J. Biochem. 119, 1019-1023 (1996)

# Analysis of the Transition State in the Unfolding of Hen Lysozyme by Introduction of Gly-Pro and Pro-Gly Sequences at the Same Site

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Received for publication, September 28, 1995

**We developed a sensitive method for analyzing the conformation of the transition state in the unfolding of hen lysozyme. The activation free energy changes of mutant lysozymes with Gly-Pro and Pro-Gly sequences at the same sites (Gly47Pro47′, Pro47Gly47′, Gly101-Pro102, Pro101Gly102, Gly117Pro118, Pro117Gly118, Gly121Pro122, and Pro121Gly122 lysozymes) were obtained for the unfolding in aqueous solution at pH 5.5 and 35°C. Since we had shown that the difference of energies of the unfolded state in lysozymes having an introduced Gly-Pro or Pro-Gly sequence at the same site was much smaller than the difference of energies of the folded states [Motoshima, H., Ueda, T., Hashimoto, Y., Tsutsumi, M., and Imoto, T. (1995) *J. Biochem.* 118, 1138-1144], we could estimate the difference of energies of the folded and the transition states unequivocally. We defined the $\phi$-value as the ratio of the difference in the free energy change in the transition state to that in the free energy change in the folded state between lysozymes with Gly-Pro and Pro-Gly sequences at the same site. The $\phi$-values gave information on how much the mutated sites retained the folded structure in the transition state. These values were 0.45 around position 47, which is located in the $\beta$-sheet structure, 0.12 at position 101-102, which is located in the loop at the upper part of the active site, 0.17 at position 117-118, which is located in the $\beta$-turn and 0.64 at position 121-122, which is located in the $3_{10}$-helix. Therefore, in the transition state in the unfolding of lysozyme, it was found that the $3_{10}$-helical region had a similar structure to the intact region, while both the $\beta$-turn and the loop at the upper part of the active site were considerably unfolded. The $\beta$-sheet structure was also moderately disrupted in the transition state.**

**Key words: Gly-Pro sequence, lysozyme, Pro-Gly sequence, stability, transition state.**

A protein is usually in equilibrium between folded and unfolded states. To stabilize a protein against reversible denaturation, the free energy change for the unfolding should be increased by stabilizing the folded state by lowering its energy level, or by destabilizing the unfolded state by raising its energy level. On the other hand, various processes can be coupled with the unfolded state of a protein. For example, digestion by a protease at physiological temperature may be one such process, and this would lead to irreversible denaturation. To stabilize a protein against irreversible denaturation coupled with the unfolded state, kinetic stabilization is important, that is, the activation free energy for the unfolding should be increased. We have recently demonstrated that, for kinetic stabilization, it is important to stabilize a protein at a site where the local structure is largely unfolded in the transition state in the unfolding. At the same time, we developed a method to find such sites by comparison of the thermodynamic stabilities and the unfolding rate constants for a set of modified proteins (*1*). Namely, it is important to determine the structure of a protein in the transition state in the unfolding in order to stabilize the protein kinetically.

A study of mutant lysozymes revealed that a Gly101-Pro102 lysozyme was more stabilized than the native lysozyme, whereas a Pro101Gly102 lysozyme was markedly destabilized, though the mutations were similar (*2*). This result indicates that the formation of the Gly-Pro sequence is effective in avoiding possible strain in the folded state caused by the introduction of a proline residue, that the energy levels in the folded state of proteins might differ considerably between mutant lysozymes with the Gly-Pro sequence and those with the Pro-Gly sequence at the same site. In a previous paper, we demonstrated by using activity and NMR spectra that all mutant lysozymes are folded, and suggested that the difference of energies of the unfolded state between lysozymes having an introduced Gly-Pro sequence and those with a Pro-Gly sequence at the same site was much smaller than the difference of energies of the

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Abbreviations: Gdn-HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; Gly47Pro47′ lysozyme, a mutant lysozyme in which Thr47 is mutated to a Gly-Pro sequence; Pro47-Gly47′ lysozyme, a mutant lysozyme in which Thr47 is mutated to a Pro-Gly sequence; Gly101Pro102 lysozyme, a mutant lysozyme in which Asp101-Gly102 is mutated to a Gly-Pro sequence; Pro101-Gly102 lysozyme, a mutant lysozyme in which Asp101-Gly102 is mutated to a Pro-Gly sequence; Gly117Pro118 lysozyme, a mutant lysozyme in which Gly117-Thr118 is mutated to a Gly-Pro sequence; Pro117Gly118 lysozyme, a mutant lysozyme in which Gly117-Thr118 is mutated to a Pro-Gly sequence; Gly121Pro122 lysozyme, a mutant lysozyme in which Gln121-Ala122 is mutated to a Gly-Pro sequence; Pro121Gly122 lysozyme, a mutant lysozyme in which Gln121-Ala122 is mutated to a Pro-Gly sequence.

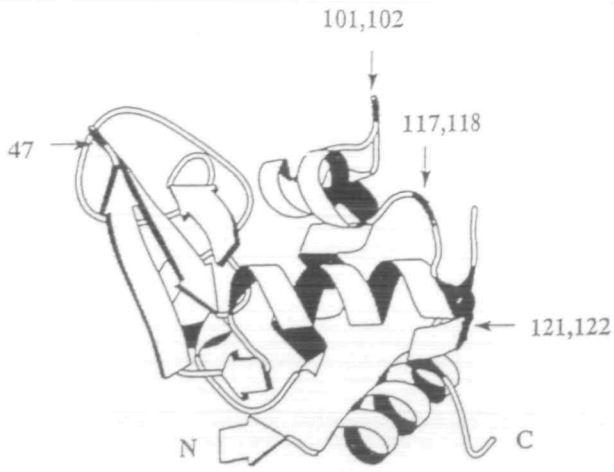


Fig. 1. **Secondary and tertiary structures in lysozyme with the mutation sites.** N and C indicate the N-terminal and C-terminal, respectively.

folded states (*3*). Therefore, by comparing the ratio of the difference in the energy level in the transition state to that in the energy level in the folded state, we can estimate how much of the folded structure is retained in the transition state at a particular site.

In this paper, we analyzed the transition state in the unfolding of hen lysozyme using lysozymes with Gly-Pro and Pro-Gly sequences at the same site. The mutations are located in the $\beta$-sheet (Thr47→Gly47Pro47′, Pro47-Gly47′), the loop region (Asp101Gly102→Gly101Pro102, Pro101Gly102), the $\beta$-turn (Gly117Thr118→Gly117-Pro118, Pro117Gly118), and the $3_{10}$-helix (Gln121Ala122 →Gly121Pro122, Pro121Gly122) (Fig. 1).

## MATERIALS AND METHODS

*Materials*—Gly101Pro102 lysozyme and Pro101Gly102 lysozyme were obtained as described before (*2*). Mutant lysozymes (Gly47Pro47′ lysozyme, Pro47Gly47′ lysozyme, Gly117Pro118 lysozyme, Pro117Gly118 lysozyme, Gly-121Pro122 lysozyme, and Pro121Gly122 lysozyme) were obtained as described before (*3, 4*). Guanidine-hydrochloride was purchased from Kanto Chemical. All other chemicals were of analytical grade for biochemical use.

*Unfolding Kinetic Experiment*—All experiments were performed at 35°C. Unfolding was initiated by rapid dilution. Experiments were done using 1 volume of protein (9 $\mu$M) in 0.1 M acetate buffer at pH 5.5, with 10 volumes of 0.1 M acetate buffer at pH 5.5 containing concentrated Gdn-HCl. This resulted in a final Gdn-HCl concentration between 3.5 and 6.5 M. The unfolding was followed by monitoring the intrinsic fluorescence of lysozyme using a Hitachi F-2000 fluorescence spectrophotometer (band width, 10 nm; excitation wavelength, 280 nm; emission wavelength, 350 nm). The sample solution was mixed using a mixing device fitted to the fluorescence spectrophotometer. The dead time was estimated to be 2 s.

## RESULTS

*Evaluation of Unfolding Rate Constants of Wild Type and Mutated Lysozymes*—The unfolding rate constant of lysozymes at pH 5.5 and 35°C was measured by Gdn-HCl jumps according to the literature (*5*). Even though an extra proline residue was introduced into the mutant lysozymes, their kinetic progress curves were monophasic under the conditions employed. Figure 2 shows plot of the logarithm of the apparent rate constants for unfolding *versus* the final Gdn-HCl concentration in the concentration jumps. On the unfolded side ([Gdn-HCl] $\geqq$ 3.5-4.5 M) in each figure, plots of $\log k_{app}$ *versus* [Gdn-HCl] give straight lines, indicating that $k_{app}$ corresponds to $k_u$ in the unfolding side (*5*). Thus, we obtained the unfolding rate constant in water, $k_u(H_2O)$, by fitting the data to the equation,

$$\log k_u = \log k_u(H_2O) - m_{ku}[\text{Gdn-HCl}] \quad (1)$$

where $m_{ku}$ is a measure of the dependence of $\log k_u$ on Gdn-HCl concentration (*6*). The values of $\log k_u(H_2O)$ for the lysozymes and the correlation factor ($r$) are summarized in Table I. Moreover, the activation free energy change ($\Delta G$) is obtained by the following equation

$$\Delta G^* = -RT\ln(hk)/(k_B T) \quad (2)$$

where $k_B$, $h$, $R$, and $T$ are the Bolzmann constant, the Planck constant, the gas constant, and temperature, respectively. The activation free energy change is also shown in Table I. All errors are calculated from the best fit of data.

The polypeptide chain of hen lysozyme is randomly coiled in the concentrated Gdn-HCl solution (*7*); therefore, the restriction of the movement of the main chain should fully affect the chain entropy of the polypeptide in the unfolded state. Moreover, we had shown that the difference of energies of the unfolded state in lysozymes having an introduced Gly-Pro or Pro-Gly sequence at the same site was much smaller than the difference of energies of the folded states (*3*). We can equate the energy level of the unfolded state of each mutant lysozyme ($D_1 \doteqdot D_2$) under the condition where $\Delta G_N \gg \Delta G_D$ (Fig. 3). In human lysozyme, which is homologous to hen lysozyme, a stable intermediate that is thermodynamically more stable than the fully unfolded state and less stable than the folded state was observed in the unfolding coordinate between the transition state and the fully unfolded state (*5*). A similar stable intermediate may be also present in the unfolding of hen lysozyme. However, when we focus on the unfolding process, we can discuss the transition state in the unfolding without considering the contribution of the stable intermediate. Thus, free energy diagrams at pH 5.5 and 35°C for the unfolding of mutant lysozymes with a Gly-Pro or Pro-Gly sequence at the same site are shown in Fig. 3 using the difference in both the free energy change in the folded state ($\Delta G_N = N_2 - N_1$) and the free energy change in the transition state ($\Delta G_{TS} = TS_2 - TS_1$) between Gly-Pro and Pro-Gly lysozymes. In the diagram, when the difference in the free energy change in the transition state between these mutants is zero, the structure at the mutated site in the transition state is similar to that in the fully unfolded state. On the other hand, when the difference in the free energy change in the transition state between these mutants is identical to that in the folded state, the structure at the mutated site in the transition state is similar to that in the folded state. Therefore, we can define $\phi$-value that shows how close the structure at the mutated site in the transition state is to that in the folded state. The $\phi$-value can be

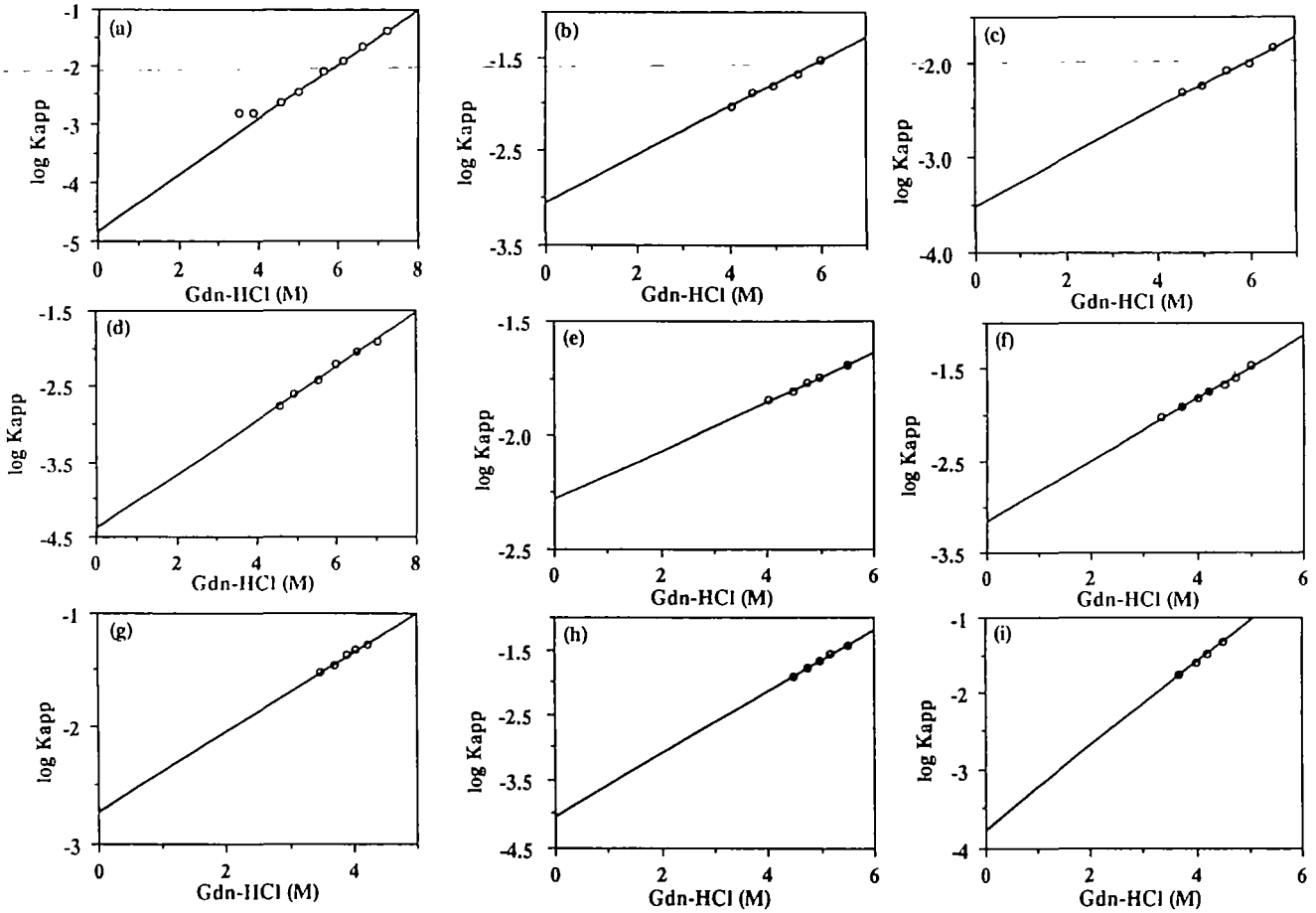


Fig. 2. **Dependence of the apparent rate constants (in $s^{-1}$) of unfolding on Gdn-HCl concentration in wild-type and mutant lysozymes in 0.1 M acetate buffer at pH 5.5 and 35°C.** The unfolding was followed by monitoring the change in the Trp and Tyr fluorescences of lysozyme (excitation wavelength, 280 nm; emission wavelength, 350 nm). (a) Wild-type lysozyme, (b) Gly47Pro47′ lysozyme, (c) Pro47Gly47′ lysozyme, (d) Gly101Pro102 lysozyme, (e) Pro101Gly102 lysozyme, (f) Gly117Pro118 lysozyme, (g) Pro117-Gly118 lysozyme, (h) Gly121Pro122 lysozyme, (i) Pro121Gly122 lysozyme.

TABLE I. **Kinetic parameters characterizing the Gdn-HCl denaturation of wild type and mutant lysozymes at pH 5.5 and 35°C.**

| Lysozyme | $\log k_u(H_2O)$ ($s^{-1}$) | $m_{ku}$ ($M^{-1}$) | $\Delta G^*(H_2O)$ (kcal/mol) | $r$ |
|---|---|---|---|---|
| Wild | −4.85±0.08 | 0.48±0.01 | 24.9±0.1 | 0.997 |
| Gly47Pro47′ | −3.06±0.06 | 0.25±0.01 | 22.4±0.1 | 0.993 |
| Pro47Gly47′ | −3.53±0.10 | 0.26±0.02 | 23.0±0.2 | 0.985 |
| Gly101Pro102 | −4.38±0.06 | 0.36±0.01 | 24.2±0.1 | 0.997 |
| Pro101Gly102 | −2.28±0.03 | 0.11±0.01 | 21.3±0.1 | 0.991 |
| Gly117Pro118 | −3.10±0.06 | 0.32±0.01 | 22.4±0.1 | 0.999 |
| Pro117Gly118 | −2.74±0.05 | 0.35±0.02 | 21.9±0.1 | 0.991 |
| Gly121Pro122 | −4.05±0.06 | 0.48±0.01 | 23.8±0.1 | 0.999 |
| Pro121Gly122 | −3.78±0.09 | 0.54±0.02 | 23.4±0.1 | 0.995 |

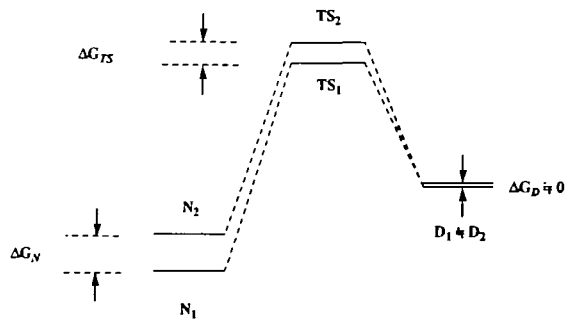


Fig. 3. **Free energy diagrams for the unfolding of mutant lysozymes at pH 5.5 and 35°C.** $N_1$, $D_1$, and $TS_1$ and $N_2$, $D_2$, and $TS_2$ indicate the energy level of the folded state, the unfolded state, and the transition state in mutant lysozymes in which the Gly-Pro (subscript 1) or Pro-Gly (subscript 2) sequence was introduced at the same site.

expressed by evaluating how much strain energy in the folded state is retained in the transition state. Namely,

$$\phi = |\Delta G_{TS}|/|\Delta G_N| \quad (3)$$

$$= |(TS_2 - TS_1)|/|(N_2 - N_1)| \quad (4)$$

$$= |[(\Delta G^*_{GP} - \Delta G^*_{PG}) - (\Delta G_{GP} - \Delta G_{PG})]|/|(\Delta G_{GP} - \Delta G_{PG})| \quad (5)$$

where $\Delta G_N$ is the difference in the free energy change for the unfolding between Gly-Pro ($\Delta G_{GP}$) and Pro-Gly ($\Delta G_{PG}$) mutants at each site, and $\Delta G_{TS}$ is the difference in the free energy change between $\Delta G_N$ and the difference in the activation free energy change between Gly-Pro ($\Delta G^*_{GP}$)

TABLE II. **Differences in free energy change and in activation free energy change and $\phi$-values between Gly-Pro and Pro-Gly lysozyme at pH 5.5 and 35°C.**

| Site | $\lvert\Delta G_N\rvert$[a] (kcal/mol) | $\lvert\Delta G_{TS}\rvert$ (kcal/mol) | $\phi$-value |
|---|---|---|---|
| 47 | 1.1 | 0.6 | 0.45 |
| 101-102 | 3.3 | 0.4 | 0.12 |
| 117-118 | 0.6 | 0.1 | 0.17 |
| 121-122 | 1.1 | 0.7 | 0.64 |

[a]The free energy changes of mutant lysozymes were obtained from the previous paper (*3*).

and Pro-Gly ($\Delta G^{\ddagger}_{PG}$) lysozymes at each site (Table II). $\Delta G_N$ was evaluated previously (*3*). For example, in mutant lysozyme in which Gln121-Ala122 was mutated to Gly121-Pro122 and Pro121Gly122, $\Delta G_N$ and $\Delta G_{TS}$ were calculated to be 1.1 and 0.7 kcal/mol, respectively, which produced $\phi$-values of 0.64. At position 121-122, 0.64 of the difference in the free energy change in the folded state between Gly121Pro122 and Pro121Gly122 lysozymes was retained in the transition state. Table II shows how much of the difference in the free energy change in the folded state was retained in the transition states at each site.

## DISCUSSION

The unfolding and refolding transition of a protein is often multiphasic due to the presence of a stable intermediate (*5, 6*). In human lysozyme, which is homologous to hen lysozyme, it was demonstrated that the unfolding rate constant in water, $k_u(H_2O)$, could be evaluated from the unfolded side ([Gdn-HCl] $\geqq$ 3 M) of the apparent rate constant even under conditions where the stable intermediate was present in the unfolding coordinate between the transition state and the fully unfolded state (*5*). Because the plots of $\log k_{app}$ *versus* [Gdn-HCl] above 3.5-4.5 M gave good straight lines for the wild-type and mutant hen lysozymes (Fig. 2), we may evaluate the unfolding rate constant in water for each mutant on the basis of the dependency of $\log k_{app}$ on the unfolded side. Thus, using $k_u(H_2O)$ for each mutant, we will discuss the kinetics of the mutant lysozymes below.

Because the polypeptide chain of hen lysozyme is randomly coiled in the concentrated Gdn-HCl solution (*7*), the restriction of movement of the main chain should fully affect the chain entropy of the polypeptide in the unfolded state. In a previous paper, we demonstrated by using activity and NMR spectra that all mutant lysozymes have a native-like conformation and showed that the difference of energies of the unfolded state in lysozymes having an introduced Gly-Pro or Pro-Gly sequence at the same site was much smaller than the difference of energies of the folded states (*3*). This finding made it easy to analyze energy levels of the proteins.

The gap between the energy levels of native states in the proteins having Gly-Pro and X(=not Gly)-Pro is large. The gaps (0.6-3.3 kcal/mol, Table II) may be large enough to allow calculation of the $\phi$-value to analyze the transition state in the unfolding of lysozyme. Matouschek *et al.* (*6*) analyzed the transition state in the unfolding of barnase using a series of barnases mutated at the same site. In these cases, the difference in the free energy change in the folded state was derived from the difference in stability between the wild-type and each mutant, and the differences in the free energy change in the folded state were not large enough for $\phi$ to be calculated in some mutations. Therefore, the present method may have another advantage in the large difference in the free energy change in the folded state.

From the $\phi$-values, it was found that the structure at residues 121-122, which is located in the $3_{10}$-helix, had a similar structure to the intact one. On the other hand, the structure at residues 117-118, is located in the $\beta$-turn, and at residues 101-102, located in the loop at the upper part of the active site, are both largely unfolded in the transition state for the unfolding of lysozyme (Table II). Moreover, since the $\phi$-value was 0.45 around residue 47, which is located in the $\beta$-sheet, the $\beta$-sheet structure was also moderately disrupted in the transition state (Table II). From analysis of the folding pathway of unfolded lysozyme obtained by hydrogen-exchange labeling techniques and the change in ellipticity in circular dichroism spectra, the $\alpha$-helical structures have been shown to form faster than the $\beta$-sheet structure before the formation of the tertiary structure (*8*). Although we do not know whether the transition state is in the coordinate of the folding pathway, the result of Radford *et al.* (*8*) was consistent with the present result that a considerable portion of the structure at residues 121-122 in the $3_{10}$-helix was retained in the transition state, but the structure around residue 47 in the $\beta$-sheet was not. Moreover, since the formation of a considerable amount of secondary structure was estimated from the $\phi$-values, the structure in the transition state was suggested to be compact, as has been reported previously (*9, 10*).

From the above results, the present method was concluded to be effective for analyzing the transition state for the unfolding of lysozyme. Hereafter, using other mutant lysozymes with Gly-Pro and Pro-Gly sequences at the same site, we may be able to elucidate the whole structure in the transition state of lysozyme. Moreover, the method should be applicable for analyzing the transition states of other proteins.

The authors would like to thank Y. Hashimoto, M. Tsutsumi, and M. Mukae for preparing mutant lysozymes.

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