Hydrogen-Exchange Kinetics of the Indole NH Proton of the Buried Tryptophan in the Constant Fragment of the Immunoglobulin Light Chain

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ABSTRACT: The constant fragment of the immunoglobulin light chain (type λ) has two tryptophyl residues at positions 150 and 187. Trp-150 is buried in the interior, and Trp-187 lies on the surface of the molecule. The hydrogen–deuterium exchange kinetics of the indole NH proton of Trp-150 were studied at various pH values at 25 °C by ^{1}H nuclear magnetic resonance. Exchange rates were approximately first order in hydroxyl ion dependence above pH 8, were relatively independent of pH between pH 7 and 8, and decreased below pH 7. On the assumption that the exchange above pH 8 proceeds through local fluctuations of the protein molecule, the exchange rates between pH 7 and 8 through global unfolding were estimated. The exchange rate constant within this pH range at 25 °C thus estimated was consistent with that of the global unfolding of the constant fragment under the same conditions as those reported previously [Kikuchi, H., Goto, Y., & Hamaguchi, K. (1986) *Biochemistry 25*, 2009–2013]. The activation energy for the exchange process at pH 7.8 was the same as that for the unfolding process by 2 M guanidine hydrochloride. The exchange rates of backbone NH protons were almost the same as that of the indole NH proton of Trp-150 at pH 7.1. These observations also indicated that the exchange between pH 7 and 8 occurs through global unfolding of the protein molecule and is rate-limited by the unfolding. At around pH 9, on the other hand, the activation energy for the exchange process of the indole NH proton of Trp-150 was smaller than that for the unfolding process, and the exchange rates differed according to the different signals of backbone NH protons. These findings together with the pH dependence of the rate constant indicated that exchange due to local fluctuations is predominant above pH 8.

The constant domain of the immunoglobulin light chain consists of two β-sheets and has only one intrachain disulfide bond buried in the hydrophobic interior between the two sheets (Beale & Feinstein, 1976; Amzel & Poljak, 1979). Previously, Kikuchi et al. (1986) studied the kinetics of reduction with dithiothreitol of the disulfide bond in various concentrations of Gdn-HCl[1] at pH 8.0 and 25 °C. It was found that the disulfide bond is reduced even in the absence of Gdn-HCl. Comparison of the results of the reduction kinetics with those of the unfolding and refolding kinetics by Gdn-HCl of the C_L fragment (Goto & Hamaguchi, 1982) showed that the reduction of the disulfide bond proceeds through a species with a conformation very similar to that of the fully unfolded one and that the C_L fragment undergoes global transition even in water.

Although hydrogen isotope exchange has been used extensively as one of the methods for studying the fluctuations of the protein molecule, it is not clear whether the exchange proceeds through global unfolding or local fluctuations of the protein molecule under physiological conditions (Woodward et al., 1982; Wedin et al., 1982; Delepierre et al., 1983; Wagner, 1983; Akasaka et al., 1985; Roder et al., 1985). The C_L fragment has two tryptophyl residues at positions 150 and 187.[2] Trp-150 is buried in the interior of the molecule and is located very close to the intrachain disulfide bond, while Trp-187 lies on the surface of the protein molecule. In the present study, we examined the hydrogen–deuterium exchange kinetics of the indole NH proton of Trp-150 at various pH values at 25 °C, which is much lower than the transition temperature (60 °C) of the thermal unfolding of the C_L fragment (Ashikari et al., 1985; Goto & Hamaguchi, 1987). The exchange rates between pH 7 and 8 were consistent with the reduction rate of the disulfide bond reported previously (Kikuchi et al., 1986). The activation energy for the exchange at pH 7.8 was the same as that for the global unfolding by Gdn-HCl. These findings demonstrate that the exchange of the indole NH proton of the buried Trp-150 of the C_L fragment between pH 7 and 8 proceeds through a fully unfolded state.

Materials and Methods

Materials. The C_L fragment of Bence Jones protein Nag (type λ) was obtained by digestion with papain as previously described (Goto & Hamaguchi, 1979). ^2H_2O (99.85%) was a product of the Commissariat a L'énergie Atomique (CEA), France. Gdn-HCl (specially purified grade) was obtained from Nakarai Chemicals. Other reagents were from Nakarai Chemicals and Wako Pure Chemicals and were used without further purification.

NMR Measurements. ^{1}H NMR measurements were performed on a JEOL GX-500s NMR spectrometer operating at 500 MHz. All chemical shifts are given in parts per million (ppm) from external 4,4-dimethyl-4-silapentane-1-sulfonate (5% ^2H_2O). The buffers used for the exchange kinetics were 50 mM phosphate buffers at various p^{2}H values containing

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[1] Abbreviations: CIDNP, chemically induced dynamic nuclear polarization; Gdn-HCl, guanidine hydrochloride; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

[2] The numbering system used in the present paper is based on the type λ light chain (Kol) (Marquart et al., 1980).

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0.15 M KCl prepared with ^2H_2O. Ten milligrams of the C_L fragment was dissolved in 0.5 mL of a buffer at a given p^{2}H. Just before the proton-exchange measurement, the best resolution was adjusted by using a protein solution under the same conditions as those used for the exchange experiment. Immediately after the protein had been dissolved in a buffer at a given p^{2}H and the temperature had become constant, the first accumulation was started. A 90° sampling pulse with a total repetition of 1.5 s was applied. For each measurement, 600 transients were collected. Because the signal of the indole NH proton of Trp-150 was well resolved (see Figure 2), the exchange rate constant was determined accurately by plotting the peak height against the mid-time of the accumulation period. The pH of the sample was measured after the experiments. A Radiometer PHM84 meter equipped with a long glass combination electrode 3 mm in diameter was used. The pH values were uncorrected meter reading of ^2H_2O solutions made by using an electrode standardized with H_2O buffer. For NMR measurements of the protein in H_2O, 6 mg of the C_L fragment was dissolved in 0.5 mL of 50 mM NH_4HCO_3/H_2O containing 10% ^2H_2O at pH 8.1, and the 1 – 1 pulse sequence (Hore, 1983) was applied with a total repetition of 2.2 s; 1000 scans were collected. NOE measurements were carried out under the same conditions.

Photo CIDNP spectra were taken on the same NMR spectrometer using a specially designed probe with a 3-mm quartz rod to introduce the laser light. 3-*N*-(Carboxymethyl)lumiflavin was added to the protein solution, and the sample tube was irradiated in the probe for 100 ms by the total light from an NEC GLC-3300 argon ion laser prior to data acquisition.

Unfolding Kinetic Measurements. The buffers used for the unfolding kinetic measurements were 50 mM phosphate buffers at various pH values containing 0.15 M KCl at final concentration. Unfolding reactions were measured on a Union Giken stopped-flow spectrophotometer, Model RA-401, using fluorescence detection. The details of the apparatus have been described previously (Goto & Hamaguchi, 1982). The unfolding was initiated by mixing a solution of the C_L fragment with Gdn-HCl solution in a 1:1 ratio. The excitation wavelength was set at 280 nm, and fluorescence at wavelengths longer than 330 nm was observed. The reservoir and the observation cell were thermostated with circulating water. The final protein concentration was about 0.02 mg/mL.

The temperature dependence of the unfolding kinetics was measured in 1.5 and 2 M Gdn-HCl and 5 M urea at pH 7.8 and in 2 M Gdn-HCl at pH 9.2. The unfolding kinetics were expressed in terms of almost one phase in the temperature range measured. All the kinetic data were analyzed as described previously (Goto & Hamaguchi, 1982). pH was measured with a Radiometer PHM26c meter at 25 °C.

RESULTS

Assignment of Indole NH Proton NMR of the Tryptophyl Residues of the C_L Fragment. Figure 1a shows the ^{1}H NMR spectrum of the low-field region of the C_L fragment in 50 mM NH_4HCO_3/H_2O containing 10% ^2H_2O at pH 8.1. The indole NH signal of tryptophan in proteins appears at around 10 ppm (Cassels et al., 1978). Parts b and c of Figure 1 show the NOE difference spectra on irradiation at peaks of 10.08 and 10.42 ppm, respectively, in spectrum a. Irradiation at the 10.08 ppm peak showed the NOE on the peaks at 6.9 and 7.1 ppm, which are assigned to the C(2)–H and C(7)–H protons of the indole. Irradiation at the 10.42 ppm peak revealed the NOE on the peaks at 7.1 and 7.6 ppm. These features of the NOE are characteristic of the tryptophan residue. No other signals in the region of 9.5–11.5 ppm showed such NOE. As shown in Figure 2, when the C_L fragment was dissolved in ^2H_2O buffer at p^{2}H 8.2, the signal at 10.42 ppm disappeared immediately, while the height of the signal at 10.08 ppm decreased slowly. On the basis of the X-ray crystallographic data for the immunoglobulin light chain (Beale & Feinstein, 1976; Amzel & Poljak, 1979) indicating that Trp-150 is located in the interior of the molecule and Trp-187 lies on the surface of the molecule, we were able to assign the signal at 10.08 ppm to the indole NH proton of Trp-150 and that at 10.42 ppm to the indole NH proton of Trp-187. In order to distinguish the surface tryptophan from the internal tryptophan, the photo-CIDNP spectra (Kaptain, 1982) were measured. The photo-CIDNP difference spectrum of the C_L fragment (not shown) showed a signal at 7.1 ppm, which corresponds exactly with the signal derived from the indole C(2)–H proton of Trp-187 in the NOE difference spectrum (Figure 1c). This finding also supports the assignment of the signal at 10.42 ppm to the indole NH proton of Trp-187, which may be exposed to the solvent.

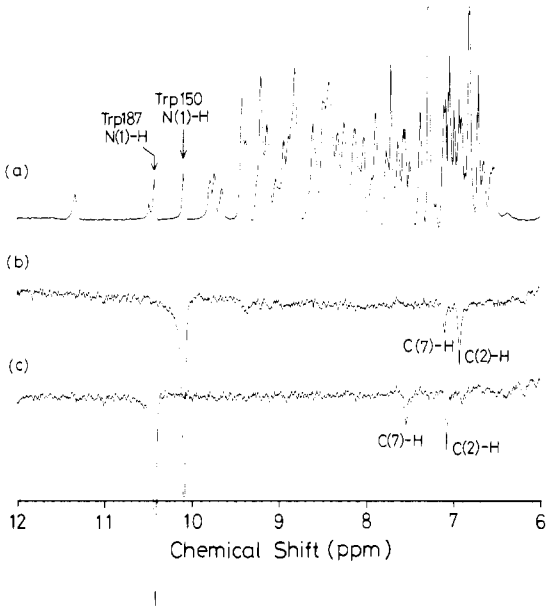

FIGURE 1: 500-MHz ^{1}H NMR spectrum of the C_L fragment in H_2O and its NOE difference spectra. (a) C_L fragment (6 mg in 0.5 mL of 50 mM NH_4HCO_3/10% ^2H_2O) at pH 8.1 and 25 °C. The 1 – 1 pulse sequence was applied, and 1000 transients were acquired. The NOE difference spectra of irradiation of peaks at 10.08 and 10.42 ppm in spectrum a are presented in (b) and (c), respectively. An attenutation of 280 for irradiation was applied for 600 ms, and 2000 transients were acquired. The difference spectrum was obtained by subtraction of free induction decays. C(2)–H and C(7)–H represent protons at the indole C(2) and C(7) positions, respectively. The peaks in (b) were derived from Trp-150 and those in (c) from Trp-187 (see text).

A signal at 11.33 ppm was observed for the NMR spectrum of the C_L fragment (Figure 1a). This signal became broader below pH 7.5 at 25 °C, probably due to protonation, and also became broader at 40 °C and pH 8.1, owing to an increase in the exchange rate with solvent (not shown). Since the pK_a value of His-190 has been determined to be 7.5 (Arata & Shimizu, 1979; Ashikari et al., 1985), the signal at 11.33 ppm can be assigned to the imidazole NH proton of His-190. A small signal observed at 10.48 ppm was of unknown origin.

Hydrogen–Deuterium Exchange Kinetics of the Indole NH Proton of Trp-150. As shown in Figure 2, the intensity of the signal at 10.08 ppm, which had been assigned to the indole NH proton of Trp-150, decreased with time when the C_L fragment was exposed to ^2H_2O. The exchange kinetics fol-

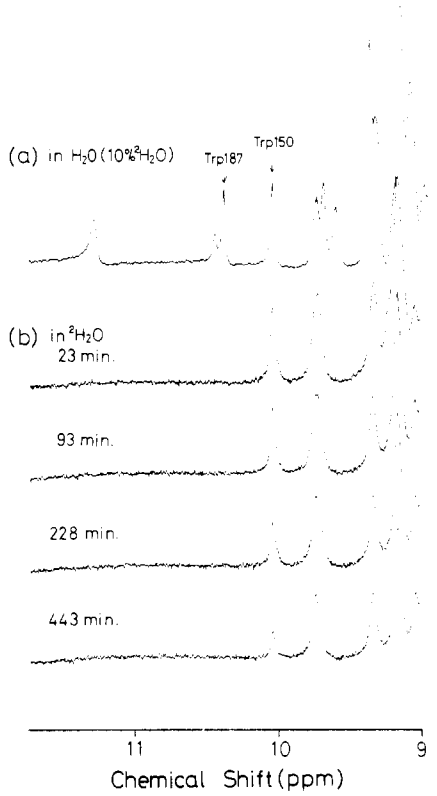

FIGURE 2: Proton exchange profile of the C_L fragment at p^2H 8.2 and 25 °C. (a) C_L fragment (6 mg in 0.5 mL of 50 mM NH_4HCO_3/10% ^2H_2O) at pH 8.1 and 25 °C. Spectral conditions are the same as in Figure 1a. (b) C_L fragment (10 mg in 0.5 mL of 50 mM phosphate buffer containing 0.15 M KCl/^2H_2O) at p^2H 8.2 and 25 °C. The free induction decay was recorded with 16 000 data points and a spectral width of ±3500 Hz. A sampling pulse of 90° was applied with total repetition of 1.5 s. Typically, 600 transients were acquired, and a line broadening of 1 Hz was applied prior to Fourier transformation.

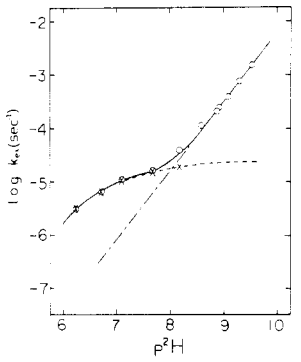

FIGURE 3: Logarithmic plot of the exchange rate constants for the Trp-150 indole NH proton against p^2H at 25 °C (○). The dashed-and-dotted line represents the contribution of the local fluctuations to the exchange, and the crosses and broken line represent that of the global fluctuations to the exchange. See text for details.

lowed a first-order process within the pH range studied. In Figure 3, the exchange rate constants (k_{ex}) are plotted against p^2H. The exchange rate constant was relatively constant between p^2H 7 and 8, decreased below p^2H 7, and increased above p^2H 8. Above p^2H 9.5, the exchange rate was too fast to be measured. A similar pH profile was obtained for bovine pancreatic trypsin inhibitor at 55–70 °C by Roder et al. (1985) and for *Streptomyces* subtilisin inhibitor at around 55 °C by Akasaka et al. (1985).

Parts a and b of Figure 4 show the exchange profiles for the slowest exchanging backbone NH protons as well as the indole NH proton of Trp-150 of the C_L fragment at p^2H 7.1

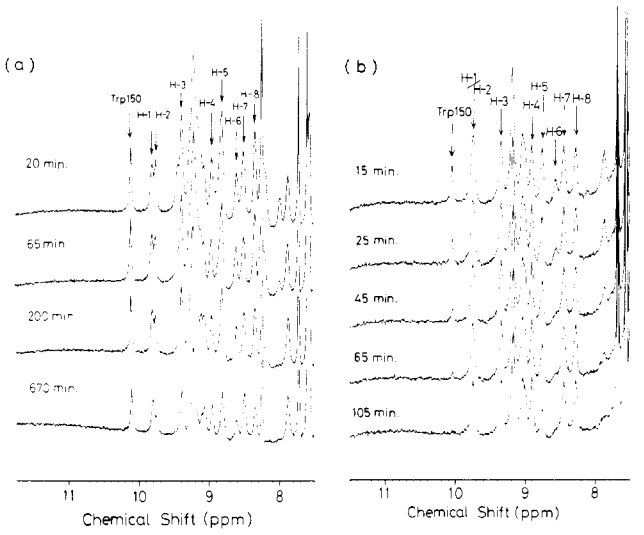

FIGURE 4: Exchange profile of the Trp-150 indole NH proton and other backbone amide protons [H(1)–H(8)] in the C_L fragment at p^2H 7.1 (a) and 9.3 (b) at 25 °C. Spectral conditions were the same as those described in Figure 2.

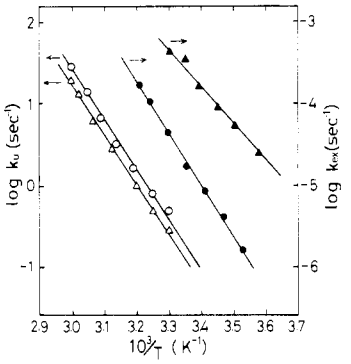

FIGURE 5: Arrhenius plots of the rate constants for the exchange (k_{ex}) of the Trp-150 indole NH proton at p^2H 7.7 (●) and 9.1 (▲) and for the global unfolding (k_u) in 2 M Gdn-HCl at pH 7.8 (○) and 9.2 (△). The experiments were performed in 50 mM phosphate buffer containing 0.15 M KCl.

Table I: Rate Constants of the Exchange of Trp-150 Indole NH Proton and Other Backbone Amide Protons in the C_L Fragment at p^2H 7.1 and 9.3 at 25 °C

	$10^5 k_{ex}$ (s^{-1})	
	p^2H 7.1	p^2H 9.3
Trp-150 indole NH	1.2	57.7
H-1	1.1	5.8[a]
H-2	2.6	5.8[a]
H-3	2.8	2.4
H-4	1.1	1.7
H-5	1.5	13.1
H-6	2.3	43.0
H-7	0.9	1.9
H-8	2.6	1.9

[a] The value for H-1/H-2 in Figure 4b.

and 9.3, respectively. The exchange rate constants for these protons are summarized in Table I. It can be seen that at p^2H 7.1 the backbone NH protons were exchanged with deuterium at rates comparable to that for the indole NH proton of Trp-150 but that at p^2H 9.3 the exchange rates differed according to the different signals.

The temperature dependence of the exchange rate constant for the indole NH proton of Trp-150 was determined at p^2H 7.8 and 9.1 (Figure 5). The activation energy for the exchange process was found to be 27.7 ± 1.1 kcal/mol at p^2H 7.8 and 21.5 ± 1.5 kcal/mol at p^2H 9.1.

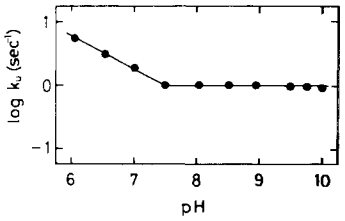

FIGURE 6: Logarithmic plot of the unfolding rate constant (k_u) in 2.5 M Gdn-HCl against pH at 25 °C. The experiments were performed by stopped-flow fluorescence measurements in 50 mM phosphate buffer containing 0.15 M KCl. See text for details.

Unfolding Kinetics of the C_L Fragment. In order to understand the mechanism of the exchange kinetics of the indole NH proton of Trp-150, we examined the kinetics of the global unfolding of the C_L fragment by Gdn-HCl. The rate constants of the unfolding by 2.5 M Gdn-HCl were the same between pH 7.5 and 10 and were increased below pH 7.5 (Figure 6).

The temperature dependence of the rate constant of the unfolding of the C_L fragment by 2 M Gdn-HCl was determined at pH 7.8 and 9.2 (Figure 5). The activation energy for the unfolding process was found to be 27.5 ± 1.2 kcal/mol at pH 7.8 and 28.2 ± 0.9 kcal/mol at pH 9.2. The activation energies for the unfolding by 5 M urea and by 1.5 M Gdn-HCl at pH 7.8 were also found to be 28.0 ± 1.0 and 28.7 ± 1.5 kcal/mol, respectively. Thus the activation energy for the unfolding was independent of the denaturing conditions used. While the activation energy for the exchange process of the Trp-150 indole NH proton at pH 7.8 was the same as that for the unfolding process, the former at pH 9.1 was smaller than the latter.

DISCUSSION

Since Trp-150 is buried in the interior of the C_L fragment molecule, its indole NH proton can be used to interpret the mechanism of hydrogen–deuterium exchange of the protein molecule. The indole NH proton signal of Trp-150 was well resolved in the NMR spectrum (see Figures 1 and 2), and the hydrogen–deuterium exchange data could be obtained accurately in the present experiments.

In order for the indole NH proton buried in the interior of the protein molecule to be exchanged with deuterium, the C_L fragment molecule must be opened and the NH proton must be accessible to solvent. Therefore, the exchange of the indole NH proton of Trp-150 with deuterium may be described on the basis of the mechanism

$$N(H) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O(H) \xrightarrow{k_3} O(D) \underset{k_1}{\overset{k_2}{\rightleftharpoons}} N(D)$$

In this mechanism, N is the native C_L fragment in which the Trp-150 indole NH proton is buried in the interior of the molecule and thus not accessible to solvent. O is any conformation of the C_L fragment in which the indole NH proton is accessible to solvent. k_1, k_2, and k_3 are the rate constants for the respective processes. We assumed a two-process model for exchange from the native protein: exchange due to local fluctuations and exchange from the unfolded state after major cooperative unfolding (Woodward et al., 1982).

In general k_2 is much larger than k_1, and the following equation holds for most proteins:

$$k_{ex} = \frac{k_1}{k_2 + k_3} k_3 \quad (1)$$

When $k_3 \gg k_2$

$$k_{ex} = k_1 \quad (2)$$

When $k_3 \ll k_2$

$$k_{ex} = (k_1/k_2)k_3 = Kk_3 \quad (3)$$

where K is the equilibrium constant between N and O (Hvidt & Nielsen, 1966; Woodward et al., 1982; Tüchsen & Woodward et al., 1987).

As shown in Figure 6, the rate constant of unfolding of the C_L fragment by 2.5 M Gdn-HCl was independent of pH between pH 7.5 and 10. This suggests that the rate constant of global unfolding of the C_L fragment molecule in the absence of denaturant is also independent of pH within this pH range. Previously, Goto and Hamaguchi (1979) and Ashikari et al. (1985) determined the free energy changes of unfolding of the C_L fragment in water by analyzing the unfolding equilibrium data with Gdn-HCl and found that the free energy change of unfolding in water is independent of pH within this pH range. Ashikari et al. (1985) also found that the ellipticity at 218 nm is independent of pH between pH 5 and 11.6. These observations show that the increase in the exchange rate above pH 8.5 (Figure 3) is due neither to an increase in the fraction of the unfolded C_L fragment molecule nor to the increase in the unfolding rate. The slope of the increase in the exchange rate above pH 8.5 was 1.1, which is very close to the slope found for the base catalysis of the chemical exchange step of the indole NH protons of derivatives of L-tryptophan (Nakanishi et al., 1978; Waelder & Redfield, 1977).

On the basis of the these findings, we assumed that the increase in the exchange rate above pH 8.5 is due to the increase with pH in the chemical exchange rate of the indole NH proton of Trp-150 exposed by local fluctuations and that the exchange is expressed by eq 3. When the contribution of this exchange through local fluctuations is subtracted from the observed exchange rates, the dotted line shown in Figure 3 is obtained. As can be seen, the exchange rate levels off above pH 7, and the rate constant at this level was found to be 2×10^{-5} s^{-1}.

Previously, Kikuchi et al. (1986) studied the kinetics of reduction with dithiothreitol of the disulfide bond of the C_L fragment at various concentrations of Gdn-HCl at pH 8.0 and 25 °C and showed that the reduction of the disulfide bond in the absence of Gdn-HCl proceeds through a species with a conformation very similar to that of the fully unfolded one. The rate constant for the unfolding of the native C_L fragment in the absence of denaturant was determined to be 7×10^{-5} s^{-1}, which is comparable to the exchange rate constant (2×10^{-5} s^{-1}) described above. This indicates that the exchange of the indole NH proton of the buried Trp-150 in the pH range between 7 and 8 proceeds through global unfolding of the protein molecule even at 25 °C and that the exchange is expressed by eq 2.

The following observations strongly support this view. (1) The activation energy for the exchange process of the indole NH proton of Trp-150 at pH 7.8 is the same as that obtained for the unfolding process of the C_L fragment by 2 M Gdn-HCl at the same pH (Figure 5). We found that the activation energies for the unfolding process of the C_L fragment by 1.5 and 2 M Gdn-HCl at pH 7.8, by 2 M Gdn-HCl at pH 9.2, and by 5 M urea at pH 7.8 were all the same. Segawa and Sugihara (1984) reported that the activation energy of the unfolding of lysozyme is independent of the type and concentration of denaturant used. Thus, the activation energy for the exchange process and that for the unfolding process may be compared directly, though the conditions under which these two values were determined are different. The finding that the activation energies for the exchange process and unfolding process at pH 7.8 are the same indicates that the

exchange process of the indole NH proton of Trp-150 at this pH is rate-limited by the global unfolding of the C_L fragment molecule. (2) As shown in Figure 4 and Table I, the exchange rate constants of the slowest exchanging backbone NH protons at p^2H 7.1 are comparable to that for the indole NH proton of Trp-150. At p^2H 9.3, on the other hand, different NH signals have different rate constants. These findings indicate that at p^2H 7.1 the exchange of the indole NH proton of Trp-150 proceeds predominantly through global unfolding and is expressed by eq 2, while at p^2H 9.3 the exchange proceeds predominantly through local fluctuations and is expressed by eq 3.

The activation energy for the exchange process of the indole NH proton of Trp-150 at pH 9.1 (21.5 kcal/mol) is smaller than that for the unfolding process of the protein molecule (28.2 kcal/mol). The activation energy for the base-catalyzed exchange rate of the indole NH proton of L-tryptophan has been determined to be 15.9 kcal/mol by Nakanishi et al. (1978). The value of 5.6 kcal/mol obtained by subtracting this value from 21.5 kcal/mol may be regarded as the activation energy for the local fluctuations.

It is interesting to note that the hydrogen exchange through global unfolding, which was observed for stable *Streptomyces* subtilisin inhibitor (Akasaka et al., 1985) and bovine pancreatic trypsin inhibitor (Roder et al., 1985) only at 60–80 °C, was observed for the C_L fragment at 25 °C, far below the transition temperature (60 °C) of the thermal unfolding of the protein (Ashikari et al., 1985; Goto & Hamaguchi, 1987). The V_L domain of immunoglobulin light chain, which has a similar immunoglobulin fold and involves antigen binding, may also undergo similar fluctuations under physiological conditions. Such global fluctuations may relate to the biological functions of the antibody.

Registry No. H, 12408-02-5; L-Trp, 73-22-3.

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