The amino acid sequence of Egyptian goose lysozyme (EGL) from egg-white and its enzymatic properties were analyzed. The established sequence had the highest similarity to wood duck lysozyme (WDL) with five amino acid substitutions, and had eighteen substitutions difference from hen egg-white lysozyme (HEL). Tyr34 and Gly37 were found at subsites E and F of the active site when compared with HEL. The experimental time-course characteristics of EGL against the N-acetylglucosamine pentamer substrate, (GlcNAc)_5, revealed higher production of (GlcNAc)_4 and lower production of (GlcNAc)_2 when compared with HEL. The saccharide-binding ability of subsites A–C in EGL was also found to be weaker than in HEL. An analysis of the enzymatic reactions of five mutants in respect of positions 34, 37 and 71 in HEL indicated the time-course characteristics of EGL to be caused by the combination of three substitutions (F34Y, N37G and G71R) between HEL and EGL. A computer simulation of the EGL-catalyzed reaction suggested that the time-course characteristics of EGL resulted from the difference in the binding free energy for subsites A, B, E and F and the rate constant of transglycosylation between EGL and HEL.

Key words: lysozyme; amino acid sequence; lysozyme-catalyzed reaction; site-directed mutagenesis; subsite

Lysozyme, one of the best characterized carbohydrates, catalyzes the hydrolysis of the β-1,4-glycosidic bonds of alternating copolymers of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid in bacterial cell walls, or of the homopolymer of GlcNAc, chitin. This enzyme has three classifications, the chicken type, goose type, and phage type, on the basis of the similarity in amino acid sequences. The amino acid sequences of lysozymes have been reported for many birds, mammals, fish, and insects. Only some birds have the goose type of lysozyme, while others have the chicken type. One of the most intensive investigations on the amino acid sequences of the chicken type of lysozyme is that for avian egg white, especially for Galliforms. The amino acid sequences of phasianid birds are known for 14 lysozymes. Since the lysozyme molecule has a high evolutionary rate and is fairly small, an analysis of the amino acid sequences of lysozymes from closely related birds provides useful information on the relationship between structure and function.

Extensive studies on hen egg-white lysozyme (HEL) have revealed that this enzyme includes six sugar residue binding sites called subsites A, B, C, D, E and F. A substrate bound to these subsites was cleaved between subsites D and E through the conventional acid catalytic reaction of Glu35 and Asp52. This enzyme also exhibited high transglycosylation activity in addition to hydrolysis, although the molecular mechanism for this highly efficient transglycosylation remains unknown. It is known that the acceptor molecule binds to subsites E and F, affecting the high efficiency of transglycosylation. Subsites A, B and C have been extensively studied by means of chemical modification, substrate binding experiments, and kinetic analyses. In contrast, information on subsites D and E is relatively limited because of some difficulties; one is the lack of characteristic amino acids at subsites D and E, and the other is the rapid hydrolysis of the GlcNAc hexamer, (GlcNAc)_6, through enzymatic action. Energy minimization of HEL complexed with (GlcNAc)_6 has prompted the proposition that two binding modes are possible for subsites E and F, resulting in right-sided and left-sided binding modes. Arg45, Asn46, and Thr47 in HEL are considered to be responsible for the left-sided binding, while Phe34, Asn37, and Arg114 are for the right-sided binding.

A data-fitting method for the experimental time-course characteristics of lysozyme-catalyzed reactions with chito-oligosaccharide can be directly used to estimate the binding free energy changes of the six subsites. We can therefore directly evaluate the contributions of amino acid substitution at the active site to substrate binding and enzymatic reactions by combining the results of an HPLC analysis of the reaction products and theoretical calculation of the reaction time-course data. The naturally occurring lysozyme molecules closely and genetically related to HEL, which carry limited amino acid substitutions at the substrate binding site, are useful to evaluate the reaction mechanisms of lysozyme such as substrate binding and lysozyme catalysis.
We first determined in this present study the amino acid sequence of Egyptian goose egg-white lysozyme (EGL) and compared it with that of other lysozymes. The time-course characteristics of the EGL-catalyzed reaction on the GlcNAc pentamer substrate, (GlcNAc)$_5$, were measured for comparison with those of HEL, and the binding constants of sugar residues at each subsite and the rate constants were estimated from the experimental time-course data through computer simulation of the lysozyme-catalyzed reaction. Site-specific mutations of residues 34, 37 and 71 in HEL were also conducted, and the mutated proteins (F34Y, N37G, F34Y/N37G, G71R, and F34Y/N37G/G71R) were subjected to activity measurements. We found Ty34, Gly37, and Arg71 in EGL to be the key residues responsible for the time-course characteristics of EGL.

Materials and Methods

Materials. Egyptian goose eggs were kindly donated by Kumamoto Zoological Park (Kumamoto, Japan) and purified as previously described. Six-times recrystallized HEL was purchased from Seikagaku Kogyo (Tokyo, Japan), and lyophilized Micrococcus luteus cells were from Sigma (St. Louis, MO, USA). The restriction enzymes and DNA modifying enzymes were purchased from Takara (Kyoto, Japan) or Toyobo (Osaka, Japan). Oligonucleotide DNA primers were synthesized by Hokkaido System Science (Sapporo, Japan). HA94-1, which contains the wild type of HEL gene in a cloning vector M13 mp18 derivative, was used for the mutagenesis. E. coli strain JM109 was used for routine transformation and plasmid preparation. Multi-copy Pichia expression kits, including expression plasmid pPIC9K and host strain GS115, were obtained from Invitrogen (La Jolla, CA, USA). N-Acetylgalactosamine oligosaccharides [(GlcNAc)$_n$] were prepared according to the method of Rupley. The purity of each oligosaccharide was confirmed by using HPLC. All other reagents used were of analytical or biochemical grade.

Carboxymethylation and enzymatic digestion. EGL was reduced and carboxymethylated for a structural analysis. EGL (10 mg) was dissolved in a 1.4 M Tris–HCl buffer at pH 8.6, and then 1.2 g of urea, 100 mL of 5% EDTA, and 33 mL of $\beta$-mercaptoethanol were added. The solution was left for 1 h at 37°C in an N$_2$ atmosphere. After the reduction, 89 mg of monooctoic acid in 300 mL of 1.0 M NaOH was added, and the mixture left for 1 h at room temperature in the dark. The reaction mixture was desalted by passing through a Sephadex G-50 column (1.7 x 46 cm) in 0.2 M NH$_4$OH, and the protein fraction was lyophilized (Cm-EGL). Cm-EGL (5 mg) was dissolved in 1 mL of distilled water, and the solution digested for 4 h with trypsin (1/50, w/w, TR-TPCK, Cooper Biomedical, USA) at pH 8.0 and 37°C, using an RTR22 pH-stat (Radiometer).

Peptide mapping and sequence analysis. The tryptic peptides were separated by reversed-phase high-pressure liquid chromatography (RP-HPLC) in an ODS 120A S5 column (4.0 x 250 mm; Yamamura Chemical Co., Tokyo, Japan). The peptides were eluted with a linear gradient of 0% to 50% of solvent B. A gradient of 0.1% trifluoroacetic acid (solvent A) and 60% acetonitrile (solvent B) was used for 130 min. The tryptic peptides were hydrolyzed for 20 h in solvent A (solvent B). A gradient of 0% to 50% of solvent B was used for 130 min. The tryptic peptides were hydrolyzed for 20 h in solvent A (solvent B).

Substrate binding. Substrate binding was evaluated by measuring the fluorescence intensity standardized by N-acetyltryptophane with a Hitachi F-4500 fluorescence photometer. The GlcNac trimer, (GlcNAc)$_3$, (0.02 mM to 0.2 mM), was incubated for 5 min at 30°C with lysozyme (0.02 mM) in a 10 mM sodium acetate buffer at pH 5.0. The reaction mixture was measured at an excitation of 291 nm and emission of 360 nm. The dissociation constant ($K_D$) was calculated as the amount of the enzyme and substrate complex (the reduction of fluorescence intensity of the reaction mixture to that of lysozyme) by Scatchard plotting, using the equation $\Delta F = -K_D \times \Delta F/[S] + \Delta F_{MAX}$ (where $\Delta F$ is the reduction of fluorescence intensity of the reaction mixture to that of lysozyme; $K_D$ is the dissociation constant; $[S]$ is the concentration of the substrate).

Circular dichroism (CD) spectra. CD spectra were obtained at 25°C with a Jasco J-720 spectropolarimeter. The proteins were dissolved in a 10 mM sodium acetate buffer at pH 5.0 to a final concentration of 0.15 mg/mL. The data are expressed in terms of the mean residual ellipticity. The path-length of the cells was 0.1 cm for the far-UV CD spectra (200–250 nm), each spectrum being corrected by subtracting the spectrum of the buffer.

Enzyme activity. The enzymatic activity toward (GlcNAc)$_n$ was measured by the method of Masaki et al. with a slight modification. The reaction mixture containing 0.1 mM lysozyme and 1 mM (GlcNAc)$_n$ was incubated at 50°C in a 10 mM sodium acetate buffer at pH 5.0. After a given reaction time, 200 mL of the reaction mixture was withdrawn and rapidly chilled in a Kool Kup (Towa Co., Tokyo, Japan). The reaction mixture was centrifuged with Ultrafuge C3LCC (Millipore, Billerica, MA, USA), and the resulting filtrate was lyophilized. The dried sample was dissolved in 50 mL of ice-cold water, and then 10 mL of the solution was applied to a TSKgel G-Oligo-PW column (7.8 x 600 mm; Tosoh Co., Tokyo, Japan) in a Jasco 800 series HPLC instrument (Japan Spectroscopic Co., Tokyo, Japan), eluting with distilled water at room temperature at a flow rate of 0.3 mL/min. Each chito-oligosaccharide concentration was calculated from the peak area monitored for the ultraviolet absorption at 220 nm, using a standard curve obtained for authentic saccharide solutions. The relative error is defined as $\Delta R = x/x_0$, where $x$ is the final concentration of the substrate and $x_0$ is the recovered concentration of all chito-oligosaccharides in (GlcNAc)$_n$ units.

The reaction rate of the lysozyme-catalyzed reaction for the initial substrate (GlcNAc)$_3$ was numerically solved to obtain the calculated time-course data. A kinetic model for the lysozyme-catalyzed reaction of chito-oligosaccharides has been reported and is presented in a simplified form by omitting some details of the reaction (Fig. 1). The rate equation in the calculation was repeatedly solved while changing the value for the binding free energy or the rate constant, $k_{i1}$ (cleavage of the $\beta$-1,4 glycosidic linkage), $k_{i-1}$ (regeneration of the glycosidic linkage), or $k_{i2}$ (hydration), so that the calculated time-course data fitted with those experimentally obtained. Details of the calculation method have been previously described.

The time-course characteristics for data fitting were repeatedly calculated by varying the value for the binding free energy change at each of the subsites to obtain the minimum cost function:

$$F = \sum \left[ \text{GlcNAc}_{n_i} - \text{GlcNAc}_{n_j} \right]$$

where $e$ and $r$ are the experimental and calculated values, $n$ is the size of the chito-oligosaccharide, and $i$ the reaction time. The set of values for the reaction parameters giving the minimum value of $F$ in the equation was regarded as the most reliable for the reaction parameters. The data for (GlcNAc)$_n$ in the early stage of the reaction were not used for defining the cost function, because the chromatographic separation of (GlcNAc)$_n$ and (GlcNAc)$_3$ was not satisfactory in the early stage of the reaction and some degree of error could not be avoided.

Mutagenesis, expression, and purification of the mutant enzymes. Site-directed mutagenesis was conducted by the PCR-based megaprimer method, using HA94-1 as a template. The F34Y and N37G mutant enzymes were prepared as previously reported. The primers used for mutagenesis were 5'-GCAAAATACGAGATGGC-3' (TTCAACAC5' (F34Y/N37G) and 5'-AAAGCCCCAAAGTCCAGG-3' (G71R). The mutations in the lysozyme genes were confirmed by DNA sequencing. The G71R mutation was introduced into the gene encoding F34Y/N37G to create the gene encoding HEL with a triple mutation (F34Y/N37G/G71R). The mutant enzymes were produced and purified as previously described. The protein concentration was measured by using a BCA protein assay reagent (Pierce, Rockford, IL, USA) and authentic HEL as a standard protein.

Circular dichroism (CD) spectra. CD spectra were obtained at 25°C with a Jasco J-720 spectropolarimeter. The proteins were dissolved in a 10 mM sodium acetate buffer at pH 5.0 to a final concentration of 0.15 mg/mL. The data are expressed in terms of the mean residual ellipticity. The path-length of the cells was 0.1 cm for the far-UV CD spectra (200–250 nm), each spectrum being corrected by subtracting the spectrum of the buffer.
Results and Discussion

Amino acid sequence of EGL

EGL was purified by the conventional method, involving the aqueous extraction of egg-white, treatment at pHs 4.0, 6.0 and 7.0, and cation-exchange chromatography. The HPLC peptide map of EGL was compared with that of HEL (Fig. 2). The amino acid compositions of all the peaks of EGL were analyzed. The peptides in each peak of EGL having both the identical amino acid composition and identical elution position to that of HEL are considered to have had the identical amino acid sequence as previously reported. The peaks indicated by arrows on the EGL peptide map were found to have substituted amino acids when compared with HEL, as judged by the elution position, amino acid composition, and amino acid sequence of the corresponding peptides. As shown in Fig. 3, we found eighteen amino acid substitutions in the EGL sequence compared with HEL, and five substitutions compared with wood duck lysozyme (WDL).

The established amino acid sequence (P84496) was closest to that of WDL previously reported (Fig. 3). Among the amino acid substitutions found in EGL, it is notable that Phe34 and Asn37 at subsites E and F in HEL were respectively replaced by Tyr and Gly. These residues are considered to be responsible for the right-sided binding mode of lysozyme catalysis. Since EGL carries the amino acid substitutions at subsites E and F, it was thought that this enzyme would be good for studying subsites E and F in lysozyme-catalyzed reactions.

Activity of EGL against the GlcNAc pentamer, (GlcNAc)$_5$

Since lysozyme has high transglycosylation activity in addition to hydrolysis, the enzyme activity cannot simply be evaluated from the hydrolytic activity toward a polymeric substrate. When investigating the lysozyme-catalyzed reactions in detail, it is important to examine

Fig. 1. Simplified Model for the Lysozyme-Catalyzed Reaction of (GlcNAc)$_5$.
In this model, $k_{+1}$, $k_{-1}$, and $k_{+2}$ are the respective rate constants for cleavage of the glycosidic linkage, transglycosylation, and hydration. Details of the calculation method have been previously described.}

Fig. 2. Comparison between the RP-HPLC Patterns for EGL and HEL.
The peaks in EGL indicated by arrows are those appearing in different positions and with different amino acid compositions when compared with HEL. See the text for details of the HPLC conditions.

Fig. 3. Amino Acid Sequence Alignment for HEL, EGL, and WDL.
The substituted amino acids are boxed. Other positions that contain no substituted amino acids are indicated by dashes.
the experimental time-course data for the oligomeric substrate degradation and product formation, because substantial information on the lysozyme catalysis, such as substrate binding and transglycosylation, can be obtained. The time-course analysis with the substrate, (GlcNAc)$_5$, reflects not only the hydrolysis of the initial substrate but also the transglycosylation produced by cleavage of the substrate and product, and enables us to evaluate the effects of different amino acids at subsites by a computer simulation analysis. We therefore analyzed the reaction time-course data for (GlcNAc)$_5$ degradation catalyzed by EGL and compared them with those of HEL and WDL.

The experimental time-course data for HEL, EGL, and WDL on the initial substrate, (GlcNAc)$_5$, are shown in Fig. 4. (GlcNAc)$_5$ was gradually degraded in the HEL-catalyzed reaction and disappeared in about 20 min, while products were formed in order of the amount as (GlcNAc)$_1$ > (GlcNAc)$_2$ > (GlcNAc)$_4$ > (GlcNAc)$_3$. The time-course data for WDL, which had the greatest sequence similarity to that of EGL, exhibited profiles similar to those of HEL, except that the amounts of (GlcNAc)$_2$ and (GlcNAc)$_3$ were slightly different. On the other hand, the profile of the time-course data for EGL differed from that for HEL, although the order of the amounts of the products was (GlcNAc)$_1$ > (GlcNAc)$_2$ > (GlcNAc)$_4$ > (GlcNAc)$_3$ for both lysozymes after 20 min of the reaction. The characteristic features of the time-course data were the increased production of (GlcNAc)$_4$ and the reduced production of (GlcNAc)$_2$. Among the amino acid substitutions found in EGL, only Tyr34 and Gly37 were located at the substrate binding site. The substitutions at positions 34 and 37 were therefore expected to contribute to the difference in product concentrations between EGL and HEL.

**Over-expression and characterization of the mutant enzymes**

To define the amino acid replacements responsible for the differences in time-course data between EGL and HEL, two mutant enzymes (F34Y and N37G) at subsites E and F were constructed, in which Phe34 and Asn37 in HEL were changed to the corresponding amino acid residues in EGL. Expression of the mutated cDNAs was achieved with the *P. pastoris* expression system, as previously described. The yield of protein from a 1-liter culture was 20–25 mg. The purity of each mutant protein was confirmed by SDS–PAGE. The N-terminal amino acid sequences of the purified mutants were identical to that of HEL. Far-UV CD spectra of the mutant enzymes were indistinguishable from that of HEL (data not shown), indicating that the global proportion had not been affected by the mutation.
We then analyzed the experimental time-course data for (GlcNAc)$_5$ degradation catalyzed by the mutant enzymes, and compared them with those for HEL and EGL (Fig. 4). Although the distribution pattern of the products formed by the F34Y and N37G mutant enzymes were different from those of HEL and EGL, the experimentally observed amounts of (GlcNAc)$_2$ and (GlcNAc)$_4$ for the mutant enzymes were close but different from those of (GlcNAc)$_1$ and (GlcNAc)$_3$ as was observed in the case of EGL. The effects of these mutations were further corroborated by constructing the mutant protein, F34Y/N37G, with two substitutions; the time-course data for the double mutant were almost the same as those for the individual mutant proteins (Fig. 4). These results indicate that the substitution of Phe34Tyr and Asn37Gly at subsites E and F can account, at least in part, for the time-course characteristics of EGL. However, the experimental time-course data for the double mutant, in which Phe34 and Asn37 in HEL were simultaneously mutated to the respective corresponding residues, Tyr and Gly, present in EGL could not be completely reproduced for the product distribution of EGL, especially the concentrations of (GlcNAc)$_2$ and (GlcNAc)$_4$. The difference in the oligomer concentration between EGL and HEL was therefore not fully accounted for by these two substitutions. This result suggests that the different profiles of HEL and EGL might have resulted not only from the substitutions at subsites E and F, but also from other factors influencing the overall structure of the proteins. Since EGL has eighteen amino acid substitutions in its molecule when compared with HEL, it was expected that these substitutions would have given rise to certain structural changes in EGL which may have contributed to the characteristic time-course data for EGL.

**CD spectra**

CD spectra for EGL and HEL were obtained to study the conformational changes accompanying the amino acid substitutions (Fig. 5). These spectra were almost identical for the two lysozymes, indicating that the amino acid substitutions found in EGL did not markedly change the global conformation of EGL. The alteration in the time-course data observed for EGL was therefore unlikely to have been due to the difference in main-chain folding between EGL and HEL.

**Subsite structures of A, B and C**

The fluorescence intensity in lysozymes derived from the Trp residues at subsites A–C (Trp62, Trp63 and Trp108) reflects the microenvironment of each of these sites. It has previously been reported that the substrate analog, (GlcNAc)$_3$, predominantly bound to subsites A–C in the ratio of 99% without hydrolysis. To investigate the microenvironments of subsites A–C, we analyzed the fluorescence spectrum of EGL caused by the Trp residues with and without (GlcNAc)$_3$, and compared it with that of HEL.

Figure 6 shows the Scatchard plot for the binding of (GlcNAc)$_3$ to EGL, as well as that to HEL. Table 1 summarizes the dissociation constant ($K_d$), association constant ($K_a$), and binding free energy for the binding of (GlcNAc)$_3$. The dissociation constant of EGL calculated from the slope of the line ($K_d = 3.64 \times 10^{-5}$ M) was substantially higher than that of HEL ($K_d = 2.09 \times 10^{-5}$ M), indicating that the binding ability of EGL for (GlcNAc)$_3$ was (by about 0.34 kcal/mol) lower than that of HEL. The environment of subsites A–C in EGL was therefore considered to be different from that in HEL. This finding, together with the identical CD spectra,
Fig. 7. Calculated Time-Course Data for HEL and EGL.
The parameter values used for this calculation are listed in Table 2. Numerals show the degree of polymerization for each reaction product species.

Table 2. Estimated Reaction Parameter Values for HEL and EGL

<table>
<thead>
<tr>
<th></th>
<th>HEL</th>
<th>EGL</th>
<th>Rate constant (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>−2.0</td>
<td>−2.1</td>
<td>0.93</td>
</tr>
<tr>
<td>B</td>
<td>−3.0</td>
<td>−2.6</td>
<td>40.0</td>
</tr>
<tr>
<td>C</td>
<td>−5.0</td>
<td>−5.0</td>
<td>0.30</td>
</tr>
<tr>
<td>D</td>
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<td>4.5</td>
<td></td>
</tr>
<tr>
<td>E</td>
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<td>−2.35</td>
<td></td>
</tr>
<tr>
<td>F</td>
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<td>−1.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>k⁺₁</td>
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<tr>
<td>k⁻₁</td>
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</tr>
<tr>
<td>k⁺₂</td>
<td>0.34 kcal/mol</td>
<td>0.34 kcal/mol</td>
<td></td>
</tr>
</tbody>
</table>

k⁺₁, k⁻₁, and k⁺₂ are the rate constants for cleavage of glycosidic linkage, transglycosylation, and hydration.

strongly suggested that the observed differences in time-course data between EGL and mutant F34Y/N37G may be attributable to the decreased affinity of subsites A–C in EGL due to some other amino acid substitutions in the molecule than those in HEL.

We have already mentioned that the experimental time-course data for WDL showed a profile quite similar to that for HEL. The observation that there was little difference in the reaction time-course data between WDL and HEL suggested that the amino acid substitutions between WDL and HEL contributed little, if at all, to the lysozyme-catalyzed reactions. We therefore directed our attention to the other amino acids that were not conserved among HEL, WDL, and EGL. A comparison of the amino acid sequence of EGL with those of HEL and WDL showed Asn37 → Gly and Gly71 → Arg to be the only substitutions found in EGL (Fig. 3). It was thus assumed that, besides Tyr34 and Gly37, Arg71 was also responsible for the characteristic time-course data for EGL.

Enzymatic activities of the position 71 mutant proteins toward (GlcNAc)₅
To confirm the involvement of the Gly71 → Arg substitution in the change in the time-course data for EGL, two mutant enzymes (G71R and F34Y/N37G/G71R), in which the Arg residue was respectively introduced at position 71 in HEL and mutant F34Y/N37G, were prepared, and the resulting mutants were again characterized with respect to their enzymatic activities. Figure 4 shows time-course data for the G71R mutant having profiles similar to those of HEL, although the enhanced production of (GlcNAc)₁ did not occur (Fig. 3). It was thus assumed that, besides Tyr34 and Gly37, Arg71 was also responsible for the characteristic time-course data for EGL.

EGL were the critical residues governing the characteristic time-course data for EGL.

Estimation of the reaction parameters for EGL
To evaluate the contribution of amino acid substitutions to the substrate-binding and lysozyme-catalyzed reaction, the experimental time-course data for EGL were analyzed by a computer simulation in order to obtain the rate constants and binding free energy values for the individual subsite. The calculated time-course data are shown in Fig. 7, and the reaction parameters obtained are summarized in Table 2. The binding free energy values for subsites C and D, rate constant k⁺₁ for cleavage of the glycosidic linkage, and rate constant k⁺₂ for hydration were identical between HEL and EGL. On the other hand, the respective binding free energy values for subsites A and B for EGL were higher by 0.1 kcal/mol and lower by 0.4 kcal/mol, when compared with those of HEL. This is consistent with the decreased binding strength (−0.34 kcal/mol) of EGL to (GlcNAc)₅. In addition, the respective binding free energy for sites E and F of EGL was found to be −2.35 kcal/mol and −1.45 kcal/mol being −0.15 and −0.05 kcal/mol lower than the respective HEL values. These data indicate that the substrate binding for EGL was different from that for HEL at or near subsites A, B, E and F, and that the characteristic time-course data for EGL would have been due to the difference in affinity of the substrate for these sites from those of HEL.

The results of the X-ray crystallographic study enable the main-chain carbonyl oxygen of Phe34 to be proposed to form a hydrogen bond with the hydroxyl group at position 6 of the sugar ring in subsite F and to participate in substrate binding at this site. The results of model-building studies based on the X-ray structure enable the postulation of Asn37 from the side-chain amide nitrogen of Asn37 to interact with the hydroxyl oxygen at position 6 of the sugar ring in subsite F through hydrogen bonding. It is thus suggested that the decrease in substrate affinity at subsite F observed for EGL may have been caused by the loss of the
hydrogen bond between the side chain of Asn37 and substrate due to the Asn37 to Gly mutation. However, the effects of the mutations on substrate binding at positions 34 and 37 were not restricted to subsite F, but were spread over the neighboring site. Since the specific hydrogen bonding interaction between the sugar residue at subsite F and Phe34 would be conserved in EGL, the decreased affinity at subsite E may have originated from the phenolic hydroxyl group of substituted Tyr34. Alternatively, the fine structure around subsite E may have been slightly altered by the simultaneous substitutions at positions 34 and 37. This situation may have lead to the reduction of substrate affinity at subsite E that was observed for EGL.

Furthermore, the rate constant of transglycosylation, $k_{-1}$, also differed between the two lysozymes: the $k_{-1}$ value for EGL was found to be 34.0 s$^{-1}$, which is 6.0 s$^{-1}$ lower than that for HEL. This suggests that the binding ability of the acceptor for transglycosylation was lower than that with HEL. This result is supported by the fact that lysozyme catalyzed a series of reactions: cleavage of the glycosidic linkage, hydration of a reaction intermediate, and transglycosylation of the intermediate to the acceptor. All of the reactants, i.e., the initial substrate, substrate fragments, hydrolysis products, and transglycosylation products, could bind to the binding site of the enzyme in various modes to give either productive or nonproductive complexes, and these reaction products could repeatedly serve as substrates, inhibitors, and acceptors in the enzymatic reaction. Each product generated from the lysozyme-catalyzed reaction of (GlcNAc)$_3$ is thus characterized by both hydrolysis and transglycosylation reactions. In the transglycosylation reaction, after the formation of a reaction intermediate (Fig. 1), the substrate fragment occupying subsites E and F must be released into the medium and an acceptor molecule must be accommodated on these sites. The result was a complex ratio of products (GlcNAc)$_{1-4}$ generated by the lysozyme-catalyzed reaction. Since the binding of an acceptor molecule to subsites E and F should be necessary for transglycosylation, the decrease in substrate affinity at subsites E and F that was observed for EGL would affect the acceptor binding ability. It is thus speculated that the slightly altered topography of subsites E and F induced by the mutations at positions 34 and 37 may have caused differences in the substrate and acceptor binding at subsites E and F, leading to lower efficiency for the transglycosylation activity of EGL.

Residue 71 (Gly71), which is highly conserved in the chicken type of lysozymes, is located in a large loop (residues 61–80) containing Trp62 and Trp63 which are important for substrate binding at subsites B and C (Fig. 8). This loop does not contain any typical secondary structure such as an $\alpha$-helix or $\beta$-strand. It has previously been reported that this loop region was structurally changed when HEL bound to (GlcNAc)$_3$.51) In particular, in the binding of the substrate analogue to the active site of HEL, the indole ring of Trp62 was observed to show considerably induced fitting and to make extensive contact with the non-polar surface of the sugar residue in subsite B.52) The crystal structure of HEL shows that the Pro70-Gly71 sequence is positioned at a sharp turn in the loop, and this region of conformational space can be expected to be energetically favorable only for the glycine residue. Hence, the replacement of Gly71 by Arg in the turn structure of EGL may give rise to certain structural changes which decrease the flexibility of the main-chain in the loop region. This situation, caused by some changes in the degree of freedom of the loop region of EGL, might induce local structural changes around Trp62 and Trp63, which in turn might lead to the decreased binding strength to (GlcNAc)$_3$ of EGL. An X-ray crystallographic analysis of EGL, both free and complexed with (GlcNAc)$_3$, should provide valuable information to elucidate the relationship between the altered properties and structural changes of the loop region containing Trp62 and Trp63 of EGL. This is now being investigated in our laboratory.
References