu$M (F)].

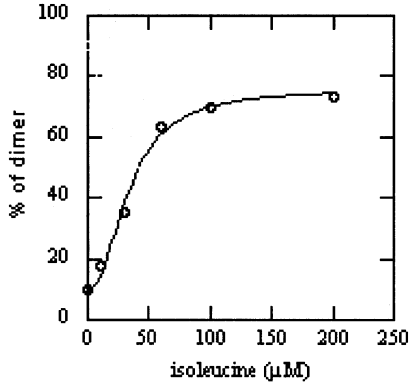


FIGURE 8: Determination of the effect of increasing concentrations of isoleucine on the dimerization of TD by mass spectrometry under nondenaturant conditions. Plot of the molecular mass as a function of isoleucine concentration. The data were fitted with a Hill equation ($K_d$ = 34.04 ± 3.47 $\mu$M; $n_H$ = 2.17 ± 3.47).

*Effect of Isoleucine Concentration on TD Quaternary Structure Determined by MS under Nondenaturant Conditions*. Study of the TD oligomeric equilibrium by MS, with a stepwise increase in the isoleucine concentration (10, 30, 60, 100, and 200 $\mu$M) at a fixed protein concentration (30 $\mu$M), showed a progressive dissociation of the tetramer into the dimer form. From the data shown in Figure 7, the effect of isoleucine on the dimerization of TD can be fitted by a cooperative equation allowing determination of an apparent $K_d$ value for isoleucine of 34 $\mu$M (Figure 8). We noticed that the proportion of dimer did not reach more than 75% of the TIC, even with a high isoleucine concentration.

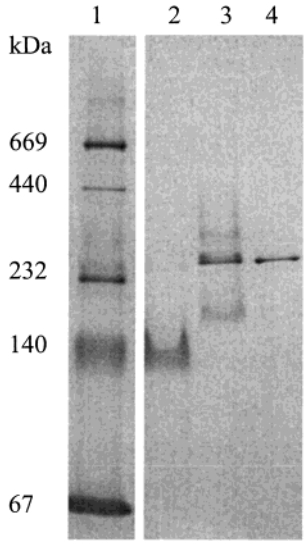


FIGURE 9: Native polyacrylamide gel electrophoreses of the mutants and the wild-type enzyme in the presence of isoleucine (1 mM): lane 1, markers; lane 2, wild-type TD (20 $\mu$g); lane 3, TD Y449L (20 $\mu$g); and lane 4, TD Y543L (20 $\mu$g). Electrophoreses were performed as described in Experimental Procedures.

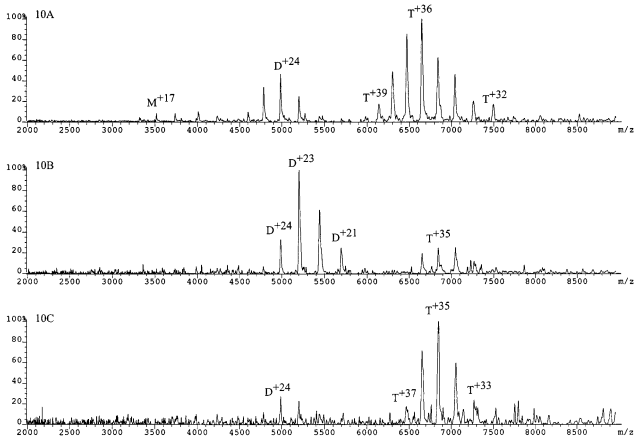


FIGURE 10: Mass spectra of the TD Y543L mutant under nondenaturant conditions (A) or in the presence of 100 $\mu$M isoleucine (B) or valine (C).

*Characterization of the Y449L and Y543L TD Mutants by nd-PAGE and nd-MS.* As previously demonstrated, Y449 and Y543 belong to high-affinity and low-affinity binding sites for isoleucine, respectively (*18*). With a view of determining if dimerization was induced by the binding of isoleucine on Y449, Y543, or both binding sites, characterization of the Y449L and Y543L TD mutants was undertaken by nd-PAGE and nd-MS. Analysis by PAGE under native conditions clearly showed that migration of both mutants was insensitive to the addition of isoleucine (Figure 9).

Unfortunately, the Y449L mutant precipitated under the experimental conditions imposed by nd-MS. Nonetheless, analysis of the Y543L mutant by nd-MS in the absence of an effector showed the presence of tetramer (73%), dimer (21%), and a small proportion of monomer (6%) (Figure 10A). Experiments carried out in the presence of isoleucine (100 $\mu$M) led to further dissociation of the Y543L mutant, yielding a majority of dimer (73%) and a low proportion of tetramer (27%) (Figure 10B). Addition of valine (100 $\mu$M) to the Y543L mutant led to a higher proportion of tetramer (82%) compared with results obtained for the mutant without the effector (Figure 10C). In the presence of ligand (isoleucine or valine), no monomer was found in the mass spectra.

*Assessment of the Cofactor Binding Stoichiometry on TD.* Whereas the PLP cofactor involving an imine bond (Schiff base) was not detected in the mass spectrum recorded under denaturing conditions (acidic conditions), mass spectra obtained under nondenaturant solvent conditions permitted an assessment of the stoichiometry of this cofactor. As the errors on the measured masses were low (from 0.03 to 0.1%) and reproducible, it could be confirmed that the PLP (PM PLP, 265.1 Da) was incorporated at a 1/1 ratio per monomer. Indeed, the experimentally measured mass of the dimer (119 832 $\pm$ 12 Da) was in agreement with the calculated molecular mass (119 834 Da) when PLP was incorporated at a 1/1 ratio per monomer. However, none of these experiments showed a mass increase that revealed the presence of supplementary isoleucine or valine on TD. A rationale for this lack of additional isoleucine or valine on TD will be described in detail in a specific paragraph dealing with the advantages and drawbacks of each technique.

## DISCUSSION

As shown by PAGE, GF, and MS under nondenaturant conditions, TD is mainly represented by a tetramer in the absence of any allosteric effector. Experiments carried out on nd-PAGE clearly showed that isoleucine can bring about the disappearance of a band corresponding to the tetramer in favor of a band corresponding to a dimer. Thus, this result demonstrates, for the first time, that isoleucine induces dimerization of the plant TD. It must be pointed out that our observations are not in agreement with studies conducted on the bacterial TD, which was reported to exist as a tetramer in the presence or absence of isoleucine (*6*).

Interestingly, nd-GF experiments performed with isoleucine never led to the elution of TD under two different peaks corresponding to the dimer and the tetramer. In fact, isoleucine only delays the elution of TD under a single peak. This behavior strongly suggests that the dimer form is in equilibrium with the tetramer, and this dimer−tetramer equilibrium would be a fast process. However, PAGE and GF under nondenaturant conditions were not suitable for characterizing such a rapid equilibrium. In contrast, mass spectrometry seemed to be ideally suited for studying this phenomenon as the mass spectra allowed the simultaneous detection of both the dimer and the tetramer, thereby confirming the occurrence of an equilibrium between these two species.

Our attention was next focused on the effect of isoleucine concentration upon the TD oligomeric equilibrium. With increasing concentrations of isoleucine, the tetramer progressively dissociates into the dimer form. However, as shown with experiments carried out on nd-GF, even a high isoleucine concentration failed to produce total conversion of the enzyme to the dimeric form. In fact, the lowest apparent molecular mass determined by GF (150 kDa) was reached at 30 $\mu$M isoleucine and did not decrease with higher effector concentrations (up to 5 mM). Accordingly, the highest proportion of the dimer obtained with the highest concentration of isoleucine (200 $\mu$M), used for nd-MS, did not reach more than 75%. Since TD is completely inhibited

at these isoleucine concentrations (*18*), this result indicates that the inhibition is not a consequence of the dimerization of TD. This demonstrates therefore that binding of isoleucine on TD induces conformational changes, leading simultaneously to enzyme inhibition and dimerization.

From a comparison of the binding parameters of isoleucine previously determined with the wild-type enzyme ($K_d = 16$ $\mu$M) (*18*) and on the Y543L TD mutant, having only one available binding site of isoleucine on Y449 ($K_d = 7$ $\mu$M) (*18*), with parameters presently determined for the dimerization of the wild-type enzyme [$K_d$(nd-GF) $= 9.6$ $\mu$M; $K_d$(nd-MS) $= 34$ $\mu$M (this work)], it is difficult to rule out the role of each of the two isoleucine binding sites in the dimerization process. With a view of elucidating the dimerization mechanism, we chose to study the Y449L and Y543L TD mutants. As shown by native PAGE, their migration is similar in the presence and absence of isoleucine, suggesting that the binding of isoleucine on both sites is required for dimerization. Analysis of these mutants by nd-MS was found to be more complex. Thus, for the Y543L TD mutant, nd-MS showed the presence of tetramer (73%), dimer (21%), and monomer (6%). As nd-MS experiments carried out on the wild-type enzyme exhibited a majority of tetramer (90.7%) with only a low proportion of dimer (9.3%) and no monomer, our results suggest that the Y543L TD mutant underwent to some extent a dissociation in solution. We attributed this dissociation to the mutation itself, which seems to induce a structural destabilization of the Y543L TD mutant under our MS experimental conditions. This finds further support from the disappearance of the monomer and from the specific reassociation into the dimer or the tetramer form when ligands were added. Strong structural destabilization of the Y449L TD mutant also seems to occur since the mutant enzyme precipitated, rendering MS experiments impractical, in this case.

Nonetheless, we continued our study on the Y543L TD mutant by testing the ligand effect on quaternary structure changes by MS. In contrast to the result obtained by nd-PAGE, mass spectra recorded under nondenaturing conditions demonstrated that the binding of isoleucine on Y543L TD induced dimerization. In this case, the increase in the proportion of the dimer form for the mutant Y543L, in the presence of isoleucine (100 $\mu$M), demonstrates that the high-affinity binding site (Y449) is involved in the dimerization process. Thus, the appearance of the dimer form in the mass spectra was related to an additional loss of stability (conformational changes) for the mutant enzyme after the binding of isoleucine on the high-affinity binding site. This suggests that Y449 isoleucine binding site plays a role in the dimerization process. The importance of Y449 in the dimerization process was also assessed with valine. Indeed, when valine (100 $\mu$M) was added to the Y543L TD mutant solution (in the absence of isoleucine), mass spectra displayed a shift of the dimer form toward the tetramer form. This result confirms that the high-affinity binding site (Y449) is also involved in the fixation of valine and that valine promotes dimer reassociation to the tetramer. However, the proportion of the tetramer was lower than for the WT, which is probably due to a structural destabilization. Thus, it seems that the TD allosteric regulation involved a synergic role of the two effector-binding sites in producing both TD dimerization and inhibition.

*Comparison of PAGE, GF, and MS under Nondenaturant Conditions*. In this study, we have employed three different techniques to determine the effect of isoleucine and valine on the oligomeric state of the plant TD. Each of the techniques that was used led to the conclusion that dimerization of plant TD is induced by isoleucine, whereas valine restores the tetrameric form. These mutually complementary techniques demonstrate that TD is in fast equilibrium between a dimeric and a tetrameric form which is dependent on the presence of isoleucine or valine. However, it is clear that each technique had its advantages and limits. Whereas advantages and limits of PAGE and gel filtration under nondenaturant conditions are well-documented in the literature, there is only scant reporting of the use of MS for studying the oligomeric status of proteins under nondenaturant conditions. Mass spectrometry is a valuable tool for investigating macromolecular noncovalent complexes. This technique requires the use of the soft electrospray ionization (ESI) mode and of specific experimental conditions such as aqueous solutions with neutral pH and low ionic strengths as well as high-mass range analyzers. Thus, MS enables the gas phase transfer of these fragile complexes while maintaining weak energy bonding involved in protein subunit contacts. To date, numerous publications reported the characterization of protein−protein, protein−DNA, and protein−ligand complexes (*23*−*26*). However, as a number of reports suggested that results observed in mass spectra could not necessarily reflect the solution phase equilibrium (*23*), it is necessary to assess the specificity of the complex detected on the mass spectra with respect to the solution phase. Several experimental parameters could influence the desorption−ionization process and modify physicochemical solution properties of various species during their gas phase transfer. They concern a difference in bonding energy between the gas phase and solution phase (e.g., hydrophobic or ionic interactions) (*23*), solvent (*27*), temperature (*28*), pH (*29*), solution strength (*30*), or instrumental parameters (*31*).

In the particular case of TD, all these parameters could play a role by promoting dissociation or aggregation during MS experiments. However, the observation of the predominant presence of tetramer in the mass spectra in the absence of any effector indicated that no dissociation occurred in the solution or in the gas phase. Moreover, since both ligands could modify the TD quaternary structure in solution as well as the fact that neither dissociation nor aggregation occurred in the gas phase, we assert that mass spectra reflect the solution phase oligomeric equilibrium of TD. Nonetheless, mass spectrometry was not suited for probing or to probe the reversion of the effect of isoleucine by the addition of valine, as reversion of isoleucine only occurred for a high valine concentration (2000 $\mu$M), a condition incompatible with MS studies (*24*).

An intriguing point of our study concerned the lack of the presence of any supplementary isoleucine molecule on TD species. As previously stated, the change in the oligomeric equilibrium observed in mass spectra in the presence of isoleucine constituted proof of its binding in solution. Also, isoleucine reversion only occurred for a high valine concentration, which means that in solution isoleucine is strongly bound to TD. Consequently, no TD−isoleucine complex dissociation occurred in solution. The failure to detect

additional isoleucine on TD could alternatively be explained either by a gas phase dissociation of the complex due to the hydrophobic character of isoleucine or by conformational changes of the complex during transfer in the gas phase, with the concomitant loss of the ligand. This latter hypothesis does not seem to be relevant as Mirza et al. showed that net heat transfer is insufficient for denaturation during the ESI process provided that the protein solution equilibrium is to some extent preserved in the gas phase (*32*). Whereas these data fail to provide information about the conformational state of the proteins in the gas phase, some reports in the literature gave evidence for protein gas phase structure that was close to solution structure (*33*, *34*). Consequently, we assign the absence of additional isoleucine on TD to a gas phase dissociation of the TD−isoleucine complex linked to the hydrophobic feature of the isoleucine moiety. This result is in agreement with the current knowledge about differences in bonding energy between the gas phase and solution phase (*23*).

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## REFERENCES

1. Gallagher, D. T., Gilliland, G. L., Xiao, G., Zondlo, J., Fisher, K. E., Chinchilla, D., and Eisenstein, E. (1998) *Structure 6*, 465−475.
2. Umbarger, H. E. (1956) *Science 123*, 848.
3. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88−118.
4. Einsenstein, E. (1991) *J. Biol. Chem. 266*, 5801−5807.
5. Fisher, K. E., and Eisenstein, E. (1993) *J. Bacteriol. 175*, 6605−6613.
6. Einsenstein, E. (1994) *J. Biol. Chem. 269*, 29416−29422.
7. Einsenstein, E., Yu, H. D., and Schwarz, F. P. (1994) *J. Biol. Chem. 269*, 29423−29429.
8. Einsenstein, E. (1995) *Arch. Biochem. Biophys. 316*, 311−318.
9. Einsenstein, E., Yu, H. D., Fisher, K. E., Iacuzio, D. A., Ducote, K. R., and Schwarz, F. P. (1995) *Biochemistry 34*, 9403−9412.
10. Chinchilla, D., Schwarz, F. P., and Eisenstein, E. (1998) *J. Biol. Chem. 273*, 23219−23224.
11. Hatfield, G. W., and Umbarger, H. E. (1970) *J. Biol. Chem. 245*, 1742−1747.
12. Decedue, C. J., Hofler, J. G., and Burns, R. O. (1975) *J. Biol. Chem. 250*, 1563−1570.
13. Hofler, J. G., and Burns, R. O. (1972) *J. Biol. Chem. 253*, 1245−1251.
14. Betz, J. L., Hereford, L. M., and Magee, P. T. (1971) *Biochemistry 10*, 1818−1824.
15. Dougall, D. K. (1970) *Phytochemistry 9*, 959−964.
16. Sharma, R. K., and Mazumder, R. (1970) *J. Biol. Chem. 245*, 3008−3014.
17. Mourad, G., and King, J. (1995) *Plant Physiol. 107*, 43−52.
18. Wessel, P. M., Graciet, E., Douce, R., and Dumas, R. (2000) *Biochemistry 39*, 15136−15143.
19. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
20. Scopes, R. K. (1974) *Anal. Biochem. 59*, 277−282.
21. Chua, N. H. (1980) *Methods Enzymol. 69*, 434−436.
22. Scrivens, J. H., Rollins, K., Jennings, R. K. C., Bordoli, R. S., and Bateman, R. (1992) *Rapid Commun. Mass Spectrom. 6*, 272−277.
23. Loo, J. A. (2000) *Int. J. Mass Spectrom. 200*, 175−186.
24. Loo, J. A. (1997) *Mass Spectrom. Rev. 16*, 1−23.
25. Ayed, A., Krutchinsky, A. N., Ens, W., Standing, K. G., and Duckworth, H. W. (1998) *Rapid Commun. Mass Spectrom. 12*, 339−359.
26. Laprévote, O., Serani, L., Das, B. C., Halgand, F., Forest, E., and Dumas, R. (1999) *Eur. J. Biochem. 256*, 356−359.
27. Loo, J. A., Ogorzalek Loo, R. R., Udseth, H. R., Edmonds, C. G., and Smith, R. D. (1991) *Rapid Commun. Mass Spectrom. 5*, 101−105.
28. Mirza, U. A., Cohen, S. L., and Chait, B. T. (1993) *Anal. Chem. 65*, 1−6.
29. Kashiwagi, T., Yamada, N., Hirayama, K., Suzuki, C., Kashiwagi, Y., Tsuchiya, F., Arata, Y., Kunishima, N., and Morikawa, K. (2000) *J. Am. Soc. Mass Spectrom. 11*, 54−61.
30. Wang, G., and Cole, R. B. (1994) *Anal. Chem. 66*, 3702−3708.
31. Mirza, U. A., and Chait, B. T. (1994) *Anal. Chem. 66*, 2898−2904.
32. Mirza, U. A., and Chait, B. T. (1997) *Int. J. Mass Spectrom. Ion Processes 162*, 173−181.
33. Suckau, D., Shi, Y., Beu, S. C., Senko, M. W., Quinn, J. P., Wampler, F. M., and MacLafferty, F. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 790−793.
34. He, F., Ramirez, J., and Lebrilla, C. B. (1999) *Int. J. Mass Spectrom. 193*, 103−114.

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