pH Dependence of Individual Tryptophan N-1 Hydrogen Exchange Rates in Lysozyme and Its Chemically Modified Derivatives

Toshiya Endo*

Department of Chemistry, College of Technology, Gunma University, Kiryu 376, Japan

Tadashi Ueda, Hidenori Yamada, and Taiji Imoto

Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka 812, Japan

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ABSTRACT: Nuclear magnetic resonance analyses have been made of the individual hydrogen–deuterium exchange rates of tryptophan indole N-1 hydrogens in native lysozyme and its chemically modified derivatives including lysozyme with an ester cross-linkage between Glu-35 and Trp-108, lysozyme with an internal amide cross-linking between the ϵ-amino group of Lys-13 and the α-carboxyl group of Leu-129, and lysozyme with the β-aspartyl sequence at Asp-101. The pH dependence curves of the exchange rates for Trp-63 and Trp-108 are different from those expected for tryptophan. The pH dependence curve for Trp-108 exchange exhibits the effects from molecular aggregation at pH above 5 and from a transition between the two conformational fluctuations at around pH 4. The exchange rates for tryptophan residues in native lysozyme and modified derivatives are not correlated with the thermodynamic or kinetic parameters in protein denaturation, suggesting that the fluctuations responsible for the exchange are not global ones. The exchange rates for tryptophan residues remote from the modification site are perturbed. Such tryptophan residues are found to be involved in a small but distinct conformational change due to the modification. Therefore, the perturbations of the N-1 hydrogen exchange rates are related to the minor change in local conformation or in conformational strain induced by the chemical modification.

It is generally accepted that protein molecules are inherently flexible and contain various levels of internal mobility within their structures (Gurd & Rothgeb, 1979; Frauenfelder et al., 1979; Karplus & McCammon, 1981). Such flexible and mobile nature of proteins should be important for their normal function and the stability against changes in surrounding conditions and probably determine the succeptibility to proteolysis and accordingly the life span of some proteins. The hydrogen exchange behavior of the interior labile hydrogen atoms shielded from the solvent has frequently been monitored for characterizing a dynamic fluctuation of the protein molecule, which brings the exchange sites into contact with the solvent molecules. Three models of fluctuations in the protein structure have been put forward to explain the exchange of interior hydrogens: global cooperative unfolding that allows the exchange through the denatured state of the protein, local unfolding that exposes the exchanging site to the bulk solvent, and a small-amplitude fluctuation that leads the solvent molecules to penetrate into the exchanging site in the interior of the protein (Woodward et al., 1982; Englander & Kallenbach, 1984).

Wedin et al. (1982) have reported the temperature dependence of the tryptophan indole N-1 hydrogen exchange in lysozyme at pH 3.8 as measured by proton nuclear magnetic resonance (NMR)[1] spectroscopy. Arrhenius plots of the exchange rates have shown that there are two distinct processes: the low activation energy process with $\Delta H^{\ddagger}$ of 13–40 kcal/mol below 57 °C and the high activation energy process with $\Delta H^{\ddagger}$ of 92–120 kcal/mol above 72 °C. The high activation energy process at pH and temperature near the thermal denaturation zone was found to be related to the process of cooperative unfolding. On the other hand, the mechanism of the low activation energy process still remains uncertain, although it is the low activation energy process that reflects the dynamic properties of the folded state of the protein under physiological conditions.

In this study, hydrogen exchange behaviors have been analyzed for tryptophan N-1 hydrogens in native lysozyme and modified derivatives at various pHs and temperatures. The experimental conditions correspond to the regime of the low activation energy process of the exchange. The lysozyme derivatives used are 108–35 lysozyme with an ester cross-linkage between Glu-35 and Trp-108 (Imoto & Rupley, 1973), (13–129)CL lysozyme with an amide cross-linking between Lys-13 (ϵ-NH_3^+) and Leu-129 (α-COO^-) (Yamada et al., 1983, 1985), and 101-β lysozyme in which the peptide bond inversion occurs with the β-aspartyl sequence formed at Asp-101 (Yamada et al., 1985). It will be shown that the fluctuations responsible for the tryptophan N-1 hydrogen exchange are not global ones although the exchange rates are affected by the chemical modification at a site remote from the tryptophan residues.

Materials and Methods

Five times recrystallized hen egg white lysozyme was a gift from Eisai Co. (Tokyo, Japan). The 108–35 lysozyme, (13–

* Address correspondence to this author at Biocenter, University of Basel, CH-4056 Basel, Switzerland.

[1] Abbreviations: NMR, nuclear magnetic resonance; 108–35 lysozyme, lysozyme derivative with an ester bond formed between the side-chain carboxylate of Glu-35 and the enol form of oxindolealanine-108; (13–129)CL lysozyme, lysozyme derivative with an amide bond formed between the ϵ-amino group of Lys-13 and the α-carboxyl group of Leu-129; 101-β lysozyme, lysozyme derivative with the β-aspartyl sequence at Asp-101; H–D exchange, hydrogen–deuterium exchange.

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129)CL lysozyme, and 101-β lysozyme were prepared as described previously (Imoto & Rupley, 1973; Yamada et al., 1983, 1985).

The 270-MHz proton NMR spectra were recorded on a Bruker WH-270 spectrometer. For kinetic measurements, 20 mg of lyophilized lysozyme or its derivative was dissolved in ^2H_2O (to a lysozyme concentration of 3.5 mM) with 0.1 M NaCl. pH values were measured in ^2H_2O solutions by a Radiometer PHM-26 pH meter equipped with a 180-mm Ingold combination electrode and were reported without isotope correction because, in the acidic range, the isotope effects on the glass electrode and the ionization constants for the ionizable groups nearly cancel (Bundi & Wüthrich, 1979). The exchange was carried out in the NMR spectrometer probe or in a constant-temperature water bath. At appropriate time intervals, NMR spectra were recorded, and the decrease in the peak height of the indole N-1 proton resonance was followed. A semilogarithmic plot of calibrated peak height against time gave a straight line, and an apparent first-order rate constant k_{obsd} was obtained. The pH dependence of proton chemical shifts was measured in H_2O (10% ^2H_2O for internal lock for the spectrometer) solution with 0.1 M NaCl, and the shifts were quoted in ppm from 4,4-dimethyl-4-silapentane-1-sulfonate (acetone was used as an internal reference).

Results

pH Dependence of Proton Chemical Shifts in Native Lysozyme and Modified Lysozyme. We first followed the pH dependence of chemical shifts of Trp N-1 proton resonances and methyl proton resonances in native lysozyme and its derivatives (Figure 1). The comparison of the pH titration behavior allows us to examine the effect of the chemical modification on the lysozyme conformation.

At a first look, we find that the chemical shifts and pH titration profiles of the resonances in native lysozyme and modified lysozyme are very similar, and thus, the three types of chemical modifications do not appear to induce global conformational changes. However, a closer examination shows that the pH dependence of proton chemical shifts of specific resonances in modified lysozyme are not identical with the corresponding resonances in native lysozyme. Such spectral changes may be interpreted in terms of a direct through-space effect or indirect effect through conformational perturbation on observed protons by the chemical modification.

The effect of the modification of Trp-108 and Glu-35 on proton NMR resonances was previously reported (Blake et al., 1978). Although no change in conformation apart from those of Glu-35 and Trp-108 was observed in crystal structure analyses (Beddell et al., 1975), NMR spectral perturbation was also found for resonances from residues other than those at the modification site (Blake et al., 1978). Such a widespread effect by the modification was also observed in this study; the proton NMR resonances from Trp-123, Trp-111, Trp-28, Trp-108, Ile-98, Leu-17, and Met-105 exhibit the effect of the internal cross-linking between Trp-108 and Glu-35 (Figure 1B). When the pH is raised, the Trp-108 N-1 proton resonance in native lysozyme shifts 0.05 ppm upfield at about pH 3.5 and 0.25 ppm downfield at pH 6.0. On the other hand, the Trp-108 resonance in 108–35 lysozyme shifts slightly (0.04 ppm) at pH 3–5 and does not shift at pH above 5. The ionizable groups affecting the Trp-108 resonance in native lysozyme are probably Asp-52 (with a pK_a of 3.4) and Glu-35 (with a pK_a of 6.1), the latter of which does not titrate in 108–35 lysozyme (Kuramitsu et al., 1977). The Trp-111 resonance in native lysozyme exhibits a titration shift at pH 2.1–7.4, but in 108–35 lysozyme, it shifts only at pH 2–5 and does not shift at pH above 5. This indicates that the Trp-111 N-1 proton resonance reflects the ionization of two titratable groups, one of which is the Glu-35 carboxyl group.

In (13–129)CL lysozyme, the effect of the modification on the conformation is more limited; the N-1 proton resonances of Trp-123 and Trp-108 and the methyl proton resonance from Leu-17 show smaller chemical shift changes as compared with those in native lysozyme (Figure 1C). In 101-β lysozyme, chemical shift changes from the corresponding resonances in native lysozyme are observed for the resonances from Leu-17, Leu-56, Ile-98, Met-105, Trp-108, and Trp-111 (Figure 1D). The Trp-111 N-1 proton resonance in 101-β lysozyme hardly shifts at a pH between 2.1 and 7.4, whereas that in native lysozyme shifts 0.17 ppm. Therefore, it is likely that the other group affecting the chemical shift of the Trp-111 N-1 proton resonance in native lysozyme is Asp-101, which is at the modification site in 101-β lysozyme. The Trp-108 N-1 proton resonance in 101-β lysozyme as well as in native lysozyme exhibits a pH titration shift as large as about 0.2 ppm, which is due to the ionization of Glu-35 probably via a conformational change in this region. Since, in crystalline native lysozyme, Trp-111 and Glu-35 are not close to each other, a conformational change triggered by the ionization of Glu-35 in 101-β lysozyme likely influences the chemical shift of the Trp-108 N-1 proton resonance but does not affect that of the Trp-111 N-1 proton resonance.

Blake et al. (1981) have reported comparative proton NMR and X-ray diffration analyses on the effect of the modification at Trp-62 in lysozyme. In that case, although the chemical shifts of several proton resonances were perturbed by the modification, the shift amount was limited to rather small values, i.e., within 0.08 ppm. Comparison of the NMR results with those from the X-ray diffraction analyses has shown that these shifts are due to minor conformational changes such as small changes in the relative orientation of side chains or those below the resolution of X-ray data. In the present cases of the three lysozyme derivatives, most of the spectral perturbation is also very small, within 0.11 ppm, except for the Trp-108 and Trp-111 N-1 proton resonances in 108–35 lysozyme. Thus, the conformational changes induced by the three types of chemical modification are small and do not involve any significant alteration of the polypeptide chain folding.

Tryptophan Indole N-1 Hydrogen Exchange in Native Lysozyme and Modified Lysozyme. The hydrogen–deuterium (H–D) exchange of the indole N-1 hydrogen in tryptophan is both acid and base catalyzed as in the case of the exchange of peptide amide hydrogens (Waelder & Redfield, 1977; Nakanishi et al., 1978; Wedin et al., 1982). The observed rate constant k_{obsd} is written as

$$k_{obsd} = k_H[^2H_3O^+]^a + k_{OH}[OH^-]^b \quad (1)$$

The plot of log k_{obsd} against pH gives a V-shaped curve with a slope of $a = 1$ and $b = 1$ in acidic and basic pH limbs, respectively, and a pH_{min}, pH of minimum rate, of 4.5.

In this study, the pH dependence of the tryptophan indole N-1 hydrogen exchange was followed for Trp-63, Trp-108, Trp-111, and Trp-123 in native lysozyme at four different temperatures, 25, 35, 47, and 55 °C, as shown in Figure 2. Trp-111 and Trp-123 show V-shaped pH dependence curves of H–D exchange with a slope of $a = 0.7$–1.2 in the acidic limb and with a slope of $b = 0.8$–1.2 in the alkaline limb, and the pH_{min} is ca. 3.8–4.0 for Trp-111 and Trp-123.

For Trp-63 exchange, only a limited portion of the pH–rate curve is observed because of its fast rate of exchange. However, the pH dependence of the exchange rate is smaller in the

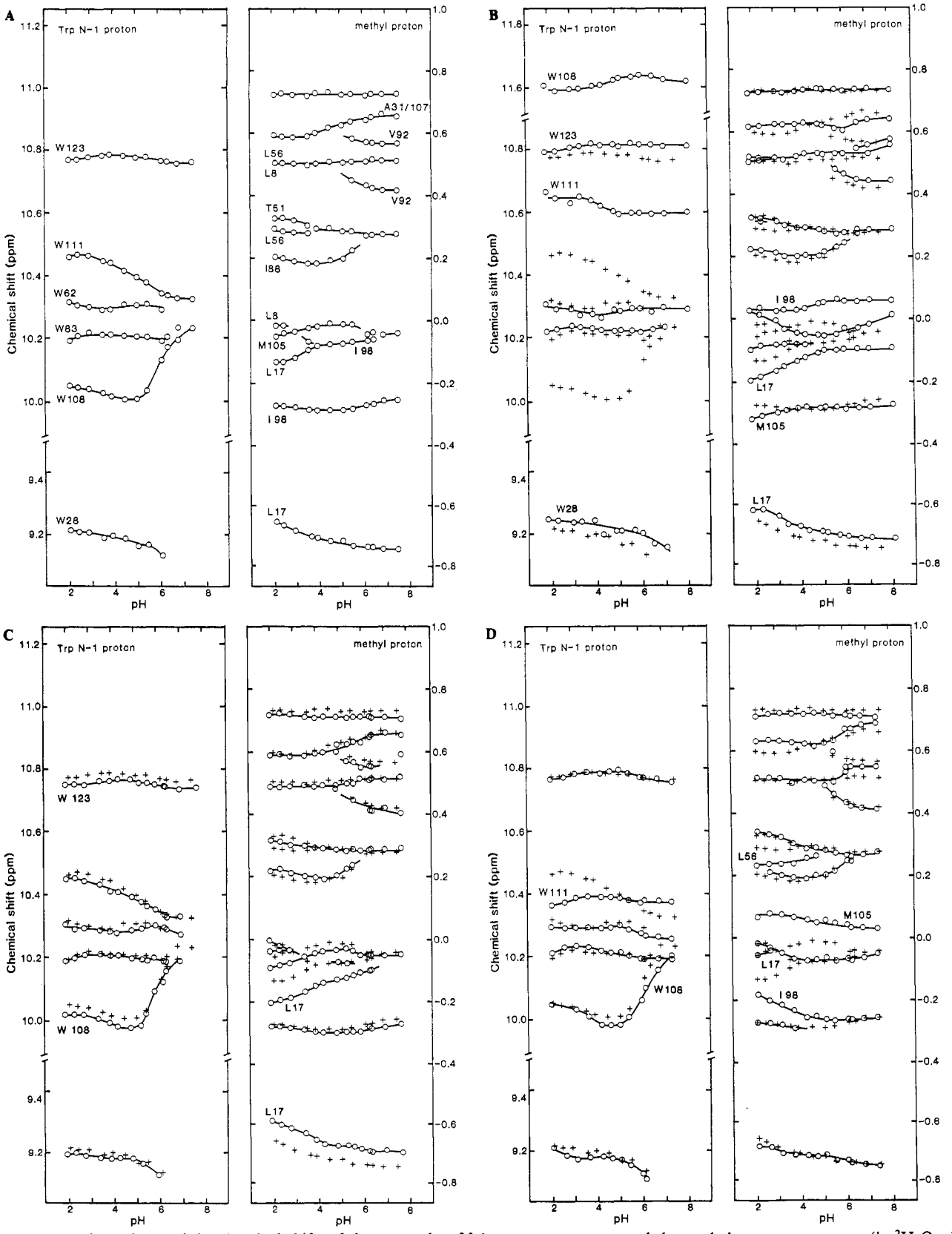

FIGURE 1: pH dependence of the chemical shifts of the tryptophan N-1 proton resonances and the methyl proton resonances (in ^2H_2O with 0.1 M NaCl, 25 °C) in native lysozyme (A), 108–35 lysozyme (B), (13–129)CL lysozyme (C), and 1C -β lysozyme (D). In (B–D), the chemical shifts of the corresponding proton resonances in native lysozyme are indicated with crosses, and assignments for the resonances whose proton chemical shifts are perturbed by the modification are shown. The resonance assignments are based on Campbell et al. (1975) and Cassels et al. (1978).

acidic limb than expected from the exchange of model compounds (tryptophan derivatives) (Waelder & Redfield, 1977). The pH–rate curve for Trp-108 cannot be fitted with a simple acid- and base-catalyzed exchange curve. The observed curves may be fitted with the assumption of at least a single ionizable group that affects the exchange. When the ionization of this titratable group causes a transition of one pH–rate curve to the other, k_{obsd} may be written as[2]

$$k_{obsd} = \frac{k_{m1} + 10^{pH-pK_a}k_{m2}}{1 + 10^{pH-pK_a}} \quad (2)$$

$$k_{m1} = k_{H1}[H^+]^{a_1} + k_{OH1}[OH^-]^{b_1}$$

$$k_{m2} = k_{H2}[H^+]^{a_2} + k_{OH2}[OH^-]^{b_2}$$

In fact, with a pK_a value of 4.1, the pH–rate curves at the four temperatures calculated with eq 2 show good agreement with the experimental results as shown in Figure 2.

From the Arrhenius plots of the exchange rates, $\Delta H^{\ddagger}$ values were obtained and are plotted against pH for the individual tryptophan residues in Figure 3. The $\Delta H^{\ddagger}$ values are 28–30 and 20 kcal/mol for Trp-111 and Trp-123, respectively, at pH 3–6, which are in good agreement with the values given by Wedin et al. (1982). On the other hand, $\Delta H^{\ddagger}$ values for Trp-63 and Trp-108 depend remarkably on pH; $\Delta H^{\ddagger}$ varies from ~10 to ~25 kcal/mol at pH 3.5–5.5 (Trp-63) and pH 5.0–6.5 (Trp-108).

The indole N-1 hydrogen exchange rates were also measured for Trp-63, Trp-108, Trp-111, and Trp-123 in the three lysozyme derivatives at 25 and 55 °C. The results are shown in Figure 4, in comparison with the exchange data for native lysozyme. The exchange for Trp-108 in 108–35 lysozyme was too slow to be followed. The pH dependence curves of exchange rates in lysozyme derivatives are basically similar in shape to those for native lysozyme. However, perturbation on the exchange rates does exist. For Trp-111 in 108–35 lysozyme, the pH dependence curve shifts to high pH. In (13–129)CL lysozyme, the exchange rates for Trp-111 (at pH ≲4) and for Trp-63 are slightly raised. In 101-β lysozyme, the N-1 hydrogens of Trp-108 and Trp-111 exchange faster (at pH ≲4) than those in native lysozyme.

Previously, it has been reported that the exchange for Trp-111 and Trp-123 in 108–35 lysozyme shows no appreciable deviation from that of native lysozyme at pH 3.8 and below 60 °C (Delepierre et al., 1983). However, Figure 4A clearly shows that it is not sufficient to check the difference in the exchange behaviors between the two proteins at a single pH. The Trp-111 N-1 hydrogen in 108–35 lysozyme happens to exchange at the same rate as that in native lysozyme at pH around 4.0–4.5, and it actually exchanges faster at the acidic-pH side and a little slower at the basic-pH side.

Discussion

Exchange in Native Lysozyme. Lysozyme has six tryptophan residues in positions 28, 62, 63, 108, 111, and 123, and indole N-1 hydrogens are partly exposed to the solvent for Trp-62, Trp-63, Trp-108, and Trp-123 but are fully buried for Trp-28 and Trp-111 in the crystal structure. The N-1 hydrogens of three of the six tryptophan residues (Trp-28, Trp-108, and Trp-111) are involved in the intramolecular hydrogen bonding in the crystal. Within 8-Å distance from the tryptophan indole N-1 atom, there are the following residues with an ionizable group: Arg-61 and Asp-101 for Trp-63, Asp-52 and Glu-35 for Trp-108, Arg-112 and Lys-116 for Trp-111, and Lys-33, Arg-5, and Asp-119 for Trp-123.

H–D exchange in globular proteins is often analyzed on the basis of the following scheme (Hvidt & Nielsen, 1966):

$$N(H) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O(H) \xrightarrow{k_3} O(^2H) \underset{k_1}{\overset{k_2}{\rightleftharpoons}} N(^2H) \quad (3)$$

[2] If one takes into account the difference in the pK_a values between the N(H) and the O(H) states (see eq 3 under Discussion), k_{obsd} is expressed in a more complex form [see eq A15 in Wagner and Wüthrich (1979)], but by use of such more precise formulation, it is difficult to determine all the adjustable parameters.

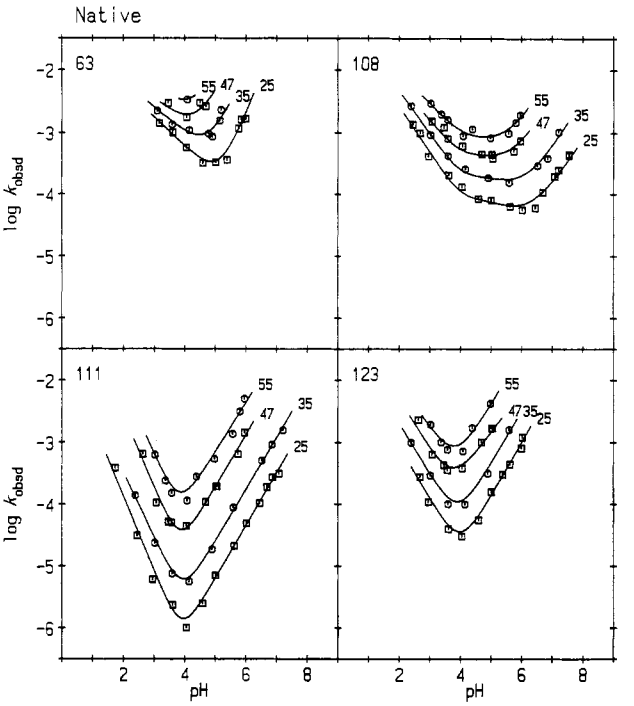

FIGURE 2: pH dependence of pseudo-first-order exchange rates, k_{obsd} (s^{-1}), for the tryptophan indole N-1 hydrogen in native lysozyme (3.5 mM) at 25, 35, 47, and 55 °C. The lines are drawn with eq 1 for Trp-63, Trp-111, and Trp-123 and with eq 2 for Trp-108 by using a pK_a of 4.1. The adjustable parameters k_H, k_{OH}, a, and b in eq 1 are obtained by a leasts-square fit in the acidic limb and in the alkaline limb for Trp-111 and Trp-123.

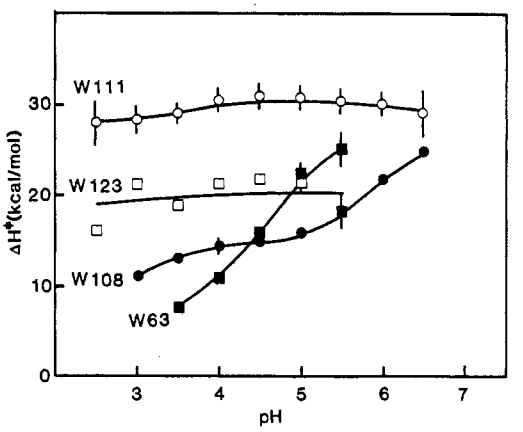

FIGURE 3: pH dependence of $\Delta H^{\ddagger}$ values for the tryptophan indole N-1 hydrogen exchange in native lysozyme. $\Delta H^{\ddagger}$ was obtained from the Arrhenius plot of the exchange rates in Figure 1A. Vertical bars indicate standard deviations.

In this scheme, N(H), the native closed form of the protein in which the tryptophan N-1 hydrogen is not accessible to solvent, is in equilibrium with O(H), the conformation in which the hydrogen is accessible to the solvent. The observed rate constant for the exchange in the scheme shown in eq 3 will be, in the two limiting cases

$$k_3 \gg k_2: \quad k_{obsd} = k_1 \qquad (EX_1 \text{ kinetics}) \quad (4)$$

$$k_3 \ll k_2: \quad k_{obsd} = (k_1/k_2)k_3 \qquad (EX_2 \text{ kinetics}) \quad (5)$$

An EX_1 process and an EX_2 process may be distinguished by the pH dependence of the exchange rate. In the EX_2 process, the exchange rate will manifest the acid and base catalysis of the exchange while the exchange rate shows no such pH dependence in the EX_1 process. The pH dependence curves of the exchange rates in Figure 2 suggest that the exchange for Trp-123 and Trp-111 is governed by the EX_2 mechanism. The

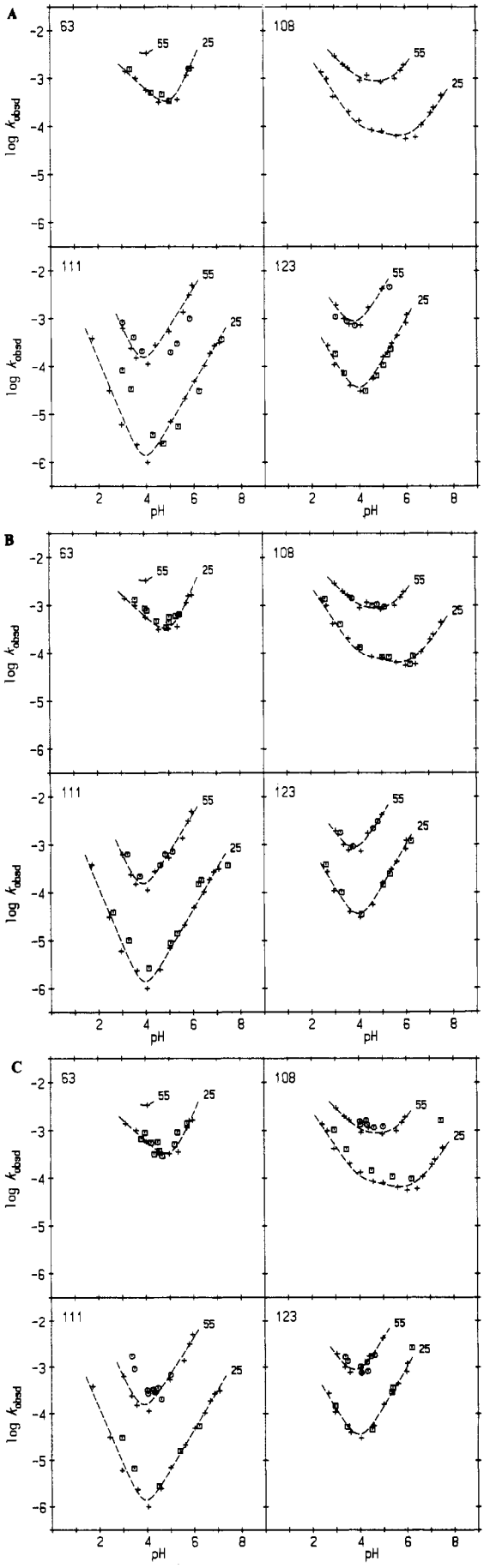

FIGURE 4: pH dependence of pseudo-first-order exchange rates, k_{obsd} (s^{-1}), for tryptophan indole N-1 hydrogens in 108–35 lysozyme (A), (13–129)CL lysozyme (B), and 101-β lysozyme (C) at 25 and 55 °C. The exchange data in native lysozyme (from Figure 2) are shown with crosses and broken lines.

pH dependence of the exchange rate for Trp-63 and Trp-108 is explained by the transition between the two acid- and base-catalyzed exchange processes as described below, and thus, the exchange of Trp-63 and Trp-108 also appears to be in the EX_2 process.

One noteworthy point in the case of the exchange in native lysozyme is that, in contrast to Trp-111 and Trp-123, Trp-108 shows an anomalous pH dependence of the exchange rate at low temperature; the exchange rate decreases only slightly with the increase of pH from 4 to 6, and then a linear base-catalyzed exchange is observed above pH 6 at 25 °C (Figure 2). With the increase of temperature, the pH of the onset of dominant base-catalyzed exchange shifts to low pH. The pH dependence curve for Trp-63 exchange also depends anomalously on temperature. The pH dependence for Trp-63 exchange is rather small in the acidic limb between pH 4.5 and pH 5.5 at 25 °C, and with increasing temperature, the pH_{min} shifts to low pH.

pH dependence curves of the hydrogen exchange deviating from the normal V-shaped curve were observed for the slowly exchanging amide hydrogens in the β-sheet of basic pancreatic trypsin inhibitor (Hilton & Woodward, 1979; Wagner & Wüthrich, 1979) and *Streptomyces* subtilisin inhibitor (Akasaka et al., 1985). In those cases, inflection occurs in the pH dependence curves of the exchange at high temperature and in the alkaline pH region and was explained by a transition among the conformational fluctuations including global fluctuations. In the present case of lysozyme, however, global fluctuation does not likely prevail in the exchange of the low activation energy process as described later, and therefore, a transition from global fluctuations to another fluctuation is not responsible for the anomaly for Trp-108 and Trp-63 exchange.

As seen in Figure 2, the unusual pH dependence curves of the Trp-108 N-1 hydrogen exchange may be expressed as a weighted sum of two pH dependence curves, which exhibit a transition to each other at pH 4.1. The transition is probably induced by the ionization of a carboxyl group of, e.g., Asp-52 located near Trp-108. Deprotonation of the Asp-52 carboxyl group may induce a transition from the conformational fluctuation around Trp-108 to that characterized by a slower exchange rate. If we assume that the exchange takes place in the conformation closely related to the folded structure, negatively charged groups, which arise in the proximity of Trp-108, will also decrease the base-catalyzed exchange rates. In both cases, the deprotonation of probably Asp-52 shifts the curve to the right gradually in the pH region of 3–5 as seen in Figure 2.

The argument against the above explanation may be that there is a difference between the apparent pK_a value (4.1) for the pH–rate curve for Trp-108 (Figure 2) and the reported pK_a value (3.4) of Asp-52 (Kuramitsu et al., 1977). Since the calculated pH–rate curves for Trp-108 exchange do not fit well the experimental data at pH 6–7 (Figure 2), it is quite likely that another mechanism in addition to the ionization of Asp-52 causes a transition between the conformational fluctuation at pH above 4.1. Native lysozyme is known to exhibit self-association at pH above 5 under the present concentration conditions, and this was confirmed by the significant pH dependence of the line widths of proton resonances at around pH 5–6 (data not shown). The self-association has been suggested to enhance the interactions among His-15, Glu-35, Trp-62, Trp-63, and Trp-108 (Shindo et al., 1977). The self-association at pH above 5 appears to slow down the exchange rate for Trp-108 that probably lies at the interface of the self-associating molecules, and such effects are more

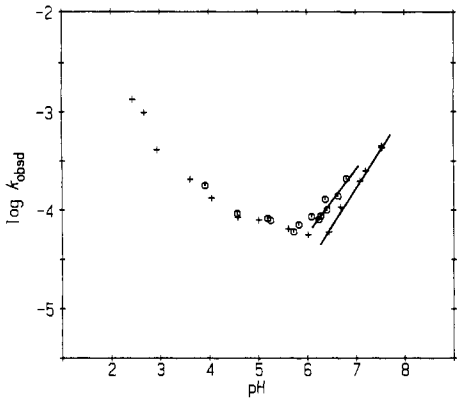

FIGURE 5: pH dependence of pseudo-first order exchange rates, k_{obsd} (s^{-1}), for Trp-108 N-1 hydrogen in native lysozyme at lysozyme concentrations of 0.7 (O) and 3.5 mM (+) at 25 °C. The straight lines were obtained from a least-squares fit for the experimental data at the basic side with eq 1.

pronounced at low temperature due to the enhanced self-association (Shindo et al., 1975). In order to examine the effect of the self-association, H–D exchange behaviors were followed at a lysozyme concentration of 0.7 mM, where the self-association is expected to be suppressed to some extent (Shindo et al., 1977). The results are shown in Figure 5. Concentration dependence was, in fact, observed for the Trp-108 exchange rates in the alkaline limb (at pH above 6) while such concentration dependence is not observed for other tryptophan residues at all (data not shown). Thus, the unusual pH dependence of the exchange rates for Trp-108 is partly due to the self-association of lysozyme molecules.

The anomaly of the pH dependence curve of Trp-63 N-1 hydrogen exchange may not be related to the self-association of the lysozyme because, in 108–35 lysozyme, which hardly tends to self-associate (Banerjee et al., 1975), the exchange for Trp-63 also exhibits similar a pH and temperature dependence to that in native lysozyme. One possible explanation is that the deprotonation of a nearby carboxyl group of probably Asp-101 with a pK_a of 4.4 (Nakae et al., 1975) may cause a transition between the conformational fluctuations around Trp-63, which are characterized by a sizeable difference in their base-catalyzed exchange rate k_{OH} at 25 °C and $\Delta H^{\ddagger}$. Then the deprotonation of the Asp-101 carboxyl group will change the pH dependence curve of the exchange for one fluctuation to that with a smaller k_{OH} for the other fluctuation, resulting in the small pH dependence of the exchange rate in the acidic limb. With a larger $\Delta H^{\ddagger}$ for the fluctuation favored by the deprotonated carboxyl group, the exchange through the fluctuation in the base-catalyzed regime will be more accelerated at higher temperature than the exchange in the acid-catalyzed regime via fluctuation in acidic pH, leading to the downward shift of the apparent pH_{min}. This model requires that the formation of the β-aspartyl sequence at Asp-101 does not significantly affect the transition between the conformational fluctuations since the Trp-63 N-1 hydrogen in 101-β lysozyme exhibits a similar pH dependence of the exchange behavior to that in native lysozyme.

The activation energy deduced from the temperature dependence of the tryptophan N-1 hydrogen exchange in native lysozyme shows that the dominant mechanism of the exchange does not change for Trp-111 and Trp-123 with pH in the temperature range of 25–55 °C. The closely related pH dependence curves of H–D exchange for Trp-111 and Trp-123 at four different temperatures (Figure 2) also indicate that common mechanisms control the exchange for each tryptophan residue in the present temperature range. On the other hand, Trp-108 shows a larger $\Delta H^{\ddagger}$ value at pH 6.5 than at pH below 5.0, suggesting different mechanisms for the exchange between pH above 6.5 and pH below 5.0. For Trp-63, $\Delta H^{\ddagger}$ is also pH dependent, and the $\Delta H^{\ddagger}$ value at pH below 4.0 appears even smaller than that (12 kcal/mol) expected for free tryptophan exchange (Nakanishi et al., 1978; Wedin et al., 1982). This apparent discrepancy probably arises from the fact that a transition between conformational fluctuations takes place at around pH 4. Thus, the pH dependence of $\Delta H^{\ddagger}$ in Figure 3 is consistent with the above model of the transition between different conformational fluctuations, which are likely induced by the self-association and deprotonation of Asp-52 for Trp-108 and deprotonation of Asp-101 for Trp-63. To further confirm the origin of the unusual pH dependence of the exchange rates for Trp-108 and Trp-63, it will be necessary to measure the tryptophan exchange rates in lysozyme derivatives where a single carboxyl group such as Asp-52 or Asp-101 is chemically modified.

Exchange in Modified Lysozymes. (a) The Exchange Is Not Correlated with Thermodynamic or Kinetic Parameters in Protein Denaturation. In the EX_1 process, k_{obsd} is determined by the rate constant of a conformational change from N(H) to O(H), k_1. In the EX_2 process, k_{obsd} is equal to k_3 multiplied by the equilibrium constant for the reaction N(H) $\rightleftharpoons$ O(H), k_1/k_2, and therefore is related to the Gibbs free energy diffrence between the states N(H) and O(H), ΔG_{NO}. If the conformational transition from N(H) to O(H) is large-amplitude unfolding, the exchange kinetics will be correlated with the rate constant of the denaturation, k_{ND} (EX_1 process), or Gibbs free energy difference between the denatured and native states, ΔG_{ND} (EX_2 process), under a given temperature and pH condition. T_m, the thermal denaturation temperature of the protein, is an index of the thermal stability of the protein molecule and is sometimes used to examine, for a series of protein homologues or derivatives, the correlation between H–D exchange rates and conformational stability against denaturation (Wüthrich & Wagner, 1979; Hilton et al., 1981; Delepierre et al., 1983). However, T_m is the temperature at which the free energy change in denaturation is equal to zero, and the protein with the lower T_m may not necessarily have a smaller ΔG_{ND} at specific temperature and pH outside the thermal denaturation zone.

Kinetic and thermodynamic parameters in the protein denaturation have been previously measured for native lysozyme and its derivatives used in this study. 108–35 lysozyme has the highest thermal stability; 108–35 lysozyme is stabilized by 5.2 kcal/mol as compared with native lysozyme at pH 2.0 and 61.8 °C in the presence of 1.94 M guanidine hydrochloride (Johnson et al., 1978). Native lysozyme and (13–129)CL lysozyme exhibit nearly the same value of the Gibbs free energy change in the denaturation, ΔG_{ND} [10.1 and 9.9 kcal/mol for native lysozyme and (13–129)CL lysozyme, respectively, at pH 8.0 and 40 °C], while 101-β lysozyme has a smaller ΔG_{ND} value (9.4 kcal/mol under the same experimental conditions) (Yamada et al., 1984). On the other hand, the unfolding rate constants for the denaturation k_{ND} have been found, from relaxation methods or protease digestion methods, to be in the order native lysozyme < 101-β lysozyme < (13–129)CL lysozyme (Yamada et al., 1984; Imoto et al., 1986). Thermal denaturation temperatures, T_ms, are 77.0, 93.4, 72.4, and 71.9 °C for native lysozyme, 108–35 lysozyme, (13–129)CL lysozyme, and 101-β lysozyme, respectively, at pH 4.25 (T. Ueda, H. Yamada, and T. Imoto, unpublished results).

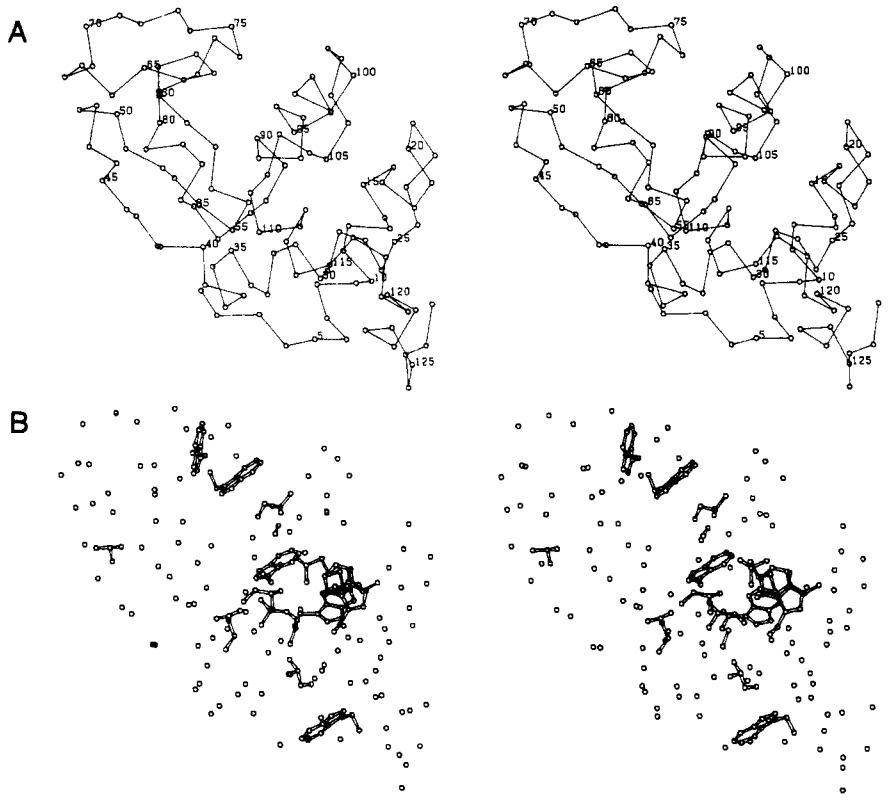

FIGURE 6: Stereoview of the crystal structure of hen egg white lysozyme (set RS5D, coordinates taken from Protein Data Bank) (Diamond, 1974). The pictures were drawn by the computer program STDRAW, which was kindly provided by Dr. Y. Mitsui. (A) Main-chain folding with α-carbons and (B) α-carbons and side chains of residues referred to in the text are shown.

Now it would be of interest to examine whether there is any correlation between the hydrogen exchange rate and T_m, ΔG_{ND}, or k_{ND}. The answer is, as seen in Figure 4, no. The Trp-123 N-1 hydrogen exchanges at the same rate for native lysozyme and the three lysozyme derivatives. The Trp-111 N-1 hydrogen in 108–35 lysozyme, which exhibits the highest thermal stability, exchanges at even a higher rate than that in native lysozyme at pH below 4.5. When compared with that in native lysozyme, the Trp-111 N-1 hydrogen exchanges faster in 101-β lysozyme, which has a smaller ΔG_{ND} value, and in (13–129)CL lysozyme, which has a larger k_{ND} but a similar ΔG_{ND} value. These observations suggest that tryptophan N-1 hydrogens in native lysozyme and its three derivatives do not exchange via globular unfolding of the molecule at temperatures below 55 °C. These findings are consistent with the conclusion drawn from the consideration of the activation energy for the exchange of tryptophan N-1 hydrogens in native lysozyme (Wedin et al., 1982). Under the condition of a low activation energy process, the conformational fluctuation, which is distinct from the globular unfolding, was also suggested from the compensation temperature of enthalpy and entropy terms to contribute to the fast exchange of hydrogens in native lysozyme (Gregory et al., 1982).

Since the conformational fluctuations we observe for Trp-63, Trp-108, Trp-111, and Trp-123 under the present pH and temperature conditions are not global and the exchange corresponds to the EX_2 exchange process, we can obtain information on the equilibrium constant $K = k_1/k_2$ or ΔG for the local structural fluctuation, N(H) $\rightleftharpoons$ O(H) (Privalov & Tsalkova, 1979). It has been suggested that the unfolded state is destabilized in 108–35 lysozyme because of the entropy loss of random coil (Johnson et al., 1979). The present results show that the tryptophan N-1 hydrogen exchange, except for that for Trp-111 at pH above 5, in 108–35 lysozyme is not slowed down as compared with that in native lysozyme. This suggests that the rotations about the side-chain bonds of the two cross-linked residues, which are expected to change the entropy of the protein, do not change between the open state for the tryptophan exchange O(H) and the folded state N(H). Therefore, the local conformational fluctuation, N(H) $\rightleftharpoons$ O(H), around Trp-63, Trp-111 (at pH below 5), and Trp-123 does not seem to involve a simultaneous unfolding process in the region around residues 35 and 108.

(*b*) *The Perturbation in the Exchange Rates Is Related to the Conformational Change by the Modification.* The polypeptide chain folding of crystalline native lysozyme is drawn in Figure 6 with the side chains of the residues studied in this study. The three types of chemical modification, which do not alter the protein folding significantly, perturb the exchange of N-1 hydrogens of not only the tryptophan residues at the modification site but also those remote from the modification site. However, these tryptophan residues whose N-1 hydrogen exchange is perturbed are in the region where a small conformational change due to the modification prevails. Figure 1 reveals that, in 108–35 lysozyme, the internal cross-link between Trp-108 and Glu-35 causes a minor conformational change in a relatively wide region in the right wing of the molecule (Figure 6). This conformationally perturbed region contains Trp-111, whose N-1 hydrogen exchange rate is affected by the modification. The N-1 hydrogen exchange is perturbed for Trp-108 and Trp-111 in 101-β lysozyme. In this case, the β-peptide linkage at the edge of the right wing induces a minor change in the conformation around the residues deep inside the molecule, where Trp-108 and Trp-111 are located. Trp-63 in the left wing and Trp-123 at the C-terminal segment are outside the conformationally perturbed region in 108–35 and 101-β lysozyme, and they are also free from perturbation in their N-1 hydrogen exchange rates.

The only exceptional case is Trp-63 in (13–129)CL lysozyme. In (13–129)CL lysozyme, a small change in the N-1

hydrogen exchange rate is observed for Trp-111 and Trp-63. Although Trp-111 is in the region where a conformational change prevails, Trp-63 is in the left wing and is out of the region. There is no indication of conformational perturbation on residues 51, 56, 62, and 63 in the left wing, as judged from the pH dependence of the chemical shifts of their proton resonances (Figure 1C). However, it should be noted that the change in the exchange rate of the Trp-63 N-1 hydrogen is so small that we cannot exclude the possibility of the very minor conformational perturbation around Trp-63 that is hardly reflected in the proton chemical shifts.

In conclusion, the perturbation in the exchange rates of the tryptophan indole N-1 hydrogens seems to be related to the small but distinct conformational change induced by the chemical modification that may be found by NMR but may not be detected by the X-ray analyses at a low resolution of, say, 2.5 Å (in the case of 108–35 lysozyme). Since this conformational changes does not likely involve a significant rearrangement of the ionizable groups (for 108–35 lysozyme; Imoto & Ono, 1980), a structural difference around tryptophan N-1 hydrogens between native lysozyme and modified lysozyme may not straightforwardly explain the difference in their exchange behaviors. Instead, the strain in the protein conformation due to the chemical modification may well alter the free energy difference between the two states N(H) and O(H) for individual tryptophan residues and thereby change the observed rate constant for the exchange. Indeed, the consideration of the thermodynamic and kinetic parameters in denaturation for (13–129)CL or 101-β lysozyme has suggested that the chemical modification changes the free energy difference of the native and denatured states due to the increase in the conformational strain in the native state caused by the modification (Yamada et al., 1984).

At last, the present interpretation of the exchange data is compatible with both of the two types of models for small-amplitude fluctuations, i.e., the solvent penetration and local unfolding models. Thus, more detailed information on the conformational change in the modified lysozyme derivatives is necessary for further distinguishing the two mechanisms. Instead, it might be said that monitoring hydrogen exchange is a sensitive method for detecting a minor conformational change or a change in the strain insofar as the exchanging site is in the region involved in the change in conformation or structural strain.

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