Analysis of Internal Motion of Single Tryptophan in *Streptomyces* Subtilisin Inhibitor from Its Picosecond Time-Resolved Fluorescence

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ABSTRACT A mode of internal motion of single tryptophan, Trp 86, of *Streptomyces* subtilisin inhibitor, was analyzed from its time-resolved fluorescence. The intensity and anisotropy decays of Trp 86 were measured in the picosecond range. These decays were analyzed with theoretical expressions derived assuming that the indole ring of tryptophan as an asymmetric rotor rotates around covalent bonds connecting indole with the peptide chain and an effective quencher of fluorescence of Trp 86 is the nearby SS bond of Cys 35—Cys 50. First, the intensity decays at 6°, 20°, and 40°C were analyzed, and then the both decays of the intensity and anisotropy at 20°C were simultaneously simulated with common parameters. Constants concerning geometrical structures of the protein used for the analysis were obtained from x-ray crystallographic data. Best fit between the observed and calculated decay curves was obtained by a nonlinear least squares method by adjusting a quenching constant averaged over the rotational angles, k_q^0 height of the potential energy, p, and three of six diffusion coefficients, D_{xx}, D_{yy}, D_{zz}, D_{xy}, D_{yz}, and D_{zx}, as variable parameters. The obtained results revealed that the internal motion of the indole ring became faster, the quenching rate of the fluorescence of Trp 86 was enhanced and the height of potential energy became lower at higher temperatures, and suggested that Trp 86 was wobbling around the long axis of the indole ring in the protein.

INTRODUCTION

It is well known that in many proteins containing a single tryptophan fluorescence intensity and anisotropy of the tryptophan decay with nonexponential functions (Beechem and Brand, 1985). It is important to clarify the origin of the nonexponentiality in the time-resolved fluorescence of the tryptophan in these systems, not only for elucidating the nature of the microenvironment surrounding tryptophan in proteins, but also for understanding the dynamic nature of the protein structure in detail. For this purpose we have derived theoretical expressions for the time-resolved intensity and anisotropy of fluorescence of the tryptophan in proteins in previous work (Tanaka and Mataga, 1987, 1992) and applied them to the analysis of the fluorescence of erabutoxin b (Tanaka and Mataga, 1992; Tanaka et al., 1987).

Streptomyces subtilisin inhibitor (SSI) was discovered and isolated by Sato and Murao (1973) and inhibits preferentially serine proteases of microbial organ, e.g., subtilisin BPN'. The protein is a dimer (mol. wt. 23,000) consisting of identical monomers, which contains a single tryptophan, Trp 86, per subunit. SSI is one of the proteins of which the structure and function are the best characterized (Hiromi et al., 1985). Three-dimensional structures of SSI and SSI-BPN' complex were solved by Mitsui et al. (1979) and Hirono et al. (1984), respectively.

In the present work we have analyzed the mode of motion of the indole ring of Trp 86 in SSI by picosecond time-resolved fluorescence spectroscopy.

MATERIALS AND METHODS

Materials

SSI was purified from the culture filtrate of *Streptomyces albogriseolus* S-3253 essentially as described previously (Sato and Murao, 1973). Protein concentration was determined from ultraviolet absorbance using $A = 0.829$ at 276 nm of the solution containing 0.1% SSI at pH 7.0.

Measurements

Fluorescence spectra were measured with a spectrofluorometer (JASCO, FP-770, Japan). Fluorescence decay curves and anisotropy were measured with a synchronously pumped, cavity-dumped dye laser and a picosecond time-correlated, single-photon counting apparatus (Yamazaki et al., 1985; Song et al., 1989). Typical time width of the instrumental response function was 30 ps. The sample was excited with a vertically pumped, cavity-dumped dye laser. Fluorescence from the sample was detected at a right angle to the excitation beam through a Polaroid HNP' B polarizer. The analyzing polarizer was set either parallel (I_V) or perpendicular (I_H) to the polarized laser light. A wedge depolarizer was inserted between the analyzing polarizer and a monochrometer. Time-dependent anisotropy, $r(t)$, was calculated according to the following equation:

$$r(t) = \frac{I_V - I_H(t)}{I_V(t) + 2I_H(t)} \quad (1)$$

The sample was excited at 295 nm and its fluorescence was monitored at 340 nm. Temperature of the sample was controlled with circulating water.

Best-fit procedure

Fluorescence intensities, I_c, were simulated by convoluting an appropriate decay function, $F(t)$, namely, multiexponential function or theoretical ex-

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pression obtained by a rotational model with observed excitation pulse $E(t)$.

$$I_c(t) = \int_0^t E(t')F(t - t')\,dt'. \quad (2)$$

The difference between the observed and calculated intensity at time t_i was expressed by Eq. 3.

$$\mathrm{Dev}(t_i) = \{I_o(t_i) - I_c(t_i)\}/\sqrt{nI_o(t_i)} \quad (i = 1, 2, 3, \cdots, n) \quad (3)$$

where $I_o(t_i)$ is observed intensity at t_i. Best-fit procedure was performed by adjusting parameters contained in the decay function so as to obtain the minimum value of a χ^2 for intensity, χ^2_I, according to a method of nonlinear least squares based on the Marquardt algorithm.

$$\chi^2_I = \sum_{i=1}^{n} \{\mathrm{Dev}(t_i)\}^2. \quad (4)$$

When both decays of intensity and anisotropy were simultaneously simulated with common parameters, χ^2 for anisotropy, χ^2_A, was calculated by the following equation:

$$\chi^2_A = \sum_{i=1}^{n} \{A_o(t_i) - A_c(t_i)\}^2/nA_o(t_i) \quad (5)$$

where $A_o(t_i)$ and $A_c(t_i)$ are observed and calculated anisotropies at $t = t_i$ with a theoretical expression stated below. In this case total value of the χ^2, χ^2_T, was obtained by a sum of those of intensity and anisotropy.

$$\chi^2_T = \chi^2_I + \chi^2_A. \quad (6)$$

To make values of χ^2_A comparable with χ^2_I, 1000 was multiplied by both $A_o(t_i)$ and $A_c(t_i)$. In this case the best-fit procedure was applied for χ^2_T.

Theoretical model

Theoretical expressions for both decays of the fluorescence intensity and anisotropy were derived according to the following models as described in detail elsewhere (Tanaka and Mataga, 1987, 1992). The indole ring of Trp 86 as an asymmetric rotor is covalently bound to the peptide chain in the spherical protein of SSI. Molecular systems are illustrated in Fig. 1. The indole ring possesses a freedom of motion of internal rotation described with Euler angles, $\omega = (\alpha\beta\gamma)$, from the spherical protein system $(x'y'z')$ to the indole system (xyz). It is assumed that an effective quencher of the fluorescence of Trp 86 is a nearby SS bond of Cys 35—Cys 50. The internal motion was described with a diffusion equation of rotational analogue of Smoluchowski equation (Favro, 1958), and therein a rotational angle-dependent quenching rate represented by Eq. 7 was introduced.

$$k_q(\alpha\beta) = k_q^0\left\{1 + \frac{4\pi}{3}\sum_{m=-1}^{1} Y^*_{1m}(\alpha_q\beta_q)Y_{1m}(\alpha\beta)\right\} \quad (7)$$

where k_q^0 is the quenching constant of the fluorescence of Trp 86 averaged over rotational angles of α and β, and $Y_{1m}(\alpha\beta)$ is the spherical harmonics. α_q and β_q are Euler angles of middle point of the S-S bond measured in the system of $(x'y'z')$. These values of $\alpha_q\beta_q$ were evaluated from the data of x-ray crystallography of SSI (Mitsui et al., 1979) and are listed in Table 1. The second term of the right side of Eq. 7 is a solid angle between a vector formed by coordinates of the origin (C_β of $C_\beta H_2$) and the middle point of the SS bond and the z axis. Eq. 7 shows that the quenching rate is maximum when the z axis of the indole ring directs to the middle point of SS bond. We implicitly assume here an electron transfer from the excited indole to the SS bond as a quenching mechanism. Probably, the electron transfer could occur from nitrogen atom of the excited indole, since the electron density is the highest at the lone pair on N of the indole ring.

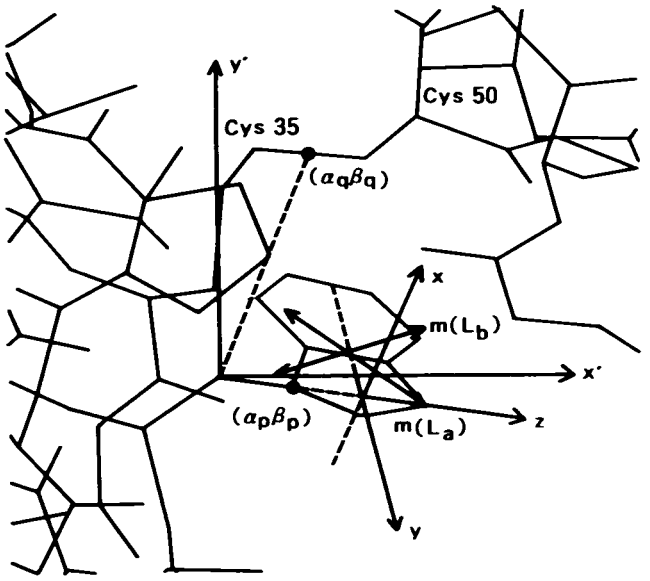

FIGURE 1 Geometrical arrangement of indole in SSI. The microenvironment of Trp 86 is illustrated according to the three-dimensional structure of SSI (Mitsui et al., 1979). Molecular coordinate system of SSI is represented with $(x'y'z')$, in which the z' axis is vertical to $x'y'$ plane (not shown). The coordinate system of indole ring of Trp 86 is denoted with (xyz). The z axis is along C_β-C_γ and y axis is in the plane of indole and perpendicular to z axis. The x axis is perpendicular to the indole ring. The directions of transition moments of L_a state and L_b state are indicated with ■ (L_a) and ■ (L_b), respectively. The direction of quencher (middle point of SS bond of Cys 35—Cys 50) is represented with $(\alpha_q\beta_q)$ of Euler angles in the system of $(x'y'z')$. The direction of C_β-C_γ is denoted with $(\alpha_p\beta_p)$ in the system of $(x'y'z')$.

A potential energy for the internal motion was also introduced in the form of Eq. 8,

$$V(\alpha\beta) = p\left\{1 - \frac{4\pi}{3}\sum_{m=-1}^{1} Y^*_{1m}(\alpha_p\beta_p)Y_{1m}(\alpha\beta)\right\} \quad (8)$$

where p is the height of the potential energy and α_p and β_p are Euler angles of C_β-C_γ expressed in terms of $(x'y'z')$ system, obtained also from the data of x-ray crystallography of SSI (see Table 1). The second term of the right side of Eq. 8 denotes a solid angle between the vector of C_β-C_γ and the z axis. Accordingly, it is assumed that the potential energy is minimal when the direction of C_β-C_γ is identical with that of the x-ray structure (Mitsui et al., 1979).

RESULTS

Analysis of the mode of motion with time-resolved intensity

Fluorescence decay curves were measured at 6, 20, and 40°C. First, we have approximated the decay curves with a multiexponential function as in most work reported (Beechem and Brand, 1985).

$$F(t) = \sum_{i=1}^{n} \alpha_i \exp\left(-\frac{t}{\tau_i}\right). \quad (9)$$

Fitting procedure was performed with decay functions of $n = 2$, 3, and 4. Best fit was obtained with $n = 3$ at temperatures of 6 and 20°C, and with $n = 4$ at 40°C. Obtained

TABLE 1 Constants used in the analysis

k_1^* (ns^{-1})	$D_p^\ddagger$ (ns^{-1})	Location of quencher§ (degree)		Location of energy¶ minimum (degree)		Direction of transition moments‖ Absorption (degree)		Emission (degree)	
		α_q	β_q	α_p	β_p	δ_a	ϵ_a	δ_e	ϵ_e
0.116	0.0	18	86	68	−40	Variable	90	50	90

* Rate constant independent of the internal rotation. The value is obtained from the longest lifetime of free tryptophan in alkaline solution (Jameson and Weber, 1981).

‡ Rotational diffusion coefficient of the spherical protein. The value of the diffusion coefficient was considered to be negligible in most analyses, because the rotational motion of the SSI (mol. wt. 23,000) as a whole could be slow enough compared with the time region of the present measurements.

§ The quencher for the fluorescence of Trp 86 was assumed to be -S-S- of Cys35—Cys50. The location of the quencher is taken to be the middle point between the two sulfur atoms which were determined by x-ray crystallography (Mitsui et al., 1979).

¶ Potential energy was introduced for the rotational motions with Euler angles of α and β. (α_p,β_p) was obtained from x-ray crystallographic data for the direction of C_β-C_γ of Trp 86.

‖ It was assumed that the fluorescence emission of Trp 86 was from the L_a-state, and light absorption (excitation at 295 nm) populated both L_a and L_b states. δ and ϵ indicate polar coordinates in the (xyz) system. The directions of the transition moments were obtained from the previous report (Yamamoto and Tanaka, 1972).

fluorescence lifetimes (τ_i) and their component fractions (α_i) are listed in Table 2. The shortest lifetimes were 130 ps at 6°C, 171 ps at 20°C, and 13 ps at 40°C, and the longest lifetimes were 1.08, 3.82, and 0.328 ns, respectively. The fractions of the shortest lifetimes were quite high, 0.424 at 6°C, 0.673 at 20°C, and 0.300 at 40°C. These decays were unusually fast compared with those of other proteins reported (Beechem and Brand, 1985; Tanaka et al., 1987). This should be due to the quenching by the SS bond of Cys 35—Cys 50, since it is known that SS bond quenches fluorescence of free tryptophan in aqueous solution (Konev, 1967). We have analyzed this quenching dynamics on the basis of our theoretical model (Tanaka and Mataga, 1987, 1992).

Indole is an asymmetric rotor and therefore diffusion process of its internal motion may be described with a tensor of second rank. The diffusion tensor contains six coefficients, D_{xx}, D_{yy}, D_{zz}, D_{xy}, D_{yz}, and D_{zx}. Free rotational motion of an asymmetric rotor can be described with only D_{xx}, D_{yy}, and D_{zz}. In the present case, however, this may no longer be true, since the internal motion is restricted by covalent bonds. Accordingly, we have chosen three of these six coefficients. k_q^0, p, and a set of the diffusion coefficients were adjusted to get the minimum value of χ_I^2.

The other constants used in the analysis are also listed in Table 1. The best-fit parameters are listed in Table 3. The fitting was much worse when D_{xx} among diffusion coefficients was adopted as one of the parameters. Furthermore, it was also worse when D_{zz} was included in the analysis. Introduction of the potential energy improved the fitting at 6°C and 20°C, but not at 40°C. The value of p which is represented as dimensionless numeric (energy divided by kT) became lower as temperature was elevated. The internal motion of the indole ring of Trp 86 should be enhanced at the higher temperature, and consequently a population of Trp 86 with higher energy in SSI could increase. This may bring about reduction of the relative height of the potential energy, p, to kT. At 40°C the value of another coefficient, D_{yz}, in addition to D_{yy} and D_{xy} increased, while it was negligible at the lower temperatures. It suggests that freedom of the internal motion increases at 40°C. It should be noted that the values of the diffusion coefficients increased as the temperature was raised. This is also reasonable, since the internal motion should become faster at higher temperature. Averaged quenching constant over rotational angles, k_q^0, also increased as the temperature was elevated, which is again very reasonable.

Analysis of mode of motion of Trp 86 from both decays of intensity and anisotropy with common parameters

Time-resolved anisotropy of the fluorescence was measured at 20°C (see Fig. 3). Apparent limiting anisotropy obtained at the shortest time (100 ps) was ~0.15. This is much lower than the limiting anisotropy of 0.4 expected when the transition moment of absorption is identical with that of emission. It decayed to <0.1 within 1 ns. The instantaneous depolarization from 0.4 to 0.15 may be due to the ultrafast internal conversion between L_a and L_b states (Ruggiero et al., 1990) since the L_a and L_b bands are overlapped at the ex-

TABLE 2 Decay parameters obtained with multiexponential decay functions

T (°C)	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3	τ_4 (ns)	α_4	χ^{2*}
6	0.130	(0.424)	0.833	(0.194)	1.08	(0.381)			2.634
20	0.171	(0.673)	0.833	(0.282)	3.82	(0.044)			1.148
40	0.013	(0.300)	0.090	(0.529)	0.328	(0.164)	1.88	(0.037)	1.284

* χ^2 between the observed and calculated intensities.

TABLE 3 Best-fit parameters obtained by the analysis of the intensity decay of SSI

T (°C)	k_q^{0*} (ns^{-1})	$p^{\ddagger}$ ($k^{-1}T^{-1}$)	Diffusion coefficients (ns^{-1})§					$\chi^{2\P}$
			D_{yy}	D_{zz}	D_{xy}	D_{yz}	D_{xx}	
6	1.51	0	0.51		0.08	0.00		4.52
	1.50	0		0.52		0.00	0.02	4.51
	3.80	1.33	2.91		0.81			2.64
20	3.17	0		0.06		0.00	0.24	2.18
	3.49	0	0.10		0.21	0.00		1.80
	3.59	0.48		0.03			0.21	1.69
	4.07	0.70	1.30		1.37			1.58
40	9.89	0	1.66	0.00	2.28			1.63
	9.80	0	3.77		3.89	1.55		1.62
	9.89	0.07	4.72		5.03	5.00		1.64

* Averaged quenching rate over the rotational angles.

‡ Height of potential energy for the internal rotation of Trp 86. The value of p is expressed in demensionless numeric which was obtained from the energy height divided by kT.

§ Diffusion coefficients of the internal rotation.

¶ χ^2 between the observed and calculated intensities.

citation wavelength (295 nm) (Weber, 1960; Mataga et al., 1964; Valeur and Weber, 1977). Very fast motion of Trp 86 may also contribute to the instantaneous depolarization. Both decays of the intensity and the anisotropy were analyzed with common parameters. Direction of transition moment of absorption was represented by polar coordinates (δ_a, ϵ_a) and that of emission by (δ_e, ϵ_e) in (xyz) system. The transition moments were considered to be in the plane of the indole ring as shown in Fig. 1. Accordingly, $\epsilon_a = \epsilon_e = 90°$ (see Table 1). It was assumed that the fluorescence of Trp 86 was emitted from the L_a state (emission was monitored at 340 nm). δ_e of L_a state was taken to be 50° (Yamamoto and Tanaka, 1972). However, δ_a is not known, because the both states of L_a and L_b are excited. δ_a should be an averaged angle in the indole plane between the transition moments of the L_a state and the L_b state. Hence, it was varied in the present analysis as an additional parameter. Obtained parameters are listed in Table 4. The fitting was improved when the diffusion coefficients, D_{yy}, D_{xy}, and D_{yz} among the six coefficients were included in the analysis of the intensity decay. D_{yz} was negligibly small. These results indicate that the internal motions of the indole ring of Trp 86 are taking place around y axis (long axis of the ring) and around an intermediate direction between y axis and x axis. The best-fit parameters were $k_q^0 = 3.64$ (ns^{-1}), $p = 0.90$ ($/kT$), $D_{yy} = 0.13$ (ns^{-1}), $D_{xy} = 0.24$ (ns^{-1}) and $\delta_e = 94°$. The values of the diffusion coefficients were quite different from those obtained by the intensity decay (see Table 3). When the potential energy was introduced, the fitting was also improved, as in the analysis of the intensity. In most analyses, the effect of the depolarization by rotational motions of the protein as a whole was ignored, since the time domain of the depolarization measured was rather short. The fitting was not improved when the rotational diffusion coefficient, $D_p = 0.023$ (ns^{-1}), was taken into account, which was evaluated from the equation of $D_p = RT/6V\eta$, where V was molar volume of SSI, and η viscosity of solvent.

DISCUSSION

The usual physicochemical properties of SSI and its complex with BPN′ are well characterized (Tonomura et al., 1985). The fluorescence emission peak of Trp 86 in SSI is observed at 340 nm upon excitation at 295 nm (Uehara et al., 1976).

TABLE 4 Best-fit parameters obtained by simultaneous simulation of the decays of both intensity and anisotropy of SSI*

k_q^{0*} (ns^{-1})	p ($k^{-1}T^{-1}$)	Diffusion coefficients (ns^{-1})					δ_a‡ (degree)	$\chi_T^{2\S}$	$\chi_I^{2\P}$	$\chi_A^{2\|}$
		D_{yy}	D_{zz}	D_{xy}	D_{yz}	D_{xx}				
3.52	0	0.21		0.35	0.086		90	4.67	1.82	11.69
3.55	0	0.11		0.23	0.095		95	3.61	1.71	8.29
3.65	0.98	0.14		0.25	0.001		94	3.52	1.67	8.06
3.65	0.98	0.14		0.25			94	3.52	1.67	8.06
3.64**	0.90	0.13		0.24			94	3.52	1.68	8.05

* Temperature was 20 °C. Notations are the same as those in Table 3.

‡ Angle between the z axis and the transition moment of absorption (excitation at 295 nm) in the indole ring.

§ χ^2 for total data points of decays of the intensity and anisotropy obtained by Eq. 6.

¶ χ^2 for the intensity decay obtained by Eq. 4.

‖ χ^2 for the anisotropy decay obtained by Eq. 5. Both values of the observed and the calculated anisotropies were multiplied by 1000 in order to obtain comparable values of χ_A^2 with those of χ_I^2.

** Rotational diffusion coefficient of spherical protein, $D_p = 0.023$ (ns^{-1}), was introduced into the theoretical equations. The value of D_p was evaluated from the equation of $D_p = RT/6V\eta$, where V is molar volume of SSI and η viscosity of solvent.

The quantum yield of the Trp 86 fluorescence is 0.014 at pH 7.0 and 0.049 at pH 2.8, while that of free tryptophan is 0.20. It drastically increases at pH below 3. Effective quencher for the fluorescence is considered to be the SS bond, since the reduction of it with $NaBH_4$ leads the fluorescence enhancement. Although the mechanism of quenching by SS the bond is not very clear at present, a weak charge transfer from the excited indole ring to the SS bond (Mataga, 1981) or heavy atom effect by sulfur or both of them might be responsible for it. Fluorescence lifetime has been also measured with a nanosecond pulse fluorometry and analyzed with a two-exponential decay function. The lifetimes reported are 0.4 ns and 5.5 ns (Hiromi and Miwa, 1985). The three-dimensional structure of SSI (Mitsui et al., 1979) is shown in Fig. 2. The extent of exposure of the indole ring to solvent molecules in crystal was calculated to be 12%. Fluorescence quenching experiments with trichloroethanol revealed that the extent of Trp exposure in the native state relative to that in the denatured state is 22% (Komiyama and Miwa, 1980).

In the present work, modes of motion of the indole ring of Trp 86 in SSI were examined on the basis of its time-resolved fluorescence. Analyses were first performed on the intensity decay and then on both intensity and anisotropy decays. From the intensity decay it was found that 1) D_{yy} and D_{xy} among the six diffusion coefficients were appreciable at 6 and 20°C and additionally D_{yz} at 40°C; 2) the introduction of potential energy in the analysis improved the fitting of the calculated decay curves with the observed curves; 3) k_q^0 and the diffusion coefficients increased and height of the potential energy, p, decreased, as temperature was raised. These results may be reasonable, since k_q^0 normally increases at higher temperatures, and the internal motion of the indole ring should become faster and easier in the protein as the temperature is raised. It should be noted that only the diffusion coefficients related to the y axis seem to be important and give definite values. This suggests that the internal motion of the indole ring is wobbling around the y axis (Kinoshita et al., 1977; Lipari and Szabo, 1982). Both decays of the intensity and anisotropy were also satisfactorily simulated with common parameters. Conclusions on the nature of the motion of the indole ring derived by the analysis of the intensity decay were not altered by this analysis, although the absolute values of the diffusion coefficients were modified. The averaged angle, δ_a, was obtained to be 94°. The angle between the z axis and transition moment of the L_a state was reported to be ~50° and that between the z axis and transition moment of the L_b state to be ~140° (Yamamoto and Tanaka, 1972). The obtained value of δ_a suggests that both L_a and L_b states are evenly excited at the excitation wavelength (Valeur and Weber, 1977).

The internal motion of Trp 86 has been also investigated by using a deuterium-labeled SSI with NMR spectroscopy (Akasaka et al., 1988). In this work, hydrogen atoms of the indole ring at 5 and 1 positions of Trp 86 were selectively labeled with deuterium. It was found that Trp 86 displays two kinds of NMR signals, a broad band with an effective correlation time of 14 ns and a narrow band with that of 0.13 ns at 25°C. It was concluded that in the solution of free SSI, "tight" and "loose" forms coexist in an equilibrium and the rate of transformation between the two conformers should be $<80\ s^{-1}$.

The following are, so far, considered to be the origin of the nonexponential decay in the fluorescence intensity observed in a protein containing the single tryptophan: 1) a hetero-

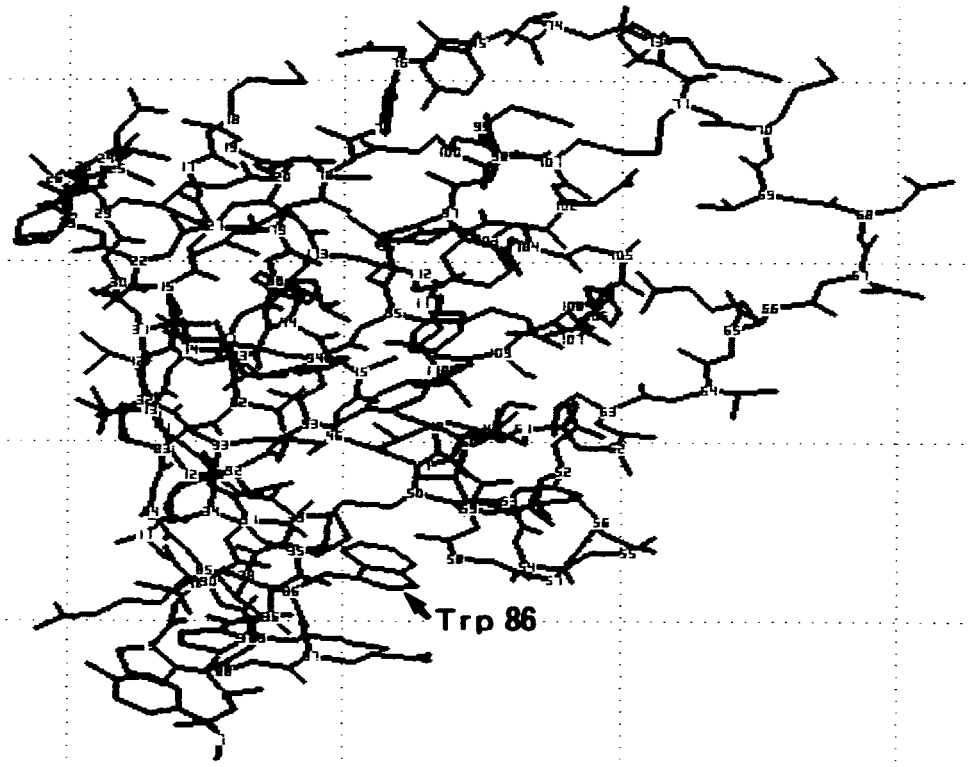

FIGURE 2 Three-dimensional structure of SSI. SSI is a dimer consisting of identical subunits. This illustrates one of the subunits. Trp 86 is in the hydrophobic environment and is covered mainly by the side chain of Gln 87 (Mitsui et al., 1979).

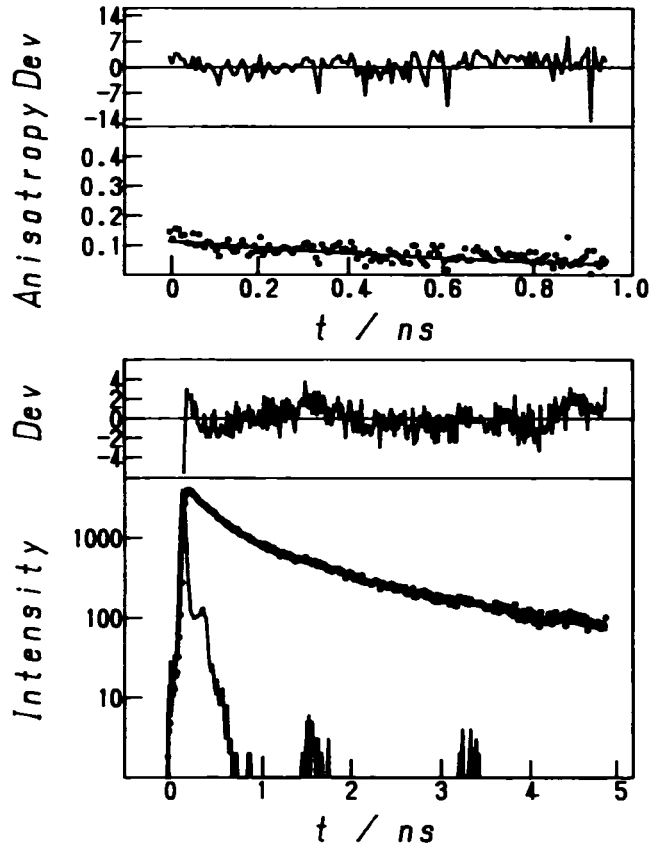

FIGURE 3 Time-resolved fluorescence of Trp 86 of SSI. Observed anisotropies and intensities are shown with closed circles. The both decays were simultaneously simulated with common parameters. Calculated anisotropies and intensities at the best-fit parameters are indicated with solid curves. Deviations of the intensities were obtained with Eq. 3. Deviations of the anisotropies were obtained with the equation

$$Dev(t_i) = \{A_o(t_i) - A_c(t_i)\}/\sqrt{nA_o(t_i)},$$

where $A_o(t_i)$ and $A_c(t_i)$ are the observed and calculated anisotropies at t_i. The both values of the observed and the calculated anisotropies were multiplied by 1000 to obtain values of χ^2_A comparable with those of χ^2_I. Obtained parameters are listed at the last line of Table 4. SSI was dissolved into 0.1 M phosphate buffer at pH 7.0 (~0.1 of absorbance at 280 nm). The temperature was 20°C.

geneous distribution in the orientation of the indole ring in the proteins with a few discrete lifetimes (Szabo et al., 1983) or continuous lifetimes (Alcala et al., 1987a,b); 2) a solvent relaxation of the surroundings of tryptophan; 3) an internal motion of the tryptophan in the protein (Tanaka and Mataga, 1982, 1987, 1992; Tanaka et al., 1987). Recently, many workers have attempted to interpret the time-resolved intensity (Henry and Hochstrasser, 1987) and anisotropy (Henry and Hochstrasser, 1987; Ichiye and Karplus, 1983; Lin et al., 1988; MacKerell et al., 1989; Axelsen et al., 1988; Axelsen and Prendergast, 1989) in terms of the method of molecular dynamics. However, it has been so far difficult to simulate quantitatively the both decays at once. It is important to point out that both observed decays of fluorescence of Trp 86 were able to be reproduced with our theoretical expressions derived for the model system stated above. The present results support the mechanism that the nonexponentiality in the intensity decays originates from the internal motion of tryptophan in proteins. In general, the quenching rate should be dependent not only on the mutual orientation but also on the distance between the quencher and the indole, whatever the quenching mechanism is. Although in the present model the dependence of the quenching rate on the distance was not taken into account, the above results suggest that the mutual orientation between the fluorescer and the quencher group is the most important factor for the quenching of tryptophan fluorescence in this protein.

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