CHAPTER 5
Molecular Dynamics of Lysozyme

5.1 Segmental Motion of Subsite C of Hen Egg-White Lysozyme: Fluorescence Depolarization Studies of Kyn62-Lysozyme as an active Analogue

5.1.1 Introduction

The spatial configuration and static interaction of hen egg-white lysozyme (HEWL) with the ligand were confirmed by X-ray crystallographic analysis, but more studies on the dynamics of the conformation should be followed to deepen our understanding of the specific enzymatic action of HEWL, because it is experimentally and theoretically accepted that the conformational flexibility and its dynamics of bioactive proteins would be closely related with completion of their biological functions.

The active site of HEWL is constituted of six subsites (Subsite A, B, C, D, E, and F) as described in Chapter 1. Trp62 mostly contributes as subsite C to the stabilization of the complex with the oligomer of N-acetyl-D-glucosamine ((NAG)_3). Therefore, the dynamical property of its segmental motion is very interesting. Usually Trp62 is itself the most important probe for the fluorescence studies of HEWL, but HEWL contains six tryptophan residues and it is difficult to separate the fluorescence of Trp62 from the others. Therefore, we present here the dynamics of subsite C through time-resolved fluorescence depolarization studies of Kyn62-lysozyme, in which Trp62 was selectively changed to kynurenine.

5.1.2 Materials and Methods

Materials

HEWL and (NAG)_3 were purchased from Sigma Chemical Co. and Seikagaku Kogyo, respectively, and used without further purification. Kyn62-lysozyme was prepared and purified as described in CHAPTER 2.

Time-resolved fluorescence anisotropy decay measurements

Time-resolved fluorescence anisotropy decay measurements were done using the technique of time-correlated single photon counting. The instrumentation and technique for the analysis are described in CHAPTER 2.

Anisotropy decay, r(t), was given with a linear combination of some
exponentials as follows,

\[ r(t) = \Sigma \beta_i \exp(-t/\phi_i) \]

where \( \phi_i \) is the rotational correlation time of the \( i \)-th component and \( \beta_i \) is the corresponding pre-exponential factor. The adequacy of the decay parameters obtained was judged by inspection of weighted residuals and other statistical parameters such as the root of the reduced chi-square (\( \sigma \)) and serial variance ratio (SVR). The reasonable decay parameters demand that the weighted residual plots be distributed homogeneously and \( \sigma \) and SVR should be in the range of 1.0-1.2 and 1.8-2.0, respectively.

5.1.3 Results and Discussion

The absorption spectrum of Kyn62-lysozyme is constituted of tryptophyl and kynurenyl bands. When excited at the kynurenyl band (300-420 nm), Kyn62-lysozyme emits the fluorescence with a maximum at 450 nm. The fluorescence anisotropy decay curves monitored and excited at 450 nm and 320 nm, respectively, are given in Fig. 5.1.1. As shown with the homogeneous distribution of weighted residuals in the residual plots (Insertion of Fig. 5.1.1) and reasonable values of \( \sigma \) and SVR (Table 5.1.1), the decay curves of free Kyn62-lysozyme was precisely described with a triple exponential function of which correlation times were \( \phi_1 = 4.2 \) ns, \( \phi_2 = 1.4 \) ns, and \( \phi_3 = 150 \) ps, respectively. Since the largest correlation time (\( \phi_1 \)) corresponds to one of the entire rotation of lysozyme, the segmental motion of kynurenine could be characterized with two faster components. The anisotropy decay function of the complexed Kyn62-lysozyme with (NAG)\(_3\) was double exponential with \( \phi_1 = 4.50 \) ns and \( \phi_3 = 170 \) ps. The slowly relaxing component of the segmental motion of kynurenine was completely suppressed by the binding of (NAG)\(_3\). Based on a theoretical model for the restricted motion of a fluorophore bound to a macromolecule, the fractional pre-exponential factor of the fluorescence anisotropy decay function, which is given in Table 5.1.1, indicates that the segmental motion of kynurenine in Kyn62-lysozyme retains the freedom of 90% in the free state and it reduced to 40% in the complex with (NAG)\(_3\).

Because the kynurenine residue of Kyn62-lysozyme is created with the cleavage of the indole ring of Trp62, we thought that kynurenine might be linked to the peptide chain in a different way with Trp62 and therefore its dynamical properties might also be different. However, the fluorescence
Fig. 5.1.1 The fluorescence anisotropy decay of Kyn62-lysozyme (A) and the complex of Kyn62-lysozyme with (NAG)$_3$ (B). Excitation wavelength, 320 nm; emission wavelength, 450 nm; band-pass of Ex./Em., 5 nm/5 nm; concentration of Kyn62-lysozyme, $5\times10^{-5}$ M. The ratio of Kyn62-lysozyme/(NAG)$_3$ is 1/100 at (B). Temperature, 25°C.

Insertion, the weighted residual plots giving the best fit, to the decays of parallel (a) and perpendicular (b) component. The weighted residuals divided by the root mean square of the residuals (RMSR), which was equivalent to $\sigma$, were plotted against full channels covering the fluorescence decay of Kyn62-lysozyme.
Table 5.1.I  The Dynamical Parameters of the Segmental Motion of Kynurenine of Kyn62-lysozyme.

<table>
<thead>
<tr>
<th></th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\beta_3$</th>
<th>$\phi_1$(ns)</th>
<th>$\phi_2$(ns)</th>
<th>$\phi_3$(ps)</th>
<th>$\epsilon^*$</th>
<th>$\sigma$</th>
<th>SVR</th>
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<td>0.13</td>
<td>0.16</td>
<td>4.20</td>
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<td>150</td>
<td>0.9</td>
<td>1.07</td>
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<td>-</td>
<td>170</td>
<td>0.4</td>
<td>1.05</td>
<td>2.00</td>
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</table>

*$\epsilon^*$, the degree of the motional freedom defined as $\epsilon^* = 1 - (\beta_1/\Sigma \beta_i)$
Excitation wavelength, 320 nm; Emission wavelength, 450 nm.
Table 5.1.II  The Fluorescence Anisotropy Decay Parameters of HEWL.

<table>
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<tr>
<th></th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\beta_3$</th>
<th>$\phi_1$(ns)</th>
<th>$\phi_2$(ns)</th>
<th>$\phi_3$(ps)</th>
<th>$\epsilon^*$</th>
<th>$\sigma$</th>
<th>SVR</th>
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<td>Lysozyme'</td>
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<td>0.45</td>
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<td>0.53</td>
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<td>Lysozyme - (GlcNAc), complex'</td>
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<td>-</td>
<td>230</td>
<td>0.35</td>
<td>1.05</td>
<td>1.92</td>
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</table>

#, Excitation and Emission wavelengths, 295 nm and 380 nm.
+, Excitation and Emission wavelengths, 295 nm and 340 nm.
*, See Table I.
anisotropy decay function of free HEWL was triple exponential and it switched to double exponential by the interaction with (NAG)₃, as shown in Table 5.1.II. Besides, it was suggested that the subsite C of HEWL also reduced its motional freedom. Although we cannot conclude definitely because the fluorescence of Trp62 cannot be separated from the others, especially of Trp108, this result demonstrates the subsite C of Kyn62-lysozyme has quite similar dynamics to Trp62 of HEWL. From this point of view, the results on the temperature and ligand size dependence of the segmental motion of kynurenine are also interesting. They showed that the subsite C moved more rapidly with increasing in the temperature without changing the motional freedom, and that the pentamer of N-acetyl-D-glucosamine allowed a larger freedom to subsite C than a monomer or trimer in the Kyn62-lysozyme-ligand complex. Reports on the dynamical properties around the active site of HEWL was discussed in next part of this chapter.

5.1.4 Conclusion

The dynamical properties of Subsite C of hen egg-white lysozyme were investigated using Kyn62-lysozyme as an active analogue. Time-resolved fluorescence depolarization studies showed that the segmental motion of kynurenine which was important in subsite C was described with two components of which the rotational correlation times were $\tau_1 = 150$ ps and $\tau_2 = 1.4$ ns, respectively. Although these two segmental motions retained 90% of motional freedom, the slower motion was completely restricted and the degree of freedom was lost to 40% during the interaction with oligomers of N-acetyl-D-glucosamine.

5.1.5 References

5.2 Molecular Dynamics of Lysozyme Studied by Time-Resolved Fluorescence Depolarization of Tryptophan Residues.

5.2.1 Introduction

Proteins are aperiodic long-chain polymers and their structures are determined by weak, noncovalent, interactions among the elements of the polypeptide. Therefore, they are expected to fluctuate intrinsically around the average structure. How does the protein fluctuations take place? It has been interesting and fundamental subject from the standpoint of chemical physics. Recently, the molecular dynamics of proteins has became a matter of the great concern among many molecular biologist. Because most globular proteins alterate their own conformations under the interactions with small molecules and other proteins and the conformational flexibility exhibited in protein is frequently essential in many biological systems. For example, in hormone-receptor binding, the conformational transition is important to transmission of information, the ligand-induced conformational change is the main part in the cooperativity exhibited by hemoglobin, and small motions in some enzyme systems such as ribonuclease and lysozyme involve in the catalytic action. Thus, any attempts to understand more fundamentally the functions of protein require the knowledge on the molecular dynamics of the internal motion of protein.

Many physico-chemical methods and techniques are examined and applied to study the protein dynamics. X-ray analysis is essential to decide the average structure of protein on which the molecular dynamics discussion is based. It also provides the information on the magnitude of protein fluctuation as temperature factor. However, in order to know the time-scale of the internal motion of protein, we have to rely upon the other methods, such as nuclear magnetic resonance, or fluorescence depolarization studies. Although these methods are widely used according to the conditions of systems under investigation, the fluorescence depolarization is an unique method to grasp the physical images of the characteristic protein dynamics. Because the time-correlation function, which is generally used for the description of the motion, is directly connected with the measured quantity such as fluorescence anisotropy. Indeed, the fluorescence depolarization studies performed using the tryptophan or tyrosine residues as the
fluorescence probe have cleared the internal motions and conformational
dynamics of protein. In addition to the advantage mentioned above, the
fluorescence spectroscopy enables the analysis with high sensitivity and
excellent time-resolution power. Therefore, no one deny the fluorescence
spectroscopic method is one of the most powerful methods for studying the
molecular dynamics of proteins. But its validity has been considerably
limited, because this method demands specified fluorophores in protein for
quantitative discussions. In the case of single-tryptophan containing protein,
the specification of the fluorophore is easy and through the spectroscopic
analysis of the tryptophyl fluorescence, the dynamics of the internal motion
of protein can be clarified. If the X-ray crystallographic structure around the
tryptophan residue is confirmed and available, the validity of the information
would be more increased. On the other hand, when proteins contain more
than two tryptophan residues, serious ambiguities are inevitably include
because of the spectral overlap of some tryptophyl fluorescence. Although the
discrimination of the individual tryptophyl fluorescence has been a subject to
be solved for a long time, fortunately, the recent development of the genetic
techniques showed a way to overcome this problem. The mutant proteins in
which tryptophan residues are replaced with the other by site-directed
mutagenesis allows us to investigate the internal motions simultaneously at
various positions in the same protein where tryptophan residues are
located. Thus, the adaptation of mutagenesis remarkably improved the
validity of the fluorescence spectroscopy to advance in knowledge on the
protein dynamics, conformation, and function.

Hen egg-white lysozyme (HEWL) is one example of multi-tryptophan
containing protein. But it is an interesting enzyme to examine the correlation
between the protein dynamics and its enzymatic action through the
fluorescence spectroscopy if the individual tryptophan residue can be
discriminated. Because its crystallographic structure was confirmed and the
most dominant fluorophores, Trp62 and Trp108 are arranged around the
binding site. They play important roles in the binding with substrate or
inhibitor and the structural stabilization, respectively. Through the
fluorescence analysis of these tryptophan residues, the lysozyme-ligand
interaction and the ligand-induced conformational change around the
binding site have been discussed. But their results inevitably include some
ambiguities similarly with other multi-tryptophan containing proteins,
because of the spectral overlap of the fluorescence between Trp62 and Trp108, which belonged in the same domain in HEWL and between these residues and the tryptophan residues, Trp28 and Trp111 located at the hydrophobic matrix. Recently, we differentially separated the intrinsic fluorescence spectra of Trp62 and Trp108 using the mutant lysozyme and the quenching resolved techniques.\(^7\) The fluorescence maxima of Trp62 and Trp108 were seen at 352 nm and 342 nm, respectively, and the fluorescence of Trp28 and Trp111 was localized in the wavelength region of 300 nm-360 nm. These results are very useful not only to characterize the ligand-induced conformational change at the multiple site but also to trace the individual segmental motions of each tryptophan residue by selecting the emission wavelength appropriately. Hitherto, the molecular dynamics of the segmental motion of each tryptophan is not confirmed, although Ichiye and Karplus reported the result by the theoretical computer simulation.\(^8\)

We described in this section the molecular dynamics of the internal motion in hen egg-white lysozyme and its responses to the ligand-binding effect using the steady state and time-resolved fluorescence depolarization of two mutant lysozymes, W62Y- and W108Y-lysozyme, in which Trp62 or Trp108 was replaced with tyrosine residue, respectively. These two mutant lysozymes were very effective for separating the fluorescence of Trp62 and Trp108.

5.2.2 Materials and Methods

Preparation of mutant lysozymes and measurements of steady-state fluorescence spectrum and anisotropy were described in CHAPTER 2. The method of fluorescence quenching experiment was described in CHAPTER 3.

5.2.3 Results

**Fluorescence Spectrum.**

As shown in CHAPTER 3, W108Y-lysozyme gave a fluorescence maximum at 342 nm similarly with HEWL. The fluorescence spectra of W62Y-lysozyme in which Trp108 was the most dominant fluorophore was deviated to the higher energy side and its maximum wavelength was found at 336 nm. When W108Y-lysozyme formed a complex with (NAG)\(_3\), the fluorescence intensity was reduced about 10% and, at the same time, the fluorescence maximum shifted to 335 nm. On the other hand, W62Y-
lysozyme enhanced its own fluorescence remarkably at the shorter wavelength side to give a shoulder around 320 nm in the complex with (NAG)$_3$.

**Fluorescence decay kinetics.**

The fluorescence decay kinetics of the mutant lysozymes and their complexes with (NAG)$_3$ were determined through the whole spectral regions. The decay parameters at 320 nm, 340 nm, and 380 nm are listed in Table 5.2.1. Although the fluorescence decay kinetics of the free lysozyme showed three or four components according to the monitored emission wavelengths, the more components involved in the decay at the shorter wavelength region. One characteristics of the fluorescence decay of the mutant lysozyme is that very short decay components of which decay times are 40–80 ps are seen at the shorter wavelength side but not at the longer wavelengths. On the binding with (NAG)$_3$, the fluorescence decay kinetics of the mutant lysozymes became simpler, i.e., the number of components involved in the fluorescence decay were reduced. The average lifetime decided by the close measurement of the decay kinetics at the whole spectral region, which was defined as $\tau_{av} = \Sigma a_i \tau_i$, was used to evaluate the collisional fluorescence quenching constant.

**Collisional Fluorescence Quenching Constants.**

Stern-Volmer constant (K$_{SV}$) of the fluorescence of mutant lysozymes were given by the slope in the plot of $I_0/I$ against the concentration of acrylamide using Stern-Volmer equation (3.1). W108Y-lysozyme showed beautiful linear line in the Stern-Volmer plots as shown in Fig. 5.2.1 and resulting K$_{SV}$ depended on the monitored emission wavelength. Although the data are not shown, the fluorescence of W62Y-lysozyme also gave linear relation between $I_0/I$ and [Q] at various wavelengths. The collisional fluorescence quenching constants of W62Y-lysozyme, W108Y-lysozyme, and their complexes with (NAG)$_3$ calculated with the average lifetime and Stern-Volmer constants (K$_{SV}$) were given in Fig. 5.2.2 together with the fluorescence spectra. As shown in Fig. 5.2.2, the collisional quenching rate of mutant lysozyme and their complexes with (NAG)$_3$ were constant at the wavelengths longer than their fluorescence maximum. Each tryptophan residue intrinsically retains the peculiar collisional quenching constant according to the own location and its values must be constant regardless the emission wavelength. Therefore, the emission wavelength independence of k$_q$ reveals
<table>
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<th>Wavelength (nm)</th>
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<th>$\tau_3$ (ns)</th>
<th>$\tau_4$ (ns)</th>
<th>$\alpha_1$</th>
<th>$\alpha_2$</th>
<th>$\alpha_3$</th>
<th>$\alpha_4$</th>
<th>$\sigma$</th>
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<td>0.55</td>
<td>-</td>
<td>-</td>
<td>1.02</td>
<td>1.93</td>
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</table>

Excitation wavelength, 300 nm
Fig. 5.2.1 Emission wavelength dependence of Stern-Volmer plots for the fluorescence quenching of W108Y-lysozyme by acrylamide. Line a, b, c, d, e, and f are 320, 330, 340, 350, 360, and 380 nm, respectively. The concentration of acrylamide is shown by [Q]. The plot was based on Stern-Volmer equation. Excitation wavelength, 300 nm. Temperature, 25°C.
Fig. 5.2.2 The emission wavelength dependence of the collisional fluorescence quenching constant $(k_q)$ of free mutant lysozymes (A) and their complex with (NAG)$_3$ (B).

○, W108Y-lysozyme; ●, W62Y-lysozyme. Solid and broken lines in A and B are fluorescence spectra of W108Y- and W62Y-lysozyme, respectively. $k_q$ was calculated by the average lifetimes and Stern-Volmer constant $(K_{SV})$ using $k_q = K_{SV}/\tau$. 

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that the fluorescence of W62Y- and W108Y-lysozymes and their complexes originate in Trp108 or Trp62, respectively, at the wavelength region longer than 360 nm. Because Trp108 or Trp62 is surrounded in polar circumstances and their fluorescence spectra deviate to the lower energy side.

Steady-State and Time-Resolved Fluorescence Anisotropy.

Fig. 5.2.3 shows the steady-state fluorescence anisotropy of HEWL, W62Y- and W108Y-lysozyme (A) and their complexes with (NAG)₃ (B). When the fluorescence anisotropy was measured at 380 nm, on the excitation at 300 nm, W62Y-lysozyme gave the higher value than W108Y-lysozyme. Since the molecular weight of these lysozyme are same, this difference in the fluorescence anisotropy value is caused by difference in the internal motion of the tryptophan residues in lysozyme. The fluorescence of W62Y-lysozyme and W108Y-lysozyme at 380 nm are attributed to Trp 108 and Trp62, respectively. Therefore, it is concluded that Trp62 is allowed to fluctuate more freely than Trp108. The fluorescence anisotropy of HEWL reasonably showed the intermediate value between W62Y- and W108Y-lysozyme. The fluorescence of HEWL is consisted of the contribution from Trp62 and Trp108 at 380 nm. In the complex of W108Y-lysozyme with (NAG)₃, the fluorescence anisotropy of W108Y-lysozyme increased about 25% indicating the internal motion of Trp62 was considerably restricted. Although the fluorescence anisotropy of HEWL was also increased, it is very interesting to note that W62Y-lysozyme gave the smaller anisotropy in the complex with the ligand than in the free enzyme. This fact suggests that the internal motion of Trp108 is liberated from the restriction in the complex by contrast to the case of Trp62.

The fluorescence anisotropy decay profiles of two mutant lysozymes, W108Y-, W62Y-lysozyme, monitored at 380 nm were shown in Fig. 5.2.4. The fluorescence anisotropy of W108Y-lysozyme decayed faster than one of W62Y-lysozyme. When the fluorescence anisotropy decay of W108Y-lysozyme-ligand complex was plotted with log-scale, it gave slower decay curve than one of free lysozyme as shown in Fig. 5.2.5. These results support the conclusions obtained by the steady-state anisotropy studies that Trp62 fluctuates more freely than Trp108 and that the internal motion of Trp62 would be restricted by the interaction with (NAG)₃. The ligand induced change in the fluorescence anisotropy decay of W62Y-lysozyme was not so large as the clear difference could be recognized in decay profile. But the fluorescence anisotropy of W62Y-lysozyme decayed faster in the complex with
Fig. 5.2.3  Steady-state fluorescence anisotropy of HEWL, W62Y-lysozyme, and W108Y-lysozyme (A) and their complexes with (NAG)_3 (B).

\( \nabla \), W62Y-lysozyme; \( \bigcirc \), HEWL; \( \bullet \), W108Y-lysozyme. Concentration of the lysozymes, 10 \( \mu \text{M} \); concentration of (NAG)_3, 1 mM; excitation wavelength, 300 nm; emission wavelength, 380 nm.
Fig. 5.2.4 Fluorescence anisotropy decay of W108Y (upper) and W62Y-lysozyme (lower).
Excitation wavelength, 300 nm; emission wavelength, 380 nm; band width, 4 nm; channel width, 10 ps/ch. Concentration of the mutant lysozymes were 10 μM. Temperature, 25°C.
Fig. 5.2.5 Semi-log plot of the fluorescence anisotropy decay of W108Y-lysozyme (b) and the complex with (NAG)$_3$ (a). Excitation and emission wavelengths are 300 nm and 380 nm, respectively. Channel width is 10 ps/ch. Concentration of W108Y-lysozyme is 10 μM and the anisotropy decay of the complex was observed at the presence of 1 mM of (NAG)$_3$. Temperature, 25°C.
(NAG)_3. When the fluorescence anisotropy decay were described with a linear combination of some exponentials as equation (5.2.1),

\[ r(t) = \sum \beta_i \exp(-t/\phi_i) \]  

where \( \phi_i \) was the rotational correlation time of i-th component and \( \beta_i \) was the corresponding amplitude, the characteristics of the segmental motion of Trp62 and Trp108 and their responses to the ligand binding were more cleared. The fluorescence anisotropy decay parameters of mutant lysozymes were summarized in Table 5.2.II. In every fluorescence anisotropy decay kinetics, the rotational correlation time within the range of 4.0–4.6 ns were commonly found. This time scale is in the consistent range with the rotational correlation time for the entire rotation of lysozyme which was calculated by Einstein-Stokes relationship, \( \phi = \eta V/kT \), \( \eta \), viscosity of water; \( V \), hydrated volume of lysozyme; \( k \), Boltzman constant; \( T \), temperature. It is interesting to note that the correlation time for the entire rotation of W108Y-lysozyme is shorter than one of W62Y-lysozyme and that the ligand binding reduced this correlation time in spite of the practical increase in the molecular weight. These results imply that W108Y-lysozyme keeps a more densely packed shape and the ligand would make the structure of lysozyme more compact. W108Y-lysozyme gave the best fit to triple-exponential decay kinetics characterized with correlation times of \( \phi_1 = 0.05 \) ns, \( \phi_2 = 0.53 \) ns, and \( \phi_3 = 4.12 \) ns. Since the longest correlation time \( \phi_3 \) is consistent with one of the entire rotation of the lysozyme, \( \phi_1 \) and \( \phi_2 \) involved in the segmental motion of Trp62. The fractional amplitudes, \( f_1 = \beta_1/\sum \beta_i \) and \( f_2 = \beta_2/\sum \beta_i \), which were a measure for the freedom of the segmental motion, were 0.11 and 0.46, respectively. The fluorescence anisotropy decay of W62Y-lysozyme was described with double exponentials and one of two correlation times also correspond to one for the entire rotation of the lysozyme. The fraction of the amplitude of the fast decay component was small and it was estimated to be 0.22.

The fluorescence anisotropy decay of W108Y-lysozyme was drastically changed by the interaction with (NAG)_3 as seen apparently in Fig. 5.2.5. The decay kinetics was changed to double exponential characterized with the correlation times, 0.26 and 4.0 ns. Concomitantly with the change in the decay kinetics, the segmental motion of Trp62 was suppressed by the binding of (NAG)_3 as shown by the reduction of the fractional amplitude of the segmental motion. The amplitude of the fast decay component of W62Y-lysozyme was increased in the complex with (NAG)_3. This result is consistent
### Table 5.2.II  Anisotropy Decay Parameters of Mutant Lysozymes and Complexes with (NAG)$_3$

<table>
<thead>
<tr>
<th></th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\beta_3$</th>
<th>$\phi_1$ (ns)</th>
<th>$\phi_2$ (ns)</th>
<th>$\phi_3$ (ns)</th>
<th>$f_1$ (%)</th>
<th>$f_2$ (%)</th>
<th>$f$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W108Y</td>
<td>0.03</td>
<td>0.13</td>
<td>0.12</td>
<td>0.05</td>
<td>0.53</td>
<td>4.12</td>
<td>11</td>
<td>46</td>
<td>57</td>
</tr>
<tr>
<td>W108Y+(NAG)$_3$</td>
<td>0.10</td>
<td>-</td>
<td>0.20</td>
<td>0.26</td>
<td>-</td>
<td>4.00</td>
<td>33</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>W62Y</td>
<td>0.06</td>
<td>-</td>
<td>0.21</td>
<td>0.22</td>
<td>-</td>
<td>4.60</td>
<td>22</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>W62Y+(NAG)$_3$</td>
<td>0.07</td>
<td>-</td>
<td>0.22</td>
<td>0.22</td>
<td>-</td>
<td>4.50</td>
<td>24</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

Ex. = 300 nm. Em. = 380 nm. Temp. = 30 ºC
with the ligand binding effect on the steady state fluorescence anisotropy of W62Y-lysozyme. In order to obtain the more detail information on the dynamics of the internal motion of lysozyme, temperature dependence of the rotational correlation times and the corresponding amplitudes were investigated at various temperatures. Fig. 5.2.6 shows the temperature dependence of the fractional amplitude \((f=\beta_i/\Sigma \beta_i)\) and rotational correlation time of the faster decay component of W62Y-lysozyme. The fractional amplitude decreased with the temperature from 10°C to 30°C, although the rotational correlation time of the segmental motion were abruptly increased at higher temperature than 30°C.

The temperature dependence of freedom of the segmental motion of Trp62 in W108Y-lysozyme was shown in Fig. 5.2.7. Sum of the motional freedoms of two segmental motions was constantly 0.6 and it was almost independent on the temperature in the range of 10°C-40°C. However, freedom of the faster motion decreased and, contrarily, one of the slower motion increased with increasing in temperature. When the temperature dependence of the rotational correlation times of W108Y-lysozyme was investigated in order to evaluate the activation energy for the segmental motion of Trp62, both of the faster and slower components gave straight lines in Arrhenius plots as shown in Fig. 5.2.8. The activation energies estimated from the slopes were 0.8 eV (18.3 kcal/mol) and 0.35 eV (8.0 kcal/mol) for the faster and the slower segmental motions, respectively.

The fluorescence anisotropy decay kinetics of W62Y- and W108Y-lysozyme measured at 320 nm were double and triple exponentials, respectively. The rotational correlation time of the slowest decay component was also consistent with one of the entire rotation of the lysozymes. The correlation times of the faster rotational motions of these mutants were 0.18 ns for W62Y-lysozyme and 0.05 ns and 0.51 ns for W108Y-lysozyme. Also at 320 nm, the fluorescence anisotropy decay kinetics of W108Y-lysozyme changed to double exponential resulting in the reduction of the motional freedom of the tryptophan residue.

5.2.4 Discussion

The emission wavelength independence of collisional fluorescence quenching constants of W62Y- and W108Y-lysozyme definitely suggests that the fluorescence of mutant lysozyme was emitted from in single tryptophan
Fig. 5.2.6 Temperature dependence of the motional freedom and corresponding rotational correlation time of Trp108 in W62Y-lysozyme.

●, motional freedom ($f$); △, rotational correlation time ($\phi_1$). $f$ is given by an equation, $f = \beta_1 / \Sigma \beta_i$; see text.
Fig. 5.2.7 Temperature dependence of the rotational freedom of Trp62 in W108Y-lysozyme.

a, total freedom; b and c, the freedom of the faster and slower decay component. (See text.)
Fig. 5.2.8 Arrhenius plot for the reciprocals of the rotational correlation times of Trp62 in W108Y-lysozyme. 

$\phi_1$ and $\phi_2$, the rotational correlation times of the faster and slower relaxing component in the fluorescence anisotropy decay.
residue at the wavelengths longer than 360 nm. As discussed in CHAPTER 3, Trp62 or Trp108 must be the unique fluorophore in W108Y- and W62Y-lysozyme, respectively, at those wavelength. This fact demonstrates that it is possible to characterize the dynamics of the segmental motion of Trp62 or Trp108 by measuring the fluorescence anisotropy at the emission wavelength longer than 360 nm.

Although the fluorescence anisotropy decay kinetics, $r(t)$, was described with linear combination of exponential function, the corresponding decay parameters, $\phi_i$ and $\beta_i$, are connected with steady-state anisotropy $r_0$ by equation (5.2.2).

$$r_0 = \frac{\int F(t) \cdot r(t) \, dt}{\int F(t) \, dt}$$  \hspace{1cm} (5.2.2)

where $F(t)$ is the total fluorescence decay. The obtained parameters for the anisotropy decay of W108Y- and W62Y-lysozyme showed excellent fit with the steady-state anisotropy. The anisotropy just after the excitation, $r_0$, which is given by $r_0 = \sum \beta_i$, is decided by the transition moments of the absorption and fluorescence and is an important factor to discuss the depolarization process of tryptophan residues in protein. Parvalbumine and M13 coat protein of bacteriophage give the largest (0.34) and the smallest (0.14) value for $r_0$ of the tryptophan residue in protein, respectively, as far as we know.\textsuperscript{9,10} Although the excitation was performed at 300 nm to avoid the concomitant excitation of tyrosine residue, $r_0$ observed for the mutant lysozyme were within reasonable range (0.27-0.30). These results reveals that our fluorescence anisotropy measurement is accurate and any artifact is not included.

The fluorescence anisotropy decay of W108Y-lysozyme monitored at 380 nm, which corresponded to one of Trp62, was described with triple exponential decay function. As the longest rotational correlation time is consistent with one for the whole rotation of the mutant lysozyme, the other components are responsible for the segmental motion of Trp62. The rotational correlation times of these two segmental motion, one is shorter, the other longer, were in the time-range of 20–200 ps and 0.3–2.1 ns, respectively, at the investigated temperature. Based on the general consideration, the rotation of the indole moiety around $C_\alpha-C_\beta$ or wobbling of the hinge site to which Trp62 is connected are assumed for the origin of the rotational fraction of Trp62.\textsuperscript{11} Considering in the time-scale of two rotational correlation times, the faster component may correspond to the indole rotation around $C_\alpha-C_\beta$ and the slower to the wobbling of the hinge position. The activation energy for the
segmental motion suggest that wobbling of the peptide main chain resulting in the slowly rotating motion is rather feasible to the thermal agitation. When temperature is higher, this motion is more active. On the other hand, the motional freedom of the fluctuational rotation of Cα-Cφ is decreased with the increasing in the temperature. This anti-correlational relationship between these two segmental motions are interesting considering the internal motion of lysozyme.

The segmental motion of Trp108 revealed by the fluorescence anisotropy decay of W62Y-lysozyme at 380 nm was described with a single exponential function at 10–30°C. Its rotational correlation time of Trp108 correspond to one of the faster segmental motion of Trp62, therefore, it is expected that only the fluctuational rotation of indole moiety around Cα-Cφ would be allowed for Trp108 in W62Y-lysozyme. Since Trp108 is surrounded with the polar groups in a cage of the peptide chain according to the crystallographic structure and raman spectra of HEWL,\textsuperscript{12-14} it is anticipated that the segmental motion of Trp108 would be considerably restricted. Indeed, its motional freedom was small and it was within the range of f₁=0.27–0.22 at the temperature lower than 30°C. When temperature is increased higher than 35°C, the rotational correlation time and the motional freedom were increased abruptly. Considering in the similarity with the response to the temperature, it is speculated that the main segmental motion of Trp108 is switched from the rotation of Cα-Cφ bond to the wobbling of the hinge point where Trp108 is attached at this temperature. Probably, two type of internal motion would be allowed for Trp108 at higher temperature. Unfortunately we could not separate experimentally these motions anticipated for Trp108.

Because of the fluorescence spectral overlap of Trp28 and Trp111 with Trp62 or Trp108, it is difficult to describe the segmental motion of Trp28/Trp111 definitely. But it is possible to discuss them based on the well-confirmed segmental motion of Trp62 or Trp108 and the result in the close measurement of the fluorescence anisotropy decay at 320 nm. The fluorescence anisotropy decay of W62Y-lysozyme monitored at 320 nm, which was described a double exponential function, showed larger fractional amplitude than at 380 nm. This suggests that the segmental motions of Trp28 and/or Trp111 reserve the higher motional freedom than Trp108 at the hydrophobic matrix box. According to Nishimoto et al., the emissional contribution of Trp108 to the total fluorescence was 50% at 320 nm.\textsuperscript{7} If it is
allowed to assume that the measured parameters were averaged with the emissional contribution, the motional freedom of Trp28 and/or Trp111 is estimated 0.38 in the W62Y-lysozyme. In the similar consideration, the motional freedom of Trp28 and/or Trp111 of W108Y-lysozyme was smaller and its freedom was estimated to be 0.17. These results imply that the fluctuational motion at the hydrophobic matrix box is more active than the expected by the X-ray structure of HEWL. Furthermore, it is also interesting to note that the fluctuational motion at the hydrophobic matrix was suppressed by the replacement of Trp108 with tyrosine residue, more hydrophilic with smaller molecular size than tryptophan residue. Ichiye and Karplus applied the molecular dynamic simulation to the fluorescence depolarization of the individual tryptophan residue in HEWL. They showed that Trp62 reserved large motional freedom but the segmental motions of Trp28 and Trp111 were extremely suppressed.\textsuperscript{8} Their simulation result is not necessarily consistent with our result, especially a discrepancy was recognized for the motion at the hydrophobic matrix region. On the other hand, Cross and Fleming showed the fluctuational motion was active at the hydrophobic matrix box region tracing the fluorescence depolarization of eosin molecule bound to tyrosine.\textsuperscript{15} Their report is quite consistent with our result.

Recently, we have reported that HEWL delicately change its own conformational according to the interaction with the ligand such as (NAG)\textsubscript{3}.\textsuperscript{16,7} The effects of the ligand binding on the steady-state and time-resolved fluorescence anisotropy of mutant lysozyme demonstrate that the segmental dynamics of the tryptophan residues would be modified concomitantly with the conformational alteration. The increase in the steady-state anisotropy and the retardation in the anisotropy decay of W108Y-lysozyme at 380 nm consistently reveals that the segmental motion of Trp62 is restricted in the lysozyme complex with (NAG)\textsubscript{3}. The fluorescence anisotropy decay kinetics of W108Y-lysozyme-(NAG)\textsubscript{3} complex was switched to the double exponential indicating the component of the segmental motion was reduced to be single. Although it is difficult to decide which component, the faster or the slower, was restricted by the interaction, we can propose an evidence showing that the fluctuation of Trp62 by wobbling would be exclusively suppressed in the fluorescence anisotropy decay studies on Kyn62-lysozyme in which Trp62 of HEWL was chemically modified to
The segmental motion of kynurenine residue in Kyn62-lysozyme revealed by the fluorescence anisotropy decay monitored at 450 nm on the excitation at 360 nm, was described with double exponentials, one is faster, the other slow, similarly with W108Y-lysozyme. When (NAG)$_3$ was bound to Kyn62-lysozyme, only the slower segmental motion of kynurenine was completely prohibited.

The fluctuation of Trp108 was considerably limited in the cage of peptide chain in the free enzyme. But the freedom of the segmental motion was rather increased, when W62Y-lysozyme interact with (NAG)$_3$. Although this result is also supported by the steady state fluorescence anisotropy of W62Y-lysozyme, it suggests the segmental motion of Trp108 would be liberated from some restriction in the peptide cage when this mutant lysozyme bind to (NAG)$_3$.

When the degree of freedom of the segmental motion was allocated to Trp28/Trp111 and Trp62 or Trp108 in the mutant lysozyme according to the emissional contribution at 320 nm, it was reduced to 0.36 and 0.05 in the W62Y- and W108Y-lysozyme by binding of the mutant lysozymes, respectively. This result suggests that the binding effect of (NAG)$_3$ extends to the hydrophobic matrix box region to restrict the segmental motions of Trp28/Trp111. Because of the fluorescence superposition between Trp62 or Trp108 and Trp28/Trp111, the results of fluorescence quenching experiments also inevitably include some ambiguity. But the reduction of the collisional quenching constant for the fluorescence of Trp28/Trp111 in the lysozyme-(NAG)$_3$ complex is consistent with the results in the fluorescence depolarization studies.

Neither Trp108 nor Trp28/Trp111 directly takes part in the binding with (NAG)$_3$. Nevertheless, the conformations and dynamical properties of these tryptophan residues are modified by the interaction of lysozyme with the ligand. Nishimoto et al. showed a spectral alteration in the quenching resolved fluorescence spectra of Trp28/Trp111 due to the Trp108-dependent conformational deformation. Lumb et al. suggested that the ligand-induced conformational alteration around Trp28 closely related with one of the Trp108 analyzing the chemical shift in the $^1$H nuclear magnetic resonance studies. Although their works implies that the conformation of Trp108 and Trp28/Trp111 are mutually dependent with each other, the increase in the motional freedom of Trp108 may be caused by the restriction of the
fluctuational motion at the hydrophobic matrix box or vice versa. Trp28/Trp111 are arranged in the hydrophobic matrix and Trp108 is located adjacent to them to maintain a framework of lysozyme structure. Therefore, the change in the dynamical properties of these tryptophan residue concomitantly with the conformational change induced by the interaction with the ligand are very interesting to consider the relation between the protein flexibility and stability.

The motional freedom, $f_i$, is connected with the order parameter, $S^2$, which gives information on the angular displacement, $\mu$, responsible for the segmental motion of the tryptophan residue as equation (5.2.3):\begin{equation}
S^2 = 1 - \sum f_i = 1/2 \cdot \cos \mu (\cos \mu + 1) \tag{5.2.3}
\end{equation}

Fig. 5.2.9 shows the effect of (NAG)$_3$ binding on the angular displacement of the internal motion of Trp62, Trp108, and Trp28/Trp111. The angular displacement for the internal motion of Trp62 was reduced from $\mu = 56^\circ$ to $\mu = 42^\circ$ by the binding of (NAG)$_3$. Then, the static orientation of Trp62 may flip to Trp108. Because, it was shown in the energy transfer experiment using Kyn62-lysozyme that kynurenine (Trp62) changed its orientation by the binding with (NAG)$_3$, so that the energy transfer efficiency from Trp108 could be enhanced.\cite{20} Trp108 which is surrounded with polar side chain in the peptide cage is allowed to fluctuate more in the lysozyme-ligand complex. It is difficult to draw definite picture for the segmental motion of Trp28 or Trp111 because the spectral separation of these residues is not completed now. However, the fluctuational motion of Trp28/Trp111 was certainly suppressed in the hydrophobic matrix box region on the interaction of the mutant lysozyme with (NAG)$_3$.

In conclusion, we have shown that the segmental motions of four fluorescent tryptophan residues exhibited the peculiar dynamical properties according to their locations and they responded individually to the interactions with the ligand. Trp62, which was considered to associate closely with the binding with the ligand, reserve the larger motional freedom and, on the other hand, the internal motion of Trp108 was restricted considerably in the vicinity of the binding site of HEWL. Although it has been considered that the hydrophobic matrix box of HEWL had a densely packed structure, the fluctuational motions of Trp28/Trp111 were more active than one expected from the X-ray structure. On the binding with (NAG)$_3$, the segmental motions of Trp62 and Trp28/Trp111 were suppressed while one of Trp108, contrarily,
Fig. 5.2.9 The change in the segmental motion of Trp62, Trp108, and Trp28 on the formation of the (NAG)$_3$ complex.
Solid and broken line are the segmental motions of Trp residue free and bound lysozyme, respectively. Corn angle is estimated from order parameter $S^2 = \frac{\beta^2}{\sum \beta_i} = \cos \mu (\cos \mu + 1)$. (See text.) Trp62 is so drawn as it takes the parallel orientation to Trp108 on the free lysozyme. The motional freedom of Trp28/Trp111 was represented by the result in W108Y-lysozyme.
was rather amplified.

5.2.5 Conclusion
The internal motion of lysozyme was described at the multiple site of lysozyme using the steady-state and time-resolved fluorescence depolarization of tryptophan residues. The fluorescence of mutant lysozymes, W62Y- and W108Y-lysozyme, in which Trp62 or Trp108 of hen egg-white lysozyme was replaced with tyrosine residue, respectively, was exclusively ascribed to Trp108 or Trp62 at the longer wavelength region in their spectra. The segmental motion of Trp62 revealed by the anisotropy decay of the unique fluorescence was described with two components probably originated from the fluctuational rotation of indole moiety about Cα-Cβ bond and rotational wobble of the peptide portion adjacent to Trp62. Although Trp62 fluctuated freely with large motional freedom, its motion was remarkably suppressed by the interaction with trimer of N-acetyl-D-glucosamine. The segmental motion of Trp108 is hindered in the cage of the peptide chain at the temperature below 30°C. But it liberated itself from the restriction on the formation of complex with the ligand or by thermal agitation at the temperature higher than 35°C.

Because of the spectral overlaps of the tryptophyl fluorescences, it is difficult to describe definitely the segmental motion of Trp28 or Trp111. However, the close analysis of the fluorescence anisotropy decay of W62Y- and W108Y-lysozyme showed that the fluctuational motion at the hydrophobic matrix box was more active than one expected from the crystallographic structure although it was suppressed by the binding of the ligand to the active site of lysozyme.

5.2.6 References
A concept that the structural flexibility is essential for the bioactive protein to complete their biological function demands the more detail studies on the conformational change and its dynamical properties induced by the protein interaction with small molecule and other protein. As shown in the data provided by spectral analysis, quenching, decay analysis, energy transfer, and depolarization, the fluorescence spectroscopy is one of the most powerful methods for such study. But, the application of the fluorescence spectroscopy is to protein limited by a fact that this method requires a specifically designated fluorophore. Although many fluorescence spectroscopists and biochemists have been eager for the quantitative application of the fluorescence spectroscopy to the multitryptophan containing protein, recent development of the protein engineering method, such as site-directed mutagenesis or chemical modification, fortunately, showed a way to overcome the limitation of the fluorescence spectroscopy. These methods enabled the substitution of tryptophan residue with other amino acid residue or its analogue to separate the designated tryptophan residues with others.

Lysozyme is an interesting enzyme to examine the relationship among protein structure, dynamics and function through the fluorescence spectroscopy if the fluorescence of each tryptophan residue is individually identified. Because the most dominant fluorophore, Trp62 and Trp108, are arranged around binding site and other fluorescent tryptophans, Trp28 and Trp111, are located at the hydrophobic matrix box.

In the present work, two mutant lysozymes, W62Y- and W108Y-lysozyme, and one chemically modified lysozyme, Kyn62-lysozyme were used for discriminating the individual tryptophyl fluorescence in lysozyme. W62Y- and W108Y-lysozyme are hen egg-white lysozyme in which Trp62 is replaced with tyrosine residue, respectively, and Kyn62-lysozyme is an active lysozyme analogue in which Trp62 is oxidized to kynurenine. These lysozyme analogue reserve high enzyme activity and useful for the steady state and time-resolved fluorescence studies on the structure and dynamics of lysozyme.

The quenching resolved fluorescence spectra of W62Y-, W108Y-, and native lysozyme showed that Trp62 and Trp108 fluorescence maxima at 352
nm and 342 nm, respectively. Although these fluorescence maxima suggested that both of Trp62 and Trp108 are surrounded by polar circumstances, Trp108 is varied in the cage of the peptide chain. Because the collisional fluorescence quenching constant of Trp108 is estimated to be small. On the other hand, Trp62 exhibits larger collisional frequency and quenching cross section to acrylamide and its fluorescence spectral distribution was quite same to one of N-acetyltryptophanamide in aqueous solution. These fluorescence properties indicate that Trp62 is liberated from the peptide chain to be fully exposed to water molecule at the binding site.

The dynamics of the segmental motion of Trp62 and Trp108 expected by the spectroscopic properties of these two residues was consistently revealed by the result of the fluorescence depolarization studies on the mutant and chemically modified lysozyme. The segmental motion of Trp62 showed that two rotational components was caused by the rotational around $\text{C}_\alpha-\text{C}_\beta$ bond, and wobbling motion of the hinge position of the peptide where Trp62 was adjacent. Trp62 fluctuate so freely at the binding site that the motional freedom may reach to 0.52. The internal motion of Trp108 evaluated by the fluorescence anisotropy of W62Y-lysozyme suggested that only small fluctuational rotation around $\text{C}_\alpha-\text{C}_\beta$ bond was allowed for this residue in the peptide cage. Unfortunately, the fluorescence of Trp28 could not be separated from one of Trp111, because these two tryptophan residues belonged to a same domain in the lysozymes. But, the fluorescence of Trp28 and Trp111 resolved from the total fluorescence of the lysozymes gave maxima at 320 nm and 330 nm, respectively. Although the quantitative analysis was difficult because of the spectral overlap of these residues with Trp62 or Trp108, the collisional quenching constant for fluorescence of Trp28/Trp111 demonstrated that considerable fluctuation may be allowed at the hydrophobic matrix box. The fluorescence anisotropy decay kinetics of mutant lysozymes also showed that Trp28/Trp111 reserved the larger motional freedom at this region.

The fluorescence of Trp62 was quenched and shifted its spectrum to the higher energy side on the interaction of lysozyme with (NAG)$_3$, while Trp108 did not change its fluorescence spectral distribution. This observation suggests that some subtle alteration would be induced in the lysozyme conformation as previously reported by Lehrer.

The more clear conformational change in the vicinity of the binding site was shown by analysis of the intramolecular energy transfer in Kyn62-
lysozyme. The overlap integral, which is essential factor for the energy transfer, between kynurenine and tryptophan residue was large enough to form a energy donor-acceptor pair in Kyn62-lysozyme. When Kyn62-lysozyme form a complex with (NAG)$_3$, the energy transfer efficiency from Trp108 was greatly enhanced. The detail analysis using Föster formalism demonstrated that this enhancement of the energy transfer rate corresponded to such a conformational change as the kynurenine residue flopped to Trp108 to shorten the Kyn-Trp108 distance about 6Å. Since Kyn62 residue directly associates with the binding with (NAG)$_3$, just as Trp62 completes a role as subsite C in HEWL, Trp62 probably exhibits same behavior to kynurenine in Kyn62-lysozyme.

The fluorescence decay analysis of HEWL, Kyn62-lysozyme and mutant lysozyme, support a model for the ligand-induced conformational change proposed by Lehrer. According to his work, the protonated carboxylic group of Glu35 approach closer to Trp108 on the interaction with (NAG)$_3$. Since Trp62 comes closer to Trp108 in the lysozyme-ligand complex, the fluorescence states of these two tryptophan residues are destabilized by the proton donating quenching action of the protonated carboxylic group of Glu35. But, the present work demonstrate such conformational change depends on pH of the solution. The pH dependence of the fluorescence decay kinetics of the native and modified lysozymes suggest the carboxylic groups kept away from Trp108 when lysozymes form a complex with (NAG)$_3$ at pH 8.0. This finding is very interesting to consider the pH dependence of the enzymatic activity of lysozyme.

The ligand-induced conformational change around Trp62 and Trp108 may closely relate with the internal motion of lysozyme. The fluctuation of Trp62, allowed to move around freely in the unbound enzyme, was suppressed by the interaction with (NAG)$_3$. The change in the fluorescence anisotropy decay kinetics of W108Y suggested that Trp62 would be fixed to the peptide bond through the binding with (NAG)$_3$. It is difficult to give a rationalization for the liberation of the internal motion of Trp108 caused by the lysozyme-ligand interaction. But it should be noted that the motional freedom of Trp108 increased concomitantly with the conformational deformation of the hydrophobic matrix box. The hydrophobic matrix box region of lysozyme is packed more densely responding to this conformational distortion in the lysozyme-(NAG)$_3$ complex. Although more information is required, probably such re-packing of the hydrophobic matrix would give a
space for the fluctuation of Trp108. Because Trp108 is arranged at the portion adjacent to the hydrophobic matrix box.

A definite picture was not obtained for the conformation and dynamics of Trp28 and Trp111. But present results demonstrate that the ligand-binding effect extend to the conformation at the hydrophobic matrix region. Such long-range effect exerted in the ligand-protein interaction must be very interesting to consider the relationship between the conformational flexibility and function of protein.
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