Unfolding analysis of the mature and unprocessed forms of *Bacillus licheniformis* γ -glutamyltranspeptidase

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Abstract *Bacillus licheniformis* γ -glutamyltranspeptidase (*B*/GGT) undergoes an autocatalytic process to generate 44.9 and 21.7 kDa subunits; however, a mutant protein (T399A) loses completely the processing ability and mainly exists as a precursor. For a comprehensive understanding of their structural features, the biophysical properties of these two proteins were investigated by circular dichroism and fluorescence spectroscopy. Tryptophan fluorescence and circular dichroism spectra were nearly identical for *B*/GGT and T399A, but unfolding analyses revealed that these two proteins had a different sensitivity towards temperature- and guanidine hydrochloride (GdnHCl)-induced denaturation. *B*/GGT and the unprocessed T399A displayed T_m values of 61.4°C and 68.1°C, respectively, and thermal unfolding of both proteins was found to be highly irreversible. Fluorescence quenching analysis showed that T399A had a dynamic quenching constant similar to that of the wild-type enzyme. *B*/GGT started to unfold beyond ~2.14 M GdnHCl and reached an unfolded intermediate, $[\text{GdnHCl}]_{0.5, \text{N-U}}$, at 2.85 M, corresponding to free energy change ($\Delta G_{\text{H}_2\text{O}}$) of 12.34 kcal mol⁻¹, whereas the midpoint of the denaturation curve for T399A was approximately 3.94 M, corresponding to a $\Delta G_{\text{H}_2\text{O}}$ of 4.45 kcal mol⁻¹. Taken together, it can be concluded that the structural stability of *B*/GGT is superior to that of T399A.

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1 Introduction

γ -Glutamyltranspeptidase (GGT; EC, 2.3.2.2) is a member of the N-terminal nucleophile (Ntn) hydrolase superfamily [1] that catalyzes the transfer of the γ -glutamyl moiety of glutathione to an acceptor that may be an amino acid, a peptide, or water. GGT plays a vital role in the γ -glutamyl cycle, a pathway for the synthesis and degradation of glutathione, drug, and xenobiotic detoxification [2–4]. In post-translational modification, the inactive precursor of prokaryotic and eukaryotic GGTs undergoes intramolecular autoprocessing to generate the mature and catalytically active enzyme. A conserved threonine residue in the enzyme, Thr391 in *Escherichia coli* GGT (*EcGGT*) [5] and Thr380 in *Helicobacter pylori* GGT (*HpGGT*) [6], serves as the N-terminal nucleophile and is required for both maturation event and catalytic reaction.

Earlier, the gene encoding *Bacillus licheniformis* GGT (*BIGGT*) was cloned and over-expressed in recombinant *E. coli* M15 cells [7]. *BIGGT* is synthesized as a 61.3-kDa precursor consisting of a signal peptide of 25 amino acid residues, a large subunit of 374 residues, and a small subunit of 187 residues (Swiss-Prot Q65KZ6). Deletion analysis of *BIGGT* shows that removal of either the signal peptide or part of the C-terminal sequence significantly affects the functional expression of this enzyme in *E. coli* [8, 9]. Site-directed mutagenesis of the conserved Thr399 of *BIGGT* has created a mutant protein (T399A), which loses completely the capability of autocatalytic processing [10]. To date, the conformation-based information for the GGT precursors is still limited. In the present investigation, we compared biophysically *BIGGT* and T399A using circular dichroism (CD) and fluorescence spectroscopy techniques. The experimental data showed that these two proteins had a different behavior towards temperature- and GdnHCl-induced denaturation.

2 Material and methods

2.1 Chemicals

Luria-Bertani (LB) media for the cultivation of recombinant *E. coli* cells were purchased from Difco Laboratories (Detroit, MI, USA). Ni^{2+} -nitrilotriacetate (Ni^{2+} -NTA) resin was acquired from Qiagen Inc. (Valencia, CA, USA). Imidazole, 2-methyl-4-([2-methylphenyl]azo)-benzenediazonium salt (Fast Garnet GBC), L- γ -glutamyl-*p*-nitroanilide (L- γ -Glu-*p*-NA), Gly-Gly, *p*-nitroaniline (*p*-NA), acrylamide, and guanidine hydrochloride were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals were commercial products of analytical or molecular biological grade.

2.2 Expression and purification of the recombinant proteins

E. coli M15 cells carrying either pQE-*BIGGT* [8] or pQE-*BIGGT*/T399A [10] were grown aerobically in 100-mL LB medium supplemented with ampicillin ($100 \mu\text{g mL}^{-1}$) and kanamycin ($25 \mu\text{g mL}^{-1}$) until the absorbance at 600 nm reached 0.8. Isopropyl- β -D-thiogalactopyranoside was then added to a final concentration of 0.1 mM, and the culture

was incubated at 20°C in a shaking incubator for another 16 h. Cells were subsequently harvested by centrifugation, resuspended in 3 mL of binding buffer (5 mM imidazole, 0.5 M NaCl, and 50 mM NaH₂PO₄; pH 7.9), sonicated on ice (30-s bursts and pulses for 5 min), and applied onto a Ni²⁺-NTA column. Sequential washing and eluting procedures were performed according to the protocol of Qiagen to obtain the purified proteins.

2.3 Protein methods

Polyacrylamide gel electrophoresis (PAGE) was performed in a vertical mini-gel system (Mini-Protein III; Bio-Rad Laboratories, Inc., Hercules, CA, USA). GGT activity in the gel was detected according to the method described previously [11]. Briefly, a 12% non-denaturing polyacrylamide gel was run at 4°C and at a constant voltage of 100 V for 2 h. To detect the transpeptidase activity, the gel was immersed in 1 mM 5-L-glutamyl-2-naphthylamide and 50 mM Gly-Gly in 50 mM Tris-HCl buffer (pH 8.0), and incubated at 37°C for 30 min. The gel was then transferred into a solution containing 0.05% Fast Garnet GBC in 8% acetic acid until the dark blue GGT band appeared.

Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed using the Laemmli buffer system [12]. Prior to electrophoresis, the samples were heated at 100°C for 5 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol. Protein markers used were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Protein concentration was measured using a protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as the standard.

2.4 Enzyme assay

GGT activity was routinely assayed at 40°C using L-γ-Glu-*p*-NA as the substrate [13]. The reaction mixture contained 1.25 mM L-γ-Glu-*p*-NA, 30 mM Gly-Gly, 1 mM MgCl₂, 50 mM Tris-HCl buffer (pH 8.0), 20 μL enzyme solution at a suitable dilution, and enough distilled water to bring the final volume to 1 mL. After 10 min incubation, the reaction was quenched by the addition of 100 μL acetic acid (3.5 N). The formation of *p*-NA was recorded by monitoring the absorbance changes at 410 nm. One unit of GGT activity is defined as the amount of enzyme that releases 1 μmol of *p*-NA per minute under the assay conditions.

2.5 CD and spectrofluorimetric analyses

Far UV-CD spectra of BIGGT and T399A were acquired on a JASCO model J-815 spectropolarimeter (JASCO Inc., Japan) from 250 to 190 nm in cuvettes at 25°C using a 1.0-nm bandwidth, 0.1-nm resolution, 0.1-cm path length, 1.0-s response time, and a 100-nm/min scanning speed. The photomultiplier absorbance was always below 600 V in the analyzed region. Each scan was repeated ten times and an average was reported. Data were corrected for the buffer effect and the results were expressed as molar ellipticity [Θ] in the units of degrees · cm² · decimol⁻¹ according to Eq. 1.

$$[\Theta] = \frac{\Theta}{10 \cdot C \cdot l} \quad (1)$$

where *l* represents the light path length (cm), *C* is the molar concentration of protein (mol/L), and Θ represents the observed ellipticity (mdeg). Thermal denaturation

experiments were performed by monitoring the ellipticity at 222 nm. The temperature was increased with a heating rate of 2°C/min from 20 to 100°C and the temperature at which half of the protein molecules were unfolded was recorded (the transition midpoint, T_m).

Steady-state fluorescence spectra and intensities were measured on a Hitachi F-7000 fluorescence spectrophotometer with an excitation wavelength of 295 nm. The concentration of BIGGT and T399A was adjusted to have an absorbance of ~ 0.05 at the excitation wavelength. Acrylamide and Ni^{2+} quenching studies were performed by the addition of a stock quencher solution to a $5 \times 5 \text{ mm}^2$ cell containing the protein sample. The dynamic quenching constant was computed from the Stern–Volmer equation [14]:

$$F_0/F = (1 + K_{SV} [Q]) \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher at a protein concentration $[Q]$, and K_{SV} is the dynamic quenching constant.

BIGGT and T399A were unfolded with different concentrations of GdnHCl in 50 mM Tris–HCl buffer (pH 8.0) at room temperature. A 30-min incubation was employed in unfolding experiments. The unfolding of the protein was monitored by fluorescence. Fluorescence spectra of BIGGT and T399A were monitored at 25°C with an excitation wavelength of 280 nm. All spectra were corrected for buffer absorption. The fluorescence emission spectra of protein samples with a concentration of 32 μM were recorded from 300 to 400 nm at a scanning speed of 240 nm/min. The maximal peak of the fluorescence spectrum and the change in fluorescence intensity were used in monitoring the unfolding processes of the enzyme. Both the red shift and the change in fluorescence intensity were analyzed together using the average emission wavelength (AEW) [15].

2.6 Analysis of unfolding data

The unfolding data of BIGGT and T399A were treated with the thermodynamic model by global fitting of the data [16]. The two-state unfolding model (Scheme 1) is described by Eq. 3,

$$\theta_{222} = \frac{\theta_N + \theta_U \cdot \exp \left[-\frac{\Delta H_U}{RT} \cdot \left(1 - \frac{T}{T_m} \right) + \frac{\Delta C_{PU}}{RT} \cdot \left(T \ln \left(\frac{T}{T_m} \right) + T_m - T \right) \right]}{1 + \exp \left[-\frac{\Delta H_U}{RT} \cdot \left(1 - \frac{T}{T_m} \right) + \frac{\Delta C_{PU}}{RT} \cdot \left(T \ln \left(\frac{T}{T_m} \right) + T_m - T \right) \right]} \quad (3)$$

where θ_{222} is the relative ellipticity at 222 nm, θ_N and θ_U are the calculated ellipticities of the native and unfolded states, respectively, ΔH_U is the free enthalpy of unfolding, ΔC_{PU} is the heat capacity of unfolding, T is temperature, and R represents the universal gas constant.

The two-state unfolding model for GdnHCl-induced denaturation of BIGGT and T399A is described by Eq. 4,

$$y_{\text{obs}} = \frac{(y_N + m_f [D]) + (y_U + m_u [D]) \cdot \exp \left[-(\Delta G_{\text{H}_2\text{O}} - m [D]) / RT \right]}{1 + \exp \left[-(\Delta G_{\text{H}_2\text{O}} - m [D]) / RT \right]} \quad (4)$$

where y_{obs} is the observed biophysical signal, y_N and y_U are the intercepts, m_f and m_u are the slopes of the pre- and post-transition baselines, T is temperature, R is the universal gas constant, $[D]$ is the concentration of GdnHCl, $\Delta G_{\text{H}_2\text{O}}$ represents an estimate of the

conformational stability of the protein in the absence of GdnHCl, and m is a measure of the dependence of ΔG .

3 Results

3.1 Protein purification and structural analysis

*B*IGGT must undergo autocatalytic processing in order to gain enzymatic activity, but the mechanism of activation has not been elucidated. Previous studies have shown that Thr399 of this enzyme plays an important role in both autocatalytic and hydrolysis reactions [10]. For both processes, a general base mechanism has been proposed in which an adjacent residue would facilitate the attack of the threonine hydroxyl on the peptide bond [4]. Substitution of Thr399 of *B*IGGT with cysteine generates a mutant enzyme that retains the ability to carry out the autocatalytic process, suggesting that the hydroxyl group of threonine could be replaced by the sulfhydryl group of cysteine [10]. With the fact that the nucleophilic attack is necessary for the maturation of the GGT enzyme, soluble T399A is expected to be present in the unprocessed form. In order to characterize biophysically

Fig. 1 Analysis of the purified enzymes by SDS-PAGE (a) and non-denaturing PAGE (b). The protein samples were electrophoresed on 12% polyacrylamide gel and visualized by Coomassie brilliant blue staining and activity staining. Lane *M* represents molecular size marker, and lanes 1 and 2 are *B*IGGT and T399A, respectively

both *B/*GGT and T399A enzymes, the His₆-tagged proteins in the cell-free extracts were purified to homogeneity by Ni²⁺-NTA resin (Fig. 1a). The molecular masses of the subunits for *B/*GGT were approximately 45 and 20.5 kDa, but the purified T399A was present as a precursor. The specific activity for *B/*GGT was 97.2 ± 7.9 U/mg and, as expected, no GGT activity was detected in T399A. The purified enzymes were also subjected to native PAGE and activity staining. As shown in Fig. 1b, one functional band with a migration corresponding to the *B/*GGT heterodimer was observed, whereas T399A was inactive and showed a slower migration in the gel. These observations indicate that the surface charge of T399A is more negative than that of *B/*GGT.

To evaluate possible structural changes that may result from the mutation, spectrofluorimetric and CD analyses were performed in *B/*GGT and T399A. Both *B/*GGT and T399A contain a total of four tryptophanyl residues (Trp157, Trp273, Trp376, and Trp408); therefore, fluorescence spectroscopy is very suitable for the conformational study of these two proteins. As shown in Fig. 2a, the tryptophan emission fluorescence spectrum of *B/*GGT was maximized at a wavelength of 338.9 nm. Interestingly, the fluorescence intensity and emission maxima of T399A were compared well with those of the wild-type

Fig. 2 Intrinsic tryptophan fluorescence (a) and CD (b) spectra of *B/*GGT and T399A. An average of five spectra is shown for each protein was recorded at 25°C

Table 1 Percent levels of secondary structure in *B*/GGT and T399A

Protein	Secondary structure (%)			
	α -Helix	β -Strand	β -Turn	Random coil
<i>B</i> /GGT	51	16	15	18
T399A	53	15	15	16

enzyme. To analyze how secondary structures of *B*/GGT were affected by the mutation, we measured far UV-CD properties of wild-type and mutant enzymes. The CD spectrum of *B*/GGT displayed strong peaks of negative ellipticity at 208 and 222 nm, indicative of substantial α -helical content (Fig. 2b). The absence of major differences in the CD spectra of *B*/GGT and T399A further indicated that the mutation had no detrimental effect on their respective secondary structures (Fig. 2b). The secondary structural contents were estimated by the online DICHROWER server (<http://public-1.cryst.bbk.ac.uk/cdweb/html/>, accessed on December 31, 2010) [17] using the implemented CDSSTR program. The normalized root-mean-square deviation value of the data fitting for *B*/GGT and T399A were 0.017 and 0.015, respectively, which were both <0.1 and thus showed a goodness-of-fit parameter [17]. As shown in Table 1, the estimated levels of secondary structure for T399A are close to those of *B*/GGT. Taken together, these data suggest that no conclusive change in the main structure of *B*/GGT has occurred as a consequence of the mutation.

3.2 Thermal unfolding

Temperature-induced denaturation of secondary structures of *B*/GGT and T399A was monitored by following the loss of ellipticity of the CD signal at 222 nm. As shown in Fig. 3, the thermal unfolding of *B*/GGT was initiated at 56.9°C and the representative peaks for a α -helix were diminished when the denaturation temperature reached 71°C. The T_m values of *B*/GGT and T399A were calculated from the thermal denaturation curves. By fitting the experimental data with Eq. 3, the T_m value for *B*/GGT was determined to be $61.4 \pm 0.4^\circ\text{C}$, corresponding to $\Delta G_{\text{H}_2\text{O}}$ of $3.04 \pm 0.15 \text{ kcal mol}^{-1}$ for the $N \leftrightarrow U$ process. T399A had a T_m value of $68.1 \pm 0.4^\circ\text{C}$, which corresponds to a $\Delta G_{\text{H}_2\text{O}}$ of $2.94 \pm 0.24 \text{ kcal mol}^{-1}$ for

Fig. 3 Unfolding and cooling curves of *B*/GGT and T399A in 20 mM Tris-HCl buffer (pH 8.0). Transitions were obtained by recording the ellipticity of the samples at 222 nm. Protein concentration was set to 1.5 mg mL^{-1}

Table 2 Thermodynamic parameters of the thermal unfolding of *B/IGGT* and T399A

Protein	T_m^a (°C)	ΔH_m^b (kcal mol ⁻¹)	ΔS_m^c (cal mol ⁻¹ K ⁻¹)	ΔG (25°C) ^d (kcal mol ⁻¹ K ⁻¹)
<i>B/IGGT</i>	61.4 ± 0.3	65.7 ± 6.9	196.5 ± 20.5	3.04 ± 0.15
T399A	68.1 ± 0.4	57.5 ± 5.0	168.5 ± 14.7	2.94 ± 0.24

^aMidpoint of the thermal unfolding curve^bEnthalpy change at T_m ^c $\Delta S_m = \Delta H_m/T_m$ ^dCalculated with Eq. 3

the denaturation process. These differences in thermal stability between *B/IGGT* and T399A were also reflected by significant changes in enthalpic and entropic contributions to the free enthalpy of unfolding (Table 2). As shown in Table 2, the ΔH value for *B/IGGT* was calculated to be 65.7 ± 6.9 kcal mol⁻¹ but this value was decreased slightly in T399A. Additionally, the ΔS values of *B/IGGT* and T399A were computed to be 196.5 ± 20.5 and 168.5 ± 14.7 cal mol⁻¹ K⁻¹, respectively.

To further explore if the unfolding reaction was reversible or not, the sample was heated at a constant rate of 2°C/min. After the thermal denaturation transitions went to completion, the protein solutions were cooled down to 20°C at a cooling speed of 2°C/min. The experimental results showed that the transition of T399A appeared at higher temperature than that of *B/IGGT*. For both proteins, the secondary structure of the native polypeptides was not recovered after these samples were cooled down to 20°C (Fig. 3). This indicates that the thermal denaturations of both *B/IGGT* and T399A are highly irreversible.

3.3 Quenching of *B/IGGT* and T399A fluorescence by acrylamide and Ni²⁺

The accessibility of groups on a protein molecule can be measured by use of quenchers to perturb fluorophores. Quenching of Trp fluorescence by adding small solutes has been widely employed in structural studies because it can provide information on the location of Trp residues with respect to the aqueous phase and on the permeability of proteins to solutes [18, 19]. Quenching of *B/IGGT* and T399A fluorescence by acrylamide and Ni²⁺ was therefore performed at room temperature. Up to the highest concentration examined, the addition of these two quenchers did not alter the absorption or the fluorescence spectrum of *B/IGGT* and T399A. As shown in Fig. 4, the Stern–Volmer plots obtained from the decrease of fluorescence intensity followed a linear dependence on the quencher concentration. The values of K_{SV} derived from the gradient of these plots were further calculated according to the Stern–Volmer equation. In the case of acrylamide, the K_{SV} values for *B/IGGT* and T399A were 6.69 and 6.77 M⁻¹, respectively. Ni²⁺ was comparatively inefficient in quenching the protein fluorescence, while a similar K_{SV} value was observed for *B/IGGT* (3.44 M⁻¹) and T399A (3.19 M⁻¹). Taken together, it can be concluded that the accessibility of the Trp residues of *B/IGGT* to the quenchers is not changed significantly by the mutation.

3.4 Unfolding of *B/IGGT* and T399A by GdnHCl

The function of a protein depends on its ability to acquire a unique three-dimensional structure. GdnHCl is commonly used as a protein denaturant which generally brings about unfolding of proteins by disrupting their secondary and tertiary structures. Thus, the

Fig. 4 Stern–Volmer plots for the quenching of *B*/GGT and T399A by acrylamide and Ni^{2+} . Prior to fluorescence analysis, the protein samples in 50 mM Tris-HCl buffer (pH 8.0) were incubated with the quenchers at room temperature for 30 min

GdnHCl-induced unfolding is very suitable for conformational stability studies of *B*/GGT and T399A. Unfolding of these two proteins at different concentrations of GdnHCl was therefore performed and the obtained data are shown in Fig. 5. In this experiment, the AEW that reported on the changes in both fluorescence wavelength and fluorescence intensity was used to calculate the thermodynamic parameters of the unfolding process. As shown in Fig. 5a, *B*/GGT started to unfold at 2.14 M denaturant with $[\text{GdnHCl}]_{0.5, N \rightarrow U}$ of 2.85 ± 0.15 M. By fitting the data with Eq. 4, the calculated free energy change ($\Delta G_{\text{H}_2\text{O}}$) for the $N \leftrightarrow U$ process of *B*/GGT was 12.34 ± 0.46 kcal mol⁻¹. The fluorescence signal of GdnHCl-induced T399A also followed a monophasic process (Fig. 5a), which showed $[\text{GdnHCl}]_{0.5, N \rightarrow U}$ of 3.94 ± 0.12 kcal mol⁻¹, corresponding to $\Delta G_{\text{H}_2\text{O}}$ of 4.45 ± 0.11 kcal mol⁻¹ for the $N \leftrightarrow U$ process. Similar results were obtained with CD spectra of both proteins (Fig. 5b). Compared with the unfolding curves of *B*/GGT and T399A, it is obvious that a higher concentration of GdnHCl was required to start the unfolding of T399A. The $[\text{GdnHCl}]_{0.5, N \rightarrow U}$ values for *B*/GGT and T399A were 2.74 ± 0.25 and

Fig. 5 Unfolding curves of *B/IGGT* and T399A in different concentrations of GdnHCl. Protein sample in 20 mM Tris-HCl buffer (pH 8.0) was incubated with various concentrations of GdnHCl for 30 min. The protein solution was then excited at 280 nm and the emission was recorded at 25°C. **a** AEW of these two proteins was used to analyze the unfolding data. **b** CD changes at 222 nm versus GdnHCl concentration

3.75 ± 0.26 M, corresponding to free energy changes (ΔG) of 6.90 ± 0.45 and 5.25 ± 0.27 kcal mol⁻¹, respectively, for the $N \leftrightarrow U$ process. These results allow us to distinguish the conformational stabilities between these two enzymes and indicate that the protein structure of *B/IGGT* is more rigid than that of T399A.

4 Discussion

B/IGGT belongs to the Ntn hydrolase superfamily in which the inactive precursors carry out the intramolecular autoprocessing to yield a heterodimeric enzyme. The molecular masses of the L- and S-subunits of *B/IGGT* are 44,874 and 20,517 Da, respectively. The purified *B/IGGT* and T399A were expectedly migrated on a native-PAGE gel to the position corresponding to the 63-kDa heterodimer, although a slower migration was observed for the T399A protein (Fig. 1). Native gel electrophoresis is a useful tool for investigating the

oligomeric states, physio-chemical properties, and biological activities of proteins [20, 21]. It is commonly known that the electrophoretic migration of proteins closely correlates with their intrinsic or imposed electric charges. Generally, the basic proteins are difficult to migrate in a native gel if they remain positively charged in the running buffer. With respect to the mature BIGGT, it is likely that the unprocessed T399A retains much more positive surface charge and the nature of this protein might contribute to a lower mobility in native gel.

The typical fold of Ntn-hydrolases consists of a four-layered catalytically active $\alpha\beta\beta\alpha$ -core structure [22]. This core is formed by two antiparallel β -sheets packed against each other, and these β -sheets are covered by a layer of antiparallel α -helices on one side [1, 23]. As members of Ntn hydrolase superfamily, the crystal structures of *EcGGT* and *HpGGT* show a conservation of the $\alpha\beta\beta\alpha$ -core structure [6, 24]. Further studies on these two enzymes have provided structural evidence for autocatalytic processing [25, 26] and the critical roles of the C-terminal region in autocatalytic processing and catalysis [27]. In the determined crystal structure of mature *EcGGT*, the C-terminal region of the L-subunit is distant from the N-terminal region of the S-subunit [24]. This observation apparently demonstrates that a conformational change has occurred upon autocatalytic processing. In fact, structure comparison between the mature *EcGGT* and unprocessed T391A has shown that the marked structural changes in the segment corresponding to the C-terminal region of the L-subunit and in the segment that forms the substrate-binding pocket occurred during the maturation process [25]. Except for these differences, the structure of the unprocessed T391A is basically similar to that of the mature *EcGGT*. However, a comparative characterization concerning the structural stability of the mature and unprocessed GGTs is still lacking. In this work, we aimed at studying the protein stability under thermal and chemical denaturation conditions in order to gain insights into the structural integrity of the mature and unprocessed BIGGTs. Typically, the free energy change for the folding of globular proteins ranges from -2.39 to -14.34 kcal mol $^{-1}$ [28]. For protein stability, almost perfect enthalpy–entropy compensation is observed [29–32]. As a consequence of the compensation, the marginal stability of globular protein structures may be a result of very large enthalpic and entropic contributions. The global free energy for folding of BIGGT at 25°C is $\Delta G_{H_2O} = -3.0$ kcal mol $^{-1}$ K $^{-1}$, which is composed of $\Delta H_m = -65.7 \pm 6.9$ kcal mol $^{-1}$ and $\Delta S_m = -196.5 \pm 20.5$ cal mol $^{-1}$ K $^{-1}$. Interestingly, comparable kinetic parameters were observed for T399A (Table 2). It has been reported that the effects of mutations on overall protein stability are to a large degree predictable [33]. Since protein flexibility allows for reorientation, it is not simple to predict the changes in dynamics and flexibility of T399A mutation.

The structural stability of mature BIGGT and unprocessed T399A protein was also analyzed after GdnHCl-induced denaturation. The superposition of the fluorescence- and CD-monitored unfolding curves at increasing GdnHCl concentrations indicates that both proteins behave as a single cooperative unit upon chemical denaturation (Fig. 5). The unfolding curves measured with both probes have similar values of $[GdnHCl]_{0.5, N-U}$. Additionally, the thermodynamic parameters obtained from tryptophan fluorescence emission spectra ($m = 4.33 \pm 0.16$ and 1.13 ± 0.24 kcal mol $^{-1}$ M $^{-1}$; $\Delta G_{H_2O} = 12.34 \pm 0.46$ and 4.45 ± 0.11 kcal mol $^{-1}$ for BIGGT and T399A, respectively) are in the range of those reported for globular proteins [34]. Structural disruption of proteins by the chaotropic reagent occurs concomitantly with unfolding of the α -helix core or exposure of the tryptophan residues to the polar environments. The exact molecular mechanism for the denaturation process induced by GdnHCl is not yet clearly defined [35, 36]. It has been

assumed that GdnHCl unfolds proteins by solubilizing the nonpolar parts of the protein molecule along with the peptide backbone CONH groups and the polar groups in the side chains of the biomolecule [37, 38]. In this regard, the unfolding process of *B/*GGT and T399A by the denaturant might follow the above-indicated path. It should be emphasized that a clear change in conformational stability of *B/*GGT was observed upon substitution of Thr399 with alanine. This was apparently suggested by the decrease in free energy difference of the unprocessed T399A. However, detailed information about the structure–stability relationship remains to be elucidated.

In conclusion, we have shown that the mature enzyme and the unprocessed T399A protein display structural differences in thermally and chemically induced unfolding, despite the fact that they might preserve a high similarity in overall structure. The comparative studies with *B/*GGT and T399A could contribute to a better understanding of the structural stability of the GGT family of enzymes.

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