

¹H-NMR Study on the Structure of Lysozyme from Guinea Hen Egg White

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The structure of lysozyme from guinea hen egg white (GEWL), which differs from hen egg white lysozyme (HEWL) by ten amino acid substitutions, was investigated by nuclear magnetic resonance (NMR) spectroscopy. GEWL and HEWL were very similar to each other in their tertiary structure as judged from the profile of ¹H-NMR spectra, pH titration, and an *N*-acetylglucosamine trisaccharide [(GlcNAc)₃] binding experiment. However, we have noticed several characteristics which distinguish GEWL from HEWL. The signal of Trp 108 indole N1H of GEWL was shifted upfield by about 0.3 ppm when compared with that of HEWL, and its hydrogen exchange was faster than that of HEWL. The p*K*_a values of Glu 35 estimated from the pH titration curve of Trp 108 indole N1H were different between GEWL and HEWL. From a careful examination of spectral changes caused by (GlcNAc)₃ binding, the changes in the chemical shift values of Trp 28 C5H and Asn 59 αCH of GEWL were found to be slightly larger than those of HEWL. Ile 55 of HEWL is replaced by valine in GEWL. Such a replacement may affect the neighboring hydrogen bonding between the main chain C=O of Leu 56 and Trp 108 indole N1H, resulting in a change in the microenvironment of the substrate-binding site near Trp 108.

The substrate-binding site of hen egg white lysozyme (HEWL) is composed of six subsites, called subsites A, B, C, D, E, and F, and the β-1,4-glycosidic linkage of the substrate located between subsites D and E is hydrolyzed by general acid catalysis involving γ-COOH of Glu 35 (1). The enzyme has been recognized to catalyze transglycosylation in addition to hydrolysis (2-4). However, the molecular mechanism of the highly efficient transglycosylation has not been elucidated yet. Since the binding of the transglycosylation acceptor to subsites E and F should be essential for transglycosylation, more information on subsites E and F is required to explain the high efficiency of transglycosylation. Although subsites A, B, and C have been intensively studied by means of chemical modifications (5-7), substrate binding experiments and kinetic analyses (8-10), information on subsites D, E, and F is quite limited. This is because no reporter group is available which can give information on the fine structure of this region.

Lysozymes have been purified from various avian egg whites, and their amino acid sequences are now established (11). The amino acid sequences of HEWL and the lysozyme from guinea hen egg white (GEWL), which have been determined by Canfield (12) and Jolles *et al.* (13), respectively, are shown in Fig. 1. There are ten amino acid substitutions between the sequences of the lysozymes. Among these amino acid substitutions, it should be noted

that the arginine residue at position 114 of HEWL is substituted by histidine in GEWL. A conformational energy calculation and a monoclonal antibody binding experiment indicated that, in subsites D, E, and F, there are two binding modes for hexasaccharide substrate, (GlcNAc)₆, that is, the "left-sided" binding mode and the "right-sided" binding mode (14, 15). Arginine 114 in HEWL is considered to participate in the productive binding of the substrate with the "right-sided" mode (16). Therefore, GEWL should be useful for investigation of the "right-sided" binding site of subsites E and F, because the imidazole ring of histidine is an appropriate reporter group for physicochemical analysis, such as nuclear magnetic resonance spectroscopy. However, except for position 114, nine amino acids are not identical between HEWL and GEWL, indicating that local environments in the protein molecule of GEWL may be somewhat different from those of HEWL. Prior to an investigation of subsites E and F by using GEWL, it is necessary to understand differences in the local environments between the two lysozymes.

Nuclear magnetic resonance (NMR) spectroscopy is useful for investigation of the environment of a specific amino acid residue in a protein molecule. Dobson and his colleagues have intensively studied the ¹H-NMR spectrum of HEWL and assigned most of the resonances for HEWL from the two-dimensional NMR spectra (17). In this paper, we have analyzed the ¹H-NMR spectra of GEWL under various conditions on the basis of the previous assignment of the resonances for HEWL, and found differences in the local environments of some amino acid residues between GEWL and HEWL. The differences between GEWL and

Abbreviations: HEWL, lysozyme from hen egg white; GEWL, lysozyme from guinea hen egg white; NMR, nuclear magnetic resonance; DQF-COSY, double quantum filtered COSY; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; (GlcNAc)_n, β-1,4-linked oligosaccharide of GlcNAc with the polymerization degree, *n*.

										10										20
HEWL:	Lys	Val	Phe	Gly	Arg	Cys	Glu	Leu	Ala	Ala	Ala	Met	Lys	Arg	His	Gly	Leu	Asp	Asn	Tyr
GEWL:	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
																				30
HEWL:	Arg	Gly	Tyr	Ser	Leu	Gly	Asn	Trp	Val	Cys	Ala	Ala	Lys	Phe	Glu	Ser	Asn	Phe	Asn	Thr
GEWL:	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
																				40
HEWL:	Gln	Ala	Thr	Asn	Arg	Asn	Thr	Asp	Gly	Ser	Thr	Asp	Tyr	Gly	Ile	Leu	Gln	Ile	Asn	Ser
GEWL:	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	Val	+++	+++	+++	+++	+++
																				50
HEWL:	Arg	Trp	Trp	Cys	Asn	Asp	Gly	Arg	Thr	Pro	Gly	Ser	Arg	Asn	Leu	Cys	Asn	Ile	Pro	Cys
GEWL:	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
																				60
HEWL:	Ser	Ala	Leu	Leu	Ser	Ser	Asp	Ile	Thr	Ala	Ser	Val	Asn	Cys	Ala	Lys	Lys	Ile	Val	Ser
GEWL:	+++	+++	+++	Gln	+++	+++	+++	+++	+++	+++	Thr	Ala	+++	+++	+++	+++	+++	+++	+++	+++
																				70
HEWL:	Asp	Gly	Asn	Gly	Met	Asn	Ala	Trp	Val	Ala	Trp	Arg	Asn	Arg	Cys	Lys	Gly	Thr	Asp	Val
GEWL:	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	Lys	His	+++	+++	+++	+++	+++	+++
																				80
HEWL:	Gln	Ala	Trp	Ile	Arg	Gly	Cys	Arg	Leu											
GEWL:	Arg	Val	+++	+++	Lys	+++	+++	+++	+++											

Fig. 1. Amino acid sequences of GEWL and HEWL. In GEWL, +++ indicates the same amino acid as the corresponding residue in HEWL.

HEWL are discussed in terms of the amino acid substitutions.

MATERIALS AND METHODS

Materials—Six-times-recrystallized HEWL was purchased from Seikagaku Kogyo. Guinea hen egg was kindly donated from Kumamoto Zoological Park, Kumamoto, and GEWL was purified from the egg white by the method of Jolles *et al.* (13) with a slight modification. D₂O (99.96%) was purchased from MSD Isotopes, and DCl (99%), NaOD (99%), deuterioacetic acid (acetic-d₃ acid-d, 99.5%), and sodium deuterioacetate (sodium acetate-d₃, 99%) were from Aldrich Chemical. *N*-Acetylglucosamine trisaccharide [(GlcNAc)₃] was prepared by acid hydrolysis of chitin followed by charcoal chromatography (18).

NMR Measurement—Six milligrams of the lysozyme was dissolved in 1 ml of D₂O, and the solution was incubated at 80°C for several minutes to replace the exchangeable hydrogens with deuterium. After being lyophilized three times from D₂O, the lysozyme was dissolved in 0.5 ml of D₂O. The pH was adjusted to an appropriate value by addition of diluted DCl or NaOD. The pH value was determined at room temperature by direct meter reading without correction for isotope effect. ¹H-NMR spectra were recorded at 500 MHz with a spectral width of 6,250 Hz using a JEOL GX-500 spectrometer. The HDO resonance was saturated by means of selective gated irradiation. Two-dimensional double quantum filtered COSY (DQF-COSY) spectra of the lysozymes were taken in the phase-sensitive mode, with a spectral width of 6,250 Hz in each dimension. Forty-eight scans were used for each of 512 *t*₁ values with 4,096 complex points in the *w*₂ dimension. All measurements were performed at 35°C, except for the measurement of hydrogen exchange rate.

For investigation of tryptophan indole N1H, lysozyme was dissolved in 90% H₂O/10% D₂O, and the spectra were obtained under the same conditions as mentioned above except for a spectral width of 8,000 Hz.

Determination of p*K*_a from Titration Curve—For determination of p*K*_a values of the histidines, pH titration was performed at the ionic strength of 0.1. Chemical shifts of the C2H and C4H of the histidines were plotted against pH,

and p*K*_a values of the histidines were obtained from the equation,

$$\delta_i = \frac{\delta HA + \delta A^- \times 10^{(pH_i - pK_a)}}{1 + 10^{(pH_i - pK_a)}} \quad (1)$$

where δHA is the chemical shift of the C2H proton of the fully protonated histidine, δA^- is that of the fully deprotonated one, and *pH*_{*i*} and δ_i are the independent and the dependent variables, respectively. The parameters δHA , δA^- , and p*K*_a were obtained by the Gauss-Newton non-linear least-squares fitting procedure on the basis of the experimental titration data. The apparent p*K*_a of Glu 35 was also determined from the pH titration curve of the resonance of Trp 108 indole N1H using Eq. 1.

Hydrogen Exchange Rate—The rate of hydrogen-deuterium exchange of tryptophan indole N1H was determined by following the decrease in peak area of the NMR signal of individual indole N1H. The exchange reaction was started by dissolving the lyophilized lysozyme in D₂O. After standing at 27°C for an appropriate period to advance the exchange reaction, accumulation was performed at 27°C under the same conditions as used for the measurements in 90% H₂O/10% D₂O. Accumulation of a spectrum required 5 min.

(GlcNAc)₃ Binding—After replacement of exchangeable hydrogens with deuterium, lysozyme and (GlcNAc)₃ were separately dissolved in sodium deuterioacetate buffer (pH 4.3). A portion of the (GlcNAc)₃ solution was added to the lysozyme solution, and the ¹H-spectrum was obtained under the same conditions as used for the measurements in D₂O. In the case of low pH, sodium deuterioacetate-DCl buffer (pH 2.3) was used. In order to investigate the spectral change concomitant with the increase of (GlcNAc)₃ concentration, (GlcNAc)₃ solution was added stepwise, and the spectra of the lysozyme-(GlcNAc)₃ mixtures at various molar ratios were obtained. For determination of the chemical shift values for the lysozyme-(GlcNAc)₃ complex, the chemical shift values of individual resonances were plotted against the concentration of (GlcNAc)₃, and the limiting chemical shifts at high (GlcNAc)₃ concentration were estimated from the titration curves. The binding constants were estimated from Scatchard plot analysis based on the spectral change in the resonance for Trp 28

C5H, which is completely separated from other resonances and most clearly responds to the addition of $(\text{GlcNAc})_3$.

RESULTS

^1H -NMR Spectra of GEWL and HEWL in D_2O —In order to assign the resonances for GEWL, we compared the spectra of GEWL and HEWL, of which the upfield and downfield regions are shown in Fig. 2. The resonances for HEWL have been assigned by Redfield and Dobson (17) as indicated in Fig. 2B. One of two histidine C2H resonances in the GEWL spectrum (peak q) exhibits the chemical shift similar to that of His 15 C2H in the HEWL spectrum. This can be assigned to His 15 C2H of GEWL, and the other (peak p) to His 114 C2H. The other resonances of GEWL indicated in Fig. 2A exhibit profiles and chemical shift values comparable to those of the corresponding resonances of HEWL indicated in Fig. 2B. From the DQF-COSY spectra of these lysozymes, we found that the COSY connectivities of the indicated resonances in Fig. 2A are quite similar to those of the corresponding resonances in

Fig. 2B. For example, as shown in the aromatic regions of the DQF-COSY spectra (Fig. 3), similar connectivities of Trp 28, Trp 63, and Trp 108 were observed for both lysozymes. Therefore, the resonances of GEWL labeled in Fig. 2A could be directly assigned to the same hydrogens as assigned for the corresponding resonances of HEWL. This assignment was confirmed by the facts that the responses of the assigned resonances to pH titration and to $(\text{GlcNAc})_3$ binding are very similar between GEWL and HEWL. These results indicate that GEWL and HEWL are basically very similar to each other in their tertiary structure.

pH Titration—The pH titration curve of histidine resonances provides information on the electrostatic environment of the histidine side chain. The spectra for GEWL were obtained at various pH values, and the chemical shift values of the resonances of His 15 and His 114 were plotted against pH to obtain the pH titration curve (Fig. 4). In those experiments, each of the histidine C4H resonances was assigned by obtaining the pH difference spectrum (19). From the nonlinear least-squares fitting analysis, the pK_a s for His 15 and His 114 were determined to be 5.70 ± 0.01

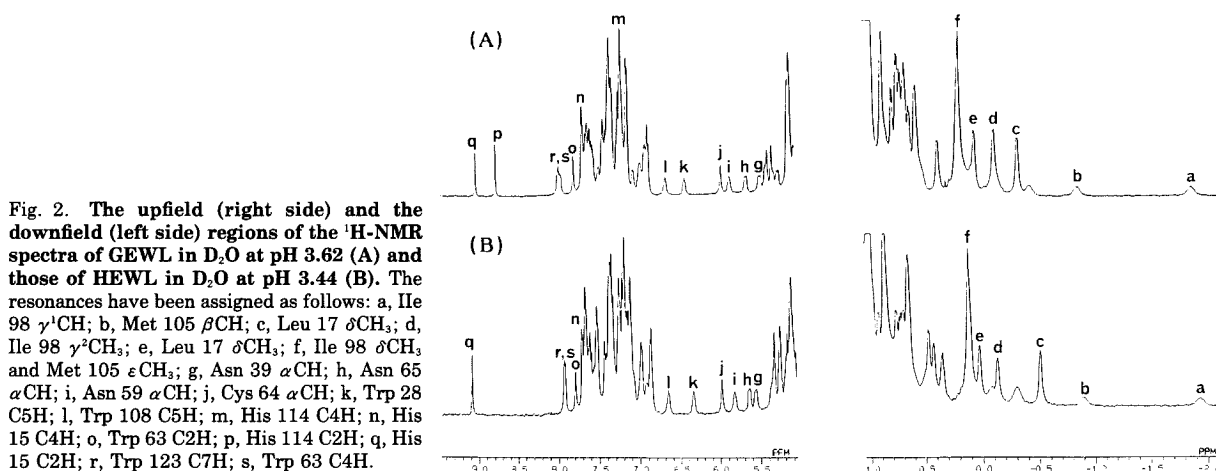


Fig. 2. The upfield (right side) and the downfield (left side) regions of the ^1H -NMR spectra of GEWL in D_2O at pH 3.62 (A) and those of HEWL in D_2O at pH 3.44 (B). The resonances have been assigned as follows: a, Ile 98 $\gamma^1\text{CH}$; b, Met 105 βCH ; c, Leu 17 δCH_3 ; d, Ile 98 $\gamma^2\text{CH}_3$; e, Leu 17 δCH_3 ; f, Ile 98 δCH_3 and Met 105 ϵCH_3 ; g, Asn 39 αCH ; h, Asn 65 αCH ; i, Asn 59 αCH ; j, Cys 64 αCH ; k, Trp 28 C5H; l, Trp 108 C5H; m, His 114 C4H; n, His 15 C4H; o, Trp 63 C2H; p, His 114 C2H; q, His 15 C2H; r, Trp 123 C7H; s, Trp 63 C4H.

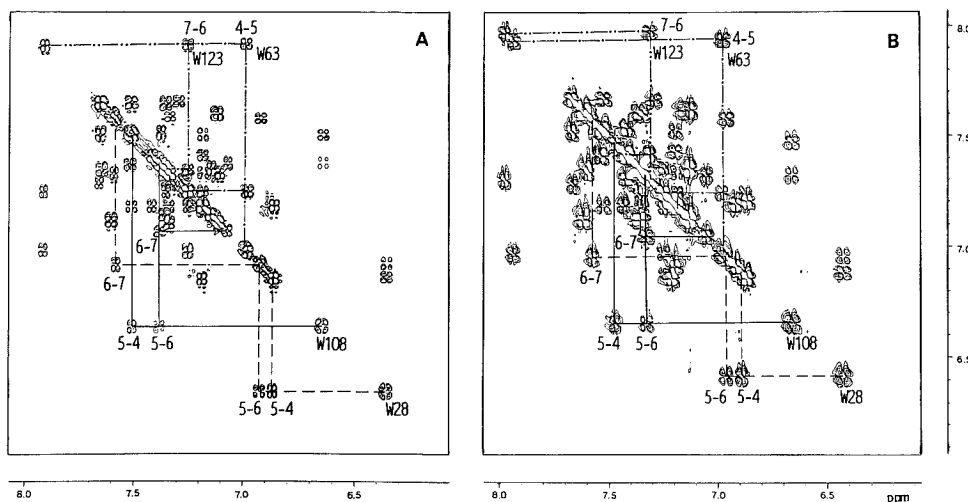


Fig. 3. Aromatic region of the phase-sensitive DQF-COSY spectra of HEWL (A) and GEWL (B).

and 6.16 ± 0.02 , respectively. Since the reported pK_a value for His 15 of HEWL is 5.8 (20), the environment of His 15 of GEWL seems similar to that of HEWL.

Tryptophan Indole N1H Hydrogens—HEWL has six tryptophan residues at positions 28, 62, 63, 108, 111, and 123, and all of the tryptophan residues are conserved in GEWL (Fig. 1). The resonances of tryptophan indole N1H hydrogens of HEWL have been intensively studied by Cassels *et al.* (21), and are assigned to individual tryptophans. On the basis of the assignment for HEWL, the

tryptophan residues in GEWL were investigated by NMR measurement in 90% $H_2O/10\%$ D_2O . As shown in Fig. 5, the profiles of four resonances which appear in the downfield region of the GEWL spectrum (10.2–11.0 ppm) are quite similar to those of Trp 62, Trp 63, Trp 111, and Trp 123 of HEWL. These resonances of GEWL can most likely be assigned to the same indole N1H hydrogens as the corresponding resonances of HEWL. However, the N1H resonance tentatively assigned to Trp 108 of GEWL appears to be shifted upfield in comparison with that of HEWL. We have obtained evidence supporting these assignments from the pH titration experiment described below.

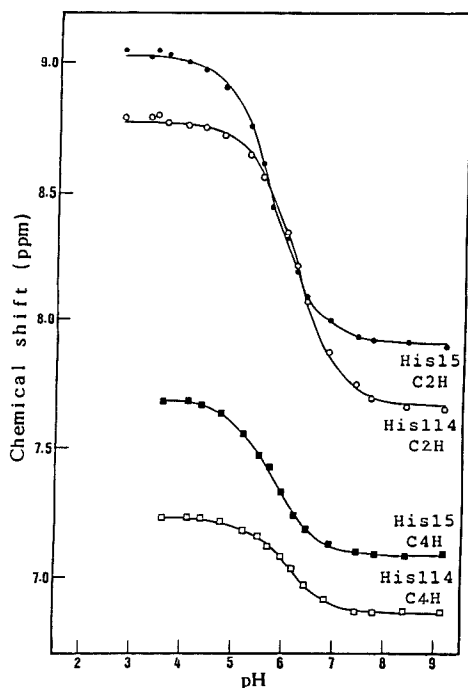


Fig. 4. pH dependence of the chemical shift values of the histidine C2H and C4H in GEWL at 35°C. The ionic strength was adjusted to 0.1 by addition of NaCl.

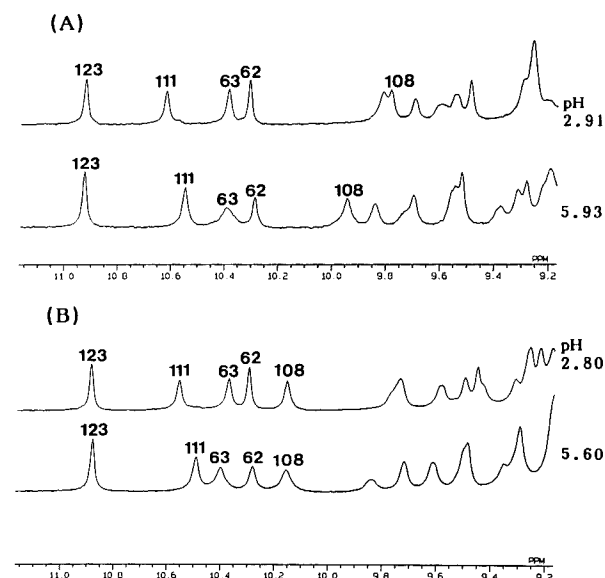


Fig. 5. Tryptophan indole N1H region of the ^1H -NMR spectra of GEWL (A) and HEWL (B) dissolved in 90% $H_2O/10\%$ D_2O at 35°C. Numerals in the figure indicate the residue numbers of the tryptophans.

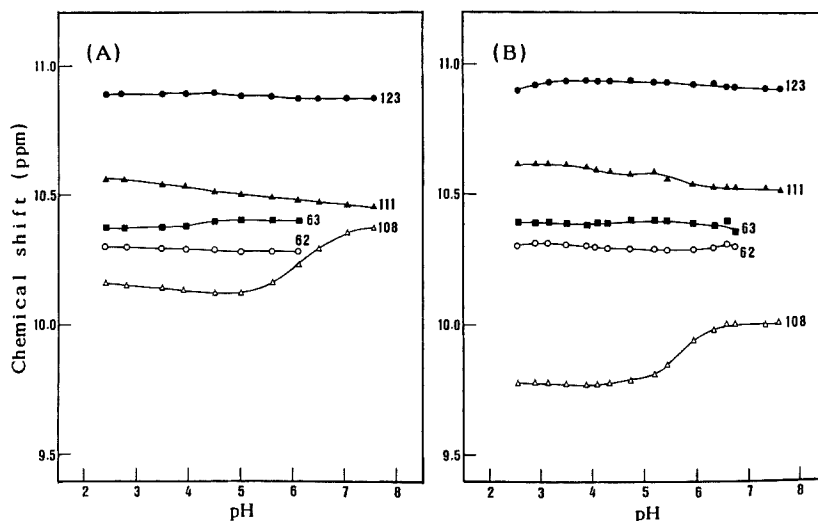


Fig. 6. pH dependence of the chemical shift values of the tryptophan indole N1H for HEWL (A) and GEWL (B) in 90% $H_2O/10\%$ D_2O at 35°C. Numerals in the figure indicate the residue numbers of the tryptophans.

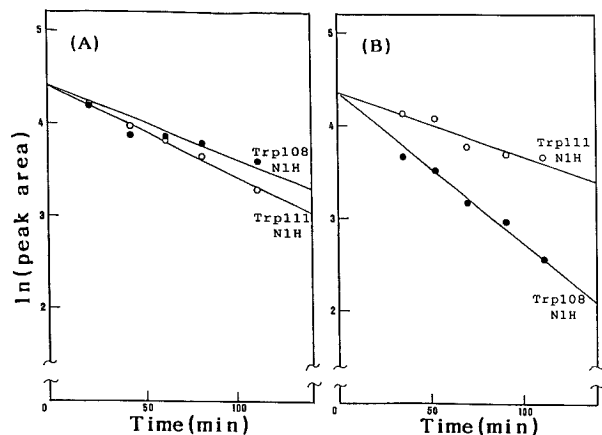


Fig. 7. First-order kinetic plots for the hydrogen exchange reaction of the tryptophan indole N1H of HEWL (A) and GEWL (B). The exchange reaction was started by dissolving the lyophilized lysozyme in D_2O and carried out at $27^\circ C$, pH 5.8.

Figure 6 shows the pH dependence of the tryptophan indole N1H resonances for HEWL and GEWL. In the higher pH region (pH 7–8), the indole N1H resonances of Trp 62 and Trp 63 of HEWL disappeared, probably due to the high exchange rate of the hydrogens. Such behavior was also observed for the identical tryptophan residues of GEWL. Tryptophan 111 and Trp 123 of GEWL responded to pH change similarly to those of HEWL. From these results, we concluded that the environments of Trp 62, 63, 111, and 123 of GEWL are closely similar to those of HEWL. In HEWL, the characteristic pH dependence of Trp 108 indole N1H is caused by the ionization of Glu 35, which is in van der Waals contact with Trp 108 indole N1H (21). Trp 108 indole N1H of GEWL also exhibits a characteristic pH dependence very similar to that of HEWL, but the chemical shift value of Trp 108 indole N1H of GEWL is smaller than that of HEWL by about 0.3 ppm over the pH range examined (pH 2.5–7.6). The pK_a values of Glu 35 estimated from the titration curve of Trp 108 are 5.66 for GEWL and 6.18 for HEWL. The difference in the local environment around Trp 108 between HEWL and GEWL may give rise to distinct pK_a values of Glu 35 by changing the hydrophobic environment around Glu 35.

Hydrogen Exchange Rate of Tryptophan Indole N1H—To obtain further information on the environment of the tryptophan residues of GEWL, we have measured the rate of hydrogen exchange with deuterium by monitoring the resonances of the tryptophan indole N1H hydrogens after dissolving GEWL in D_2O . As reported by Wedin *et al.* (22), the exchange rates of Trp 62 and Trp 63 are too fast to be measured and that of Trp 28 is extraordinarily slower than those of the other tryptophans at pH 3.8. Since the resonance of Trp 108 indole N1H of GEWL is overlapped with other resonances in the lower pH region (Fig. 5), the exchange rates were measured at pH 5.8, where the resonance of Trp 108 is completely separated from other resonances. At this pH, the exchange rate of Trp 123 also becomes too fast to be measured. Thus, only the exchange rates of Trp 111 and Trp 108 are measurable under this condition, and first-order kinetic analysis was performed for each residue. Figure 7 illustrates the first-order kinetic

TABLE I. Differences^a in chemical shift values between free lysozyme and lysozyme-(GlcNAc)₃ complex.^b

	HEWL		GEWL	
	pH		pH	
	2.6	4.4	2.7	4.8
Trp 28 C5H	−0.17	−0.22	−0.22	−0.27
Asn 59 α CH	−0.23	−0.07	−0.32	−0.10
Trp 63 C2H	+0.13	+0.18	+0.12	+0.16
Asn 65 α CH	−0.05	0.00	−0.07	−0.02
Ile 98 γ^1 CH	−0.24	−0.24	−0.26	−0.24
Ile 98 γ^2 CH ₃	−0.07	−0.10	−0.12	−0.10
Met 105 β CH	+0.02	−0.04	+0.04	−0.04
Trp 108 C5H	−0.05	−0.04	−0.02	−0.04

^aThe values are expressed in ppm. Only the resonances which exhibit differences of more than ± 0.04 ppm are listed. The positive and negative signs indicate shifts downfield and upfield, respectively.

^bThe chemical shift values of individual resonances were plotted against (GlcNAc)₃ concentration, and the limiting chemical shifts at high (GlcNAc)₃ concentration were regarded as the chemical shift values for the lysozyme-(GlcNAc)₃ complex.

plots for exchange of the indole N1H hydrogen with deuterium. The rate constants for the exchanges of Trp 111 and Trp 108 were estimated to be 1.1×10^{-4} and 2.7×10^{-4} s^{−1}, respectively, for GEWL, and 1.6×10^{-4} and 1.3×10^{-4} s^{−1} for HEWL. The rate of Trp 108 is considerably larger than that of Trp 111 in GEWL whereas the rates are comparable in HEWL. Tryptophan 108 of GEWL, therefore, seems more accessible to the solvent than that of HEWL.

(GlcNAc)₃ Binding—The resonances of many hydrogens in HEWL are perturbed by the addition of *N*-acetylglucosamine oligosaccharide (23). To examine the substrate binding properties of GEWL and HEWL, (GlcNAc)₃ was added to the lysozyme solution, and then the NMR signal responses to the ligand binding were analyzed. Major effects were observed on the resonances of Trp 28, Asn 59, Trp 63, and Ile 98 for both lysozymes. The resonances shifted gradually upfield or downfield with increase in (GlcNAc)₃ concentration, but no further spectral change was observed when the molar ratio of (GlcNAc)₃ to the lysozyme was increased above 2.0. The response of GEWL to the increase in (GlcNAc)₃ concentration was slightly more sensitive than that of HEWL as judged from the response of the Trp 28 C5H resonance. In fact, the binding constants of (GlcNAc)₃ to the lysozymes were estimated to be 3.6×10^3 M^{−1} for HEWL and 8.3×10^3 M^{−1} for GEWL, from Scatchard plot analysis based on the spectral change in the resonance for Trp 28 C5H. Table I lists differences in chemical shift values of individual resonances between the free lysozyme and the lysozyme-(GlcNAc)₃ complex. At both pH values, GEWL and HEWL are basically similar with respect to the values of the differences, indicating that both lysozymes should have similar binding modes for (GlcNAc)₃. We have found, however, that the differences in the chemical shift values of Asn 59 α CH and Trp 28 C5H of GEWL are somewhat larger than those of HEWL. The binding site of GEWL may respond to the ligand binding more flexibly than that of HEWL.

pH Titration in the Presence of (GlcNAc)₃—Since the ligand binding may alter the pK_a of two histidines, pH titration was performed in the presence of (GlcNAc)₃. The pK_a values estimated from the titration curves were 5.49 ± 0.05 for His 15 and 6.04 ± 0.02 for His 114 of GEWL. Only a slight decrease in pK_a values was observed upon addition

of (GlcNAc)₃. Since the two histidines are located somewhat far from subsites A, B, and C, the environments of two histidines in GEWL may not be affected by (GlcNAc)₃ binding.

DISCUSSION

The structure of lysozyme has been analyzed by means of physicochemical measurements, such as NMR or crystallographic analysis, because these methods are most powerful for examining the fine structure of the binding and catalytic sites. Such methods, however, require a large quantity of lysozyme. At present, it is hard to obtain a sufficient quantity of chemically modified or mutant lysozymes for studies on the structure and function of the lysozyme. On the other hand, avian lysozymes can be easily purified from their egg white in satisfactory amount. Amino acid sequences are now established for many avian egg white lysozymes, and only a limited number of amino acids are substituted in these lysozymes (11). Therefore, the avian lysozymes are very useful for investigation of the structure and function of lysozyme. In this paper, we have tried to investigate the relationship between amino acid substitutions and difference in the fine structure of the active site using GEWL and HEWL.

We have successfully assigned several resonances of the NMR spectrum of GEWL, which are labeled in Fig. 2A, by comparison with those of HEWL. As judged from the chemical shift values of the assigned resonances, the differences between HEWL and GEWL are minor, indicating that the tertiary structure of GEWL is similar to that of HEWL in most regions.

From the pH titration curve, the pK_a value of His 15 of GEWL was determined to be 5.70, which is similar to that of HEWL. An X-ray crystallographic study of HEWL showed that the imidazole ring of His 15 is partly shielded from solvent by the guanidyl group of Arg 14 and forms a hydrogen bond with Thr 89 (1). Since Arg 14 and Thr 89 are conserved in GEWL, the state of His 15 of GEWL is probably the same as that of HEWL. In Fig. 2, the resonance of the imidazole C2H of His 114 is clearly separated from the other resonances, and is suitable as a probe for investigation of the state of subsite F. The pK_a for His 114 was determined to be 6.16 and is closer to the pK_a value of the histidine derivative, *N*-acetylhistidinemethylamide (6.65), than that for His 15 (5.70). Since the low pK_a value of His 15 should be due to interactions with other amino acid residues, His 114 may be relatively free from interactions with other amino acid residues in comparison with His 15.

The profile of the resonance of His 114 and the pK_a value of the histidine residue did not change upon the addition of (GlcNAc)₃. These results indicate that the state of His 114 is not dependent upon the (GlcNAc)₃ binding to subsites A, B, and C. Alteration of the state around His 114 should require addition of (GlcNAc)₄, (GlcNAc)₅, or (GlcNAc)₆ to GEWL solution. However, since the oligosaccharides are immediately hydrolyzed by the lysozyme, it is also necessary to find experimental conditions which prevent the lysozyme from hydrolyzing the oligosaccharides.

The pH titration curves of tryptophan indole N1H hydrogens revealed that the resonance of Trp 108 indole N1H of GEWL is shifted upfield in comparison with that of

HEWL. The pK_a values of Glu 35 estimated from the pH titration curve of Trp 108 indole N1H are different between GEWL and HEWL. The measurements of the hydrogen exchange rates indicate that Trp 108 indole N1H of GEWL is more accessible to the solvent than that of HEWL. All of these results indicate that the environments of Trp 108 are considerably different between GEWL and HEWL. In order to understand the difference in the state of Trp 108 between GEWL and HEWL, we considered the hydrogen bonding between Trp 108 indole N1H and the main chain C=O of Leu 56. In GEWL, the isoleucine residue at position 55 of HEWL is replaced by valine. The replacement may loosen the hydrogen bonding between Trp 108 and Leu 56, leading to a larger accessibility of Trp 108 to the solvent. The larger accessibility of Trp 108 may also affect the hydrophobic environment of Glu 35, which is extremely close to Trp 108, resulting in the lower pK_a value of the glutamic acid. Since the deshielding of Trp 108 indole N1H by the main chain C=O of Leu 56 should be relieved by looser hydrogen bonding between Trp 108 and Leu 56 of GEWL, the resonance of Trp 108 indole N1H would shift upfield.

Data in Table I clearly indicate that the chemical shift changes induced by the ligand binding are slightly larger for Asn 59 αCH and Trp 28 C5H of GEWL than for those of HEWL. In addition, the binding constant of (GlcNAc)₃ for GEWL is somewhat larger than that for HEWL. In GEWL, the loose hydrogen bonding between Trp 108 and Leu 56 may make the structure of the (GlcNAc)₃ binding site more flexible, resulting in slightly different binding properties from those of HEWL.

The experimental time-courses of (GlcNAc)₃ degradation catalyzed by GEWL have been obtained by HPLC analysis, and analyzed by theoretical calculation based on a kinetic model for HEWL (24). From the kinetic analysis, it was found that the rate constant of glycosidic bond cleavage for GEWL is somewhat larger than that for HEWL. Since the rate constant value reflects the catalytic potency of Glu 35, it is very likely that the larger rate constant value of GEWL is caused by the different state of Glu 35 resulting from the loose hydrogen bonding between Trp 108 and Leu 56.

We have rather unexpectedly found that the amino acid substitution at position 55 significantly affects the environments of Trp 108 and Glu 35 at the active site. To obtain further information on the structure of GEWL, analysis of the two-dimensional NMR spectra including NOESY spectrum is in progress, and the results will be reported in the succeeding paper.

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