The Quenching-Resolved Fluorescence Spectrum and Its Application to Studies of the Folding/Unfolding of Trypsin Inhibitor from Seeds of the Bitter Gourd

SHUZO MATSUMOTO, ETSUKO NISHIMOTO, HIRONORI SOEJIMA, and SHOJI YAMASHITA

Institute of Biophysics, Faculty of Agriculture, Graduate School of Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Received January 29, 2010; Accepted April 16, 2010; Online Publication, July 7, 2010
[doi:10.1271/bbb.100070]

With reference to the local conformation of a protein, it is interesting to differentiate the individual fluorescence properties of included tryptophan residues without modification. The fluorescence spectrum of bitter gourd trypsin inhibitor (BGTI) was separated into two emission bands by the quenching-resolved fluorescence method. One emission band was given as a fraction with the Stern-Volmer quenching constant, 44.9 x 10^-3 M^-1, against the fluorescence quenching by KI, and it showed an emission maximum intensity at 341 nm. The fluorescence quenching constant of the other band was 1.58 x 10^-3 M^-1, and the maximum wavelength was found at 337 nm. These separated emissions were due to the fluorescence of Trp54 and Trp9 of BGTI. The quenching resolved-fluorescence spectrum was effectively applied to the precise description of the polar circumstances surrounding the Trp residues in the unfolding intermediate state of BGTI. The results suggested that the molten globule-like state of BGTI adopted such a peculiar conformation that the helix domain including Trp9 was packed more densely while the other loop domain partially unfolded.

Key words: trypsin inhibitor; fluorescence quenching; fluorescence lifetime; protein folding/unfolding

The indole moiety, which is the fluorescent side chain of the tryptophan residue, exhibits a specific photo-physical property. In the excited singlet state ascribed to 1L_α by excitation at about 295 nm, the dipole moment of the indole moiety is enormously increased and the resulting dipole-dipole interaction with the surrounding polar molecules lowers the energy level of fluorescence emitting state. In non-polar circumstances, the fluorescence of tryptophan is emitted from the 1L_α state, the energy of which level is just below that of 1L_α. Therefore, the protein with tryptophan residue surrounded by more polar circumstances gives the maximum at the longer wavelength side. Because of this specific property, the tryptophan residue is an excellent reporter of conformation change in proteins. Indeed, fluorescence spectroscopy of a protein through the tryptophan residue has provided essential information on protein structure, dynamics, and function by the advanced time-resolved technique and photo-selection rules. The higher spectroscopic sensitivity and time resolution power of fluorescence methods can detect emission even from a single molecule and resolve fast motions extending to 10^-8 to 10^-12 s. This competence of fluorescence spectroscopy has great advantages for the conformation and dynamic characterization of the protein folding/unfolding intermediate state, which is intrinsically in disorder, but the fluorescence spectroscopic study of a protein through the tryptophan residue has limits and restrictions. Many proteins contain more than one tryptophan residue. In such a case, it is difficult to distinguish the fluorescent properties of the individual tryptophan residues included in a protein. In order to overcome this limitation, replacement of specified tryptophan residue by site-directed mutagenesis is generally used, but the amino acid replacement method sometimes has serious effects on the conformation around the remaining tryptophan, depending on the location of the tryptophan residue replaced. Furthermore, the amino acid replacement method is a time-consuming task. Therefore, it is desirable to develop a method to investigate the individuality of the tryptophan residues in multi-tryptophan-containing proteins. Nishimoto et al. succeeded in describing interesting conformational changes and molecular dynamics around Trp-62 and Trp-108 of hen egg-white lysozyme by a combination of quenching resolved fluorescence with site-directed mutagenesis. The quenching resolved fluorescence method used in their work was based on the Stern-Volmer equation. Tryptophan residue exposed to the protein surface allows the easier approach of quenchers such as acrylamide and KI to quench tryptophyl fluorescence. Thus exposed tryptophan can be distinguished with a buried one by analyzing the quenching efficiency of the quencher.

Trypsin inhibitor from bitter gourd (BGTI) contains two tryptophan residues, at positions, 9 and 54. The fluorescence maximum of BGTI is at 339 nm and the spectral line shape holds greater symmetry. This suggests that two tryptophan residues are in similar polar circumstances, and it is difficult to discriminate them in the ordinary way. In the present study, we...
separated these two tryptophan residues by the quenching resolved fluorescence technique and the results compared with the fluorescence spectroscopic properties of W9F and W54F, in which Trp9 and Trp54 are replaced with phenylalanine residues. The validity of the quenching resolved fluorescence spectrum was examined, and the results were applied to an investigation of the unfolding of BGTI.

The three-dimensional structure of BGTI has been determined at very high resolution, and its structure has been characterized as a tightly packed domain consisting of two α-helices and a loosely packed loop domain. Tryptophan residues, Trp9 and Trp54, are located in the densely packed domain and loop domain, respectively (Fig. 1). Because of this exquisite arrangement of tryptophan residues, BGTI is the interesting subject in investigating the characteristic conformation and dynamics of the protein folding/unfolding intermediate through fluorescence spectroscopy. The frame structure of BGTI is very similar to that of chymotrypsin inhibitor 2, which has provided interesting information on the protein folding mechanism. Correspondingly, we have reported that the folding/unfolding intermediate state of BGTI locally retained a more densely packed structure using mutant BGTI. In the present study, the peculiar conformation and dynamics of the unfolding intermediate state of BGTI were determined by establishing the quenching-resolved fluorescence properties of BGTI.

Materials and Methods

Materials. Seeds of bitter gourd (Momordica charantia) were purchased from Nakahara (Fukuoka, Japan). Restriction enzymes and DNA modifying enzymes were from MBI Fermentas (Hanover, MD) and Toyobo (Osaka, Japan) respectively. Thermo sequenase cycle sequencing kits containing 7-deaza-dGTP were from GE Healthcare UK (Buckinghamshire, UK). A chameleon double-standard site-directed mutagenesis kit was from Novagen (Madison, WI). E. coli strains JM109 and Xl1-blue were used as host cells in producing recombinant BGTI and its mutant proteins. All other common chemicals and reagents were purchased at the highest purity available and were used without further purification.

Purification of BGTI. Crude protein including BGTI was extracted from milled and ground seeds of bitter gourd. After precipitation in saturated ammonium sulfate, the crude BGTI was separated and purified by cation-exchange chromatography using HiTrap SP and Mono S columns (GE Healthcare UK). The purified BGTI, the purity of which was confirmed by SDS–PAGE, was dialyzed against 50 mM Tris–HCl buffer, pH 7.5, and stored at −80°C until use.

The methods for the expression and purification of mutant BGTI are described in reference 5. The genes encoding the mutant BGTI were expressed in E. coli strain BL21 (DE3) using expression plasmid pET-16b. Mutant BGTIs were purified from extracts of the cells by a combination of HiTrap Chelating column (GE Healthcare UK) and ion exchange column chromatography by the Biologic DualFlow chromatography system (BioRad, Hercules, CA). First, mutant BGTIs were separated from other proteins with a His-tag trap column, which was prepared by conjugating a HiTrap Chelating column with Ni2+. After desalting and condensation, the His-tag was removed by incubating BGTI with Factor Xa for 12 h. In order to remove the His-tag and undigested BGTI, the sample solution was applied to the His-tag trap column again. The eluted sample was purified by cation exchange chromatography using a HiPrep 16/10 CM FF column (GE Healthcare UK), after desalting. The purities of the mutant BGTI were confirmed by HPLC on YMC-Pack Diol-60 (Kyoto, Japan) and SDS–PAGE using 15% polyacrylamide gel. The concentrations of BGTI and its mutants were determined by the spectrophotometric method.

The purified BGTI and its mutants, W54F-BGTI and W9F-BGTI, were prepared in 20 mM Tris–HCl buffer, pH 7.8, for unfolding experiments. Small aliquots of the sample solution were added to reaction mixtures containing the desired concentrations of guanidine hydrochloride (Gdn). These protein solutions were incubated at 25°C for 5 h to attain unfolding equilibrium under conditions including various concentrations of Gdn. Under these conditions, no further changes in any of the experimental parameters used here, such as fluorescence spectra and decay kinetics, were recognized after incubation.

Steady-state and time-resolved fluorescence measurements. Steady-state fluorescence measurements were performed on a Hitachi 850 fluorescence spectrophotometer (Tokyo). The excitation and emission spectra were strictly corrected for the excitation and detection system. The effects of undesired stray light were removed by subtraction methods. Steady-state fluorescence anisotropy was measured using the same instrument. After measurement of parallel (I∥) and perpendicular (I⊥) components against vertical excitation, fluorescence anisotropy (r) was calculated by the following equation:

\[
\eta = \frac{I_\perp - G \eta_i}{I_\parallel + 2G \eta_i}
\]

where G is the grating factor decided by the intensity ratio of the vertical component to the horizontal one against horizontal excitation.

Time-resolved fluorescence and fluorescence anisotropy were measured by the sub-picosecond laser based time-correlated single photon counting (TCSPC) method. The excitation pulse (295 nm) was generated from a combination of sub-picosecond Ti-Sapphire laser (Tsunami, Spectra-Physics, Mountain View, CA), pulse picker with second harmonic generator (model 3980, Spectra-Physics), and third harmonic generator (GWU, Spectra-Physics). The repetition rate was 800 kHz, and the full width at half maximum (FWHM) of the excitation pulse obtained was 100 fs. The fluorescence emission pulse was detected with a multi-channel plate type photomultiplier (3809U-50, Hamamatsu Photonics, Shizuoka, Japan). The output signals of a time-to-amplitude converter (TAC 457, Ortec; Oak Ridge, TN) were accumulated in 2,048 channels in a multichannel analyzer (Maestro-32, Ortec). The channel width was 11 ps/ch. The details of the apparatus and procedure are described in a previous report.

The fluorescence decay kinetics of BGTI were described by a linear combination of exponentials:

\[
F(t) = \sum_i \alpha_i \exp(-t/\tau_i)
\]

where τ is the fluorescence decay time of the i-th component and αi is the corresponding pre-exponential factor, and αi and τi were determined by the iterative convolution and non-linear curve fitting methods. The adequacy of curve-fitting was judged by residual plots and statistic parameters, such as the serial variance ratio (SVR) and the sigma value (σ).
CD spectrum measurement. The CD spectrum of BGTI and its mutant were recorded on a J-720 spectropolarimeter (Jasco, Tokyo). The sample solution of BGTI was buffered with 20 mM Tris–HCl buffer, pH 7.8. The protein concentration used for the CD spectrum was adjusted to 0.1 mg/ml. A CD cuvette with a 1-mm optical-path length was used for measurements in the far-UV region.

Quenching-resolved fluorescence spectrum. The quenching-resolved fluorescence spectrum of BGTI was obtained using a modification of the Stern-Volmer equation:

$$\frac{F_i}{F_0} = \sum \frac{f_i}{(1 + K_{SV}Q)}$$  \( \text{Eq. 3} \)

where \( F \) and \( F_0 \) are the fluorescence intensities in the presence and absence of the quencher molecule (Q), and \( K_{SV} \) and \( f_i \) are the Stern-Volmer constant and the corresponding fraction of the steady-state fluorescence intensity. The fluorescence spectra were precisely measured in the presence of the desired concentrations of KI. The fluorescence intensity, sampled at 2-nm intervals, was plotted based on eq. 3, and parameters \( K_{SV} \) and \( f_i \) were determined by simultaneous global analysis. The quenching-resolved fluorescence spectrum was obtained by assigning the fluorescence intensity according to the \( f_i \) at each wavelength.

Results

Quenching-resolved fluorescence spectrum

As shown in Fig. 2, the fluorescence spectrum of BGTI appeared as a single band with the center at 339 nm in 20 mM Tris–HCl buffer solution (pH 7.2). Since BGTI contains two tryptophan residues, Trp9 and Trp54, in the vicinity, adjacent to the hydrophobic core and near the loop structure respectively, the fluorescence of BGTI must be composed of the emissions of the two Trps, which exhibited slightly different spectrum distributions. The fluorescence of BGTI decreased in intensity without shifting of the emission maximum wavelength in the presence of 3 M of Gdn, and the spectrum eventually shifted in maximum wavelength to 355 nm, accompanying a reduction in intensity in the buffer containing 6 M Gdn.

The inset of Fig. 3 shows fluorescence spectral changes in BGTI under quenching by KI. While the fluorescence intensity fell with increases in the concentration of KI, the fluorescence maximum wavelength shifted to the blue side, because fluorescence was quenched more effectively on the longer wavelength side. The global curve-fitting results at various wavelengths deciding the Stern-Volmer quenching constant, \( K_{SV} \), and the corresponding fluorescence emission fraction, \( f_i \), are shown in Fig. 3. The fitting adequacy, chi-square, and the correlation were \( 5 \times 10^{-3} \) and 1.00 respectively. Figure 4 shows the quenching-resolved fluorescence spectrum of BGTI obtained by allocating the steady-state fluorescence intensity at the various wavelengths according to the size of the fraction, \( f_i \). The fluorescence band (\( f_1 \)), giving \( K_{SV}f_1 = 44.9 \times 10^{-3} \text{ M}^{-1} \), showed a maximum at 341 nm, and the maximum of the
fluorescence band \((f_2)\), giving the quenching constant \(K_{SVR} = 1.58 \times 10^{-3}\,\text{M}^{-1}\), was seen at 337 nm, while the total fluorescence emission spectrum showed a maximum at 339 nm. The normalized fluorescence band intensities of \(f_1\) and \(f_2\) were 0.67 and 0.33 respectively.

The fluorescence spectra of W9F- and W54F-BGTI showed fluorescence maxima at 342 nm and 336 nm respectively. Considering that the bandpath of the fluorescence spectrophotometer used was 2 nm, those values are consistent with those of components \(f_1\) and \(f_2\) in the quenching-resolved fluorescence spectrum of BGTI. The fluorescence quantum yields of W9F and W54F mutant of BGTI estimated using \(N\)-acetyl-L-tryptophanamide as fluorescence standard were 0.1 and 0.005 respectively, whereas that of BGTI was larger, estimated to be 0.14.

**Fluorescence intensity decay**

The fluorescence decay function is given by determining parameters such as the number of components, \(i\), decay time, \(\tau_i\), and the corresponding amplitude \(a_i\) in a linear combination of the exponential function. The fluorescence decay parameters of BGTI, W9F-BGTI, and W54F-BGTI are summarized in Table 1. The fluorescence decay kinetics of BGTI were described by the triple exponential, the components of which were characterized by decay times of 5.31, 0.40, and 0.05 ns. On the other hand, the fluorescence decay kinetics of the mutant BGTI showed the best fit to the double exponentials. Although the number of decay components of BGTI was expected to be 4, the shorter decay time of W9F-BGTI and the longer one of W9F-BGTI were not resolved in the decay function of BGTI. It should be noted that the longer decay time of W9F-BGTI was longer than the longest decay time of BGTI. The average lifetime of W54F-BGTI, defined as \(\tau_{sv} = \sum a_i \tau_i\), was extremely short, and the shorter decay time was estimated to be shorter than the shortest one of BGTI. As clearly shown in Table 1, the longest and shortest decay times were contributions of Trp54 and Trp9 respectively.

Table 1. Fluorescence Decay and Spectroscopic Parameters of BGTI, W9F-, and W54F-BGTI

<table>
<thead>
<tr>
<th></th>
<th>(a_1)</th>
<th>(a_2)</th>
<th>(a_3)</th>
<th>(a_4)</th>
<th>(\tau_1) (ns)</th>
<th>(\tau_2) (ns)</th>
<th>(\tau_3) (ns)</th>
<th>(\sigma)</th>
<th>SVD</th>
<th>(Em_{max}) (nm)</th>
<th>(\phi)</th>
<th>(\tau_{sv}) (ns)</th>
<th>(\tau_r) (ns)</th>
<th>(\tau_{nr}) (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGTI</td>
<td>0.47</td>
<td>0.06</td>
<td>0.47</td>
<td>—</td>
<td>5.31</td>
<td>0.40</td>
<td>0.05</td>
<td>1.05</td>
<td>1.87</td>
<td>339</td>
<td>0.14</td>
<td>2.18</td>
<td>18.4</td>
<td>2.50</td>
</tr>
<tr>
<td>W9F</td>
<td>0.97</td>
<td>0.03</td>
<td>—</td>
<td>—</td>
<td>6.64</td>
<td>0.58</td>
<td>—</td>
<td>1.05</td>
<td>1.87</td>
<td>341</td>
<td>0.10</td>
<td>6.46</td>
<td>64.6</td>
<td>7.69</td>
</tr>
<tr>
<td>W54F</td>
<td>0.33</td>
<td>0.67</td>
<td>—</td>
<td>—</td>
<td>0.16</td>
<td>0.04</td>
<td>—</td>
<td>1.10</td>
<td>1.80</td>
<td>337</td>
<td>0.005</td>
<td>0.08</td>
<td>16.0</td>
<td>0.08</td>
</tr>
</tbody>
</table>

\(\phi\): Fluorescence quantum yield
\(\tau_{sv}\): Average lifetime
\(\tau_{nr}\): Non-radiative lifetime

In order to determine the individual spectroscopic properties of the tryptophan residue in the mutant and wild-type BGTI, the radiative \((\tau_r)\) and non-radiative \((\tau_{nr})\) lifetimes were estimated by the fluorescence quantum yield \((\phi)\) and decay parameters based on the equation \(\phi = \tau_{sv}/\tau_r\) and \(1/\tau_{sv} = 1/\tau_r + 1/\tau_{nr}\). These fluorescence parameters are also summarized in Table 1. The radiative transition rate of Trp9 was 3.5 times faster than that of Trp54. As shown by the larger \(k_{sv}\), Trp9 interacts with surrounding amino acids such as Lys6 and Glu27. Their amino and carboxyl groups are dominant quenchers for tryptophyl fluorescence through the proton transfer and electron transfer mechanism.

**Protein unfolding**

Figure 5A shows the equilibratory unfolding profile of BGTI as revealed by changes in fluorescence intensity and maximum wavelength. The fluorescence intensity started to decrease at 1 M of Gdn, and reached an almost constant value at Gdn concentrations higher than 5 M. On the other hand, the fluorescence maximum wavelength was unchanged until 4 M of Gdn. These results suggest that the large conformation change that the tryptophan residues were exposed at the protein surface was initiated in the presence of Gdn at higher than 4 M. A conformational change that results in a decrease in tryptophyl fluorescence intensity should be induced in the presence of 3–4 M of Gdn. The unfolding profile, as monitored by the ellipticity of the CD spectrum, is shown in Fig. 5B. The CD spectrum of BGTI did not show the usual double minimum, but almost a single peak at 205 nm, which is attributable to the \(\pi^0-\pi^-\) transition (data not shown). When the ellipticities at 222 nm were plotted against the Gdn concentration, they were constant up to 3 M of Gdn, and then decreased to

---

**Fig. 5.** Equilibratory Unfolding Curves of BGTI. A, Fluorescence intensity and peak wavelength. Excitation wavelength, 295 nm. \(\triangle\), Fluorescence intensity; \(\bullet\), Peak wavelength in the spectrum. The solid line is for sight and is not based on any theoretical equation for the unfolding. B, Fluorescence anisotropy and ellipticity in CD. \(\bullet\), Fluorescence anisotropy; \(\bigcirc\), Relative ellipticity at 222 nm in the CD spectrum. Fluorescence anisotropy was measured at the wavelength giving maximum intensity. The solid line is just for sight and not based on any theoretical equation for the unfolding.
show a constant value at 6 M. The steady-state fluorescence anisotropy measured at 340 nm (Fig. 5B) increased and then decreased increases in the concentration of Gdn, and showed a maximum value at 3 M.

The fluorescence anisotropy depends on the viscosity of the solution. Therefore, much attention should be paid to interpretation of the results of fluorescence anisotropy. However, it is reasonable to conclude that the decrease in the fluorescence anisotropy precedes the decrease in ellipticity in CD, and the internal motion of peptide segments, including tryptophan residues, which is a controlling factor in fluorescence anisotropy, is suppressed most in the presence of about 3 M of Gdn.

The fluorescence spectra of BGTI under quenching by KI were measured in buffer solution including 2, 4, 6, and 8 M of Gdn (data not shown). The fluorescence intensities at the various wavelengths were not necessarily quenched uniformly. Quenching efficiencies were higher on the longer wavelength side. This tendency was especially pronounced solutions of 2 and 4 M of Gdn, and therefore the fluorescence spectrum of BGTI equilibrated in this concentration region of Gdn showed a blue-shifted line shape in the presence of higher concentrations of KI. The precision of global analysis of the fluorescence intensities at various wavelengths covering the full spectral region against the KI concentration was so high that the fluorescence quenching of BGTI was well described with two Stern-Volmer constants and the corresponding fractions at each wavelength. The quenching-resolved fluorescence spectrum was obtained by allocating the steady-state fluorescence intensity according to the fraction size at each wavelength. Figure 6 shows the quenching-resolved fluorescence spectra of BGTI equilibrated with 0, 2, 4, 6, and 8 M of Gdn. As shown in Fig. 4, the fluorescence spectrum of BGTI in the absence of Gdn was resolved into two sub-bands with maxima at 341 nm ($f_1$) and 337 nm ($f_2$). The $f_2$ band shifted the maximum wavelength to the blue side a little with keeping the $f_1$ band shifted the maximum wave-length to the red side at 6 M, and the bi-directional 1

![Normalized fluorescence spectrum](image)

**Fig. 6.** The Quenching-Resolved Fluorescence Spectrum of BGTI in the Presence of Various Concentrations of Gdn.

- a, Normalized fluorescence spectrum; b, the $f_1$ band; c, the $f_2$ band. The wavelength giving the maximum fluorescence intensity is indicated by the vertical dotted line.

<table>
<thead>
<tr>
<th>Gdn (M)</th>
<th>$K_{SV}^1$ ($M^{-1}$)</th>
<th>$K_{SV}^2$ ($M^{-1}$)</th>
<th>$\chi^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$4.49 \times 10^{-3}$</td>
<td>$1.58 \times 10^{-3}$</td>
<td>$0.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>2</td>
<td>$6.50 \times 10^{-3}$</td>
<td>$1.75 \times 10^{-3}$</td>
<td>$0.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>4</td>
<td>$4.17 \times 10^{-3}$</td>
<td>$1.23 \times 10^{-3}$</td>
<td>$1.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>6</td>
<td>$5.81 \times 10^{-3}$</td>
<td>$0.75 \times 10^{-3}$</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>8</td>
<td>$4.48 \times 10^{-3}$</td>
<td>—</td>
<td>$2.4 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

$^a$Stern-Volmer Constants, $K_{SV}^1$ and $K_{SV}^2$, were estimated using the equation, $F_i(\lambda)/F_0(\lambda) = f_i/(1 + K_{SV}^i \cdot [Q]) + f_2/(1 + K_{SV}^2 \cdot [Q])$, where $F_0$ and $F$ are fluorescence intensities in the absence and the presence of KI, $f_i$ is the $i$-th fraction, and $[Q]$ is the concentration of KI.

$^b$Precision parameter.
intermediate state, with the local structure densely packed around Trp9 and relaxed around Trp54 during the unfolding process of BGTI.

Discussion

The fluorescence spectrum of BGTI was separated into two bands by the quenching-resolved technique based on a modification of the Stern-Volmer equation. The separated fluorescence bands gave maximum intensities at 341 nm and 337 nm, and their corresponding quenching constants by KI were $K_{q}^{58} = 4.49 \times 10^{-3} \text{s}^{-1}$ and $1.58 \times 10^{-3} \text{s}^{-1}$ respectively. These bands are presumably to be attributed to the fluorescence of Trp54 and Trp9, because their peak wavelengths were coincident with the spectrum of mutant BGTI. The crystallographic structure of BGTI confirms this conclusion. Trp9 is located in a more densely-packed region, and hence should give a smaller quenching constant. The coincidence of the quenching-resolved fluorescence band of BGTI with mutant BGTI suggests that the polar circumstances of the two tryptophan residues are not much changed by the mutations. The present results suggest that the quenching-resolved fluorescence spectrum is a potent method of separating the tryptophan residue arranged at similar polar circumstances to specify individual fluorescence spectroscopic properties in a multi-tryptophan containing protein without amino acid replacement. However, inconsistencies in the fluorescence decay kinetics of BGTI indicate that the amino acid replacement has some effect on the local conformation around Trp9 and Trp54. The shorter decay time of the fluorescence of W54F-BGTI was prolonged and no fluorescence spectral shift to the red side of BGTI was found until the Gdn concentration was higher than 4 M. Furthermore, one fluorescence band ($f_{1}$) in the quenching-resolved fluorescence spectrum, presumably attributable to Trp54, showed a red shift, and the rotational freedom of the same tryptophan residue increased to a Gdn concentration range of 2–4 M. These features of the specific conformation state are consistent with the fundamental properties of the MG state. But it should be noted that the fluorescence band ($f_{2}$) with a smaller quenching constant and Trp9 in the W54F-BGTI shifted in maximum wavelength to 330 nm when the Gdn concentration increased from 0 to 4 M. At the same time, the rotational motion of Trp9 was restricted to the same Gdn concentration range. This suggests that a specific conformation state such that the packing of Trp54 is relaxed, and contrarily, the amino acid residues surrounding Trp9 are more compactly packed as the concentration of Gdn increases to 6 M. Such a partially structured or partially unfolded intermediate state of the protein has been established, although a tryptophan immobile state involved in the unfolding of creatin kinase was reported through special analytical methods for the fluorescence spectrum. The protein folding/unfolding intermediate state, the MG state, is stabilized through interaction between $\alpha$-helices. 4) Chymotrypsin inhibitor 2, the native structure of which was very similar to BGTI, created a nucleus site in the $\alpha$-helices domain, controlling the subsequent protein folding process. 4) Considering these experimental facts, the heterogeneous packing conformation of BGTI observed here is interesting for elucidation the protein folding/unfolding mechanism, because Trp9 is located in the adjacent part of the $\alpha$-helices, and restriction of the rotational motion and the blue shift of the fluorescence spectrum of this tryptophan residue can result from specific cohesive interactions between the surrounding $\alpha$-helices, which stabilize the BGTI structure against the destructive fluctuation of the loosely packed domain in the protein unfolding process.
References