Histidine-114 at Subsites E and F Can Explain the Characteristic Enzymatic Activity of Guinea Hen Egg-white Lysozyme

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The courses of the reaction catalyzed by guinea hen egg-white lysozyme (GHL), in which Asn113 and Arg114 at subsites E and F in hen egg-white lysozyme (HEL) are replaced by Lys and His, respectively, was studied with the substrate N-acetylglucosamine pentamer, (GlcNAc)5. Although GHL was found to retain the main-chain folding similar to HEL as judged from CD spectroscopy, the courses of GHL showed increased production of (GlcNAc)2 and reduced production of (GlcNAc)4 when compared with HEL. To identify critical residue(s) involved in the alteration in the courses of GHL, two mutant enzymes as to subsites E and F in HEL, N113K and R114H, were prepared by site-directed mutagenesis. Kinetic analysis of these mutants revealed that the mutation of Asn113 to Lys had little effect on the courses of HEL, while the Arg114 to His mutation completely reproduced the courses of GHL, demonstrating that His114 in GHL is the key residue responsible for the characteristic courses of GHL. Computer simulation of the reaction courses of the R114H mutant revealed that this substitution decreased not only the binding free energies for subsites E and F, but also the rate constant of transglycosylation. The Arg residue at position 114 may play an important role in the transglycosylation activity of HEL.

Key words: lysozyme; lysozyme-catalyzed reactions; site-directed mutagenesis; subsite; transglycosylation

Hen egg-white lysozyme (HEL; EC 3.2.1.17) is a carbohydrate hydrolase that catalyzes the hydrolysis of the β-1,4-glycosidic linkage of the polysaccharide constituting the peptideglycan of bacterial cell walls and the polymer of N-acetylglucosamine (GlcNAc), chitin. It is the first enzyme the three-dimensional structure of which was analyzed by X-ray crystallography and has been extensively studied as a model protein for elucidating enzymatic function and protein stability. Binding subsites for six monosaccharides were recognized in the active site cleft and called A to F. A substrate bound to subsites is hydrolyzed between subsites D and E by general acid catalysis involving γ-COOH of Glu35.

This enzyme is known to catalyze transglycosylation in addition to hydrolysis. However, the molecular mechanism by which such a high efficiency is achieved has not been discovered yet. The origin of this high efficiency is, at present, one of the most fundamental questions on lysozyme catalysis. Since the affinity of the acceptor molecule to subsites E and F is considered to be one of the factors controlling the efficiency of transglycosylation, it is important to investigate the structure-function relationships of subsites E and F.

On the basis of theoretical conformational energy calculations, there are two distinct binding modes for subsites E and F of HEL, "left-sided" and "right-sided" modes. In the latter mode, the terminal reducing sugar in subsite F is proposed to make contacts with such residues as Asn113 and Arg114. Among avian lysozymes sequenced thus far, guinea hen egg-white lysozyme (GHL) has substitutions at positions 113 and 114, in which Lys and His in HEL replace Asn and Arg in HEL, respectively (Fig. 1). Previously, we showed that GHL and HEL were very similar to each other in their tertiary structure as judged from the profile of 1H-NMR spectra, pH titration, and a GlcNAc trimer, (GlcNAc)3, binding experiment. Therefore, this enzyme would be one of the good materials for investigating the role of the "right-sided" binding site of subsites E and F in lysozyme-catalyzed reactions.

A data-fitting method for the experimental courses of lysozyme-catalyzed reactions with chitooligosaccharide can be directly used to estimate the binding free energy changes of the six subsites. Thus, we can directly evaluate the contributions of amino acid substitutions at the active site to substrate binding and enzymatic reactions by a combination of HPLC analysis of reaction products and theoretical calculation of reaction courses.

In this study, to gain further insight into the roles of subsites E and F, the courses of the GHL-

Abbreviations: GHL, guinea hen egg-white lysozyme; HEL, hen egg-white lysozyme; GlcNAc, N-acetylglucosamine; (GlcNAc)_n, β-1,4-linked oligosaccharide of GlcNAc; CD, circular dichroism; RP-HPLC, reversed-phase high pressure liquid chromatography

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catalyzed reaction on the substrate GlcNAc pentamer, (GlcNAc)₅, were measured in comparison with those of HEL. Based on the experimental data, the binding constants of sugar residues at each subsite and the rate constants were obtained by computer analysis. Furthermore, we produced HEL mutant enzymes at Asn113 and Arg114, and the resulting mutants were characterized. On the basis of the results, the effects of the mutations found at sub-sites E and F in GHL on lysozyme catalysis are also discussed.

Materials and Methods

Materials. Six-times recrystallized HEL was purchased from Seikagaku Kogyo (Tokyo, Japan). Guinea hen eggs were kindly donated by Kumamoto Zoological Park, Kumamoto, and GHL was purified from the egg whites by the methods of Jolles et al. with a slight modification. BCA protein assay reagent was obtained from Pierce (IL, USA). Restriction enzymes and nucleic acid modification enzymes were obtained from TaKaRa (Kyoto, Japan) or Toyobo (Osaka, Japan). The oligonucleotide primers were purchased from Hokkaido System Science (Sapporo, Japan). HA94-1, which contains HEL cDNA in a cloning vector, M13 mp18 derivative, was used as a starting material for mutagenesis. E. coli strain JM109 was used for the transformation and propagation of recombinant plasmids. The Pichia pastoris expression system including expression vector pPIC9K was purchased from Invitrogen (CA, USA). P. pastoris GS115 (Invitrogen) was used as the host strain for expression. All other reagents and chemicals were of analytical grade.

Site-directed mutagenesis and preparation of mutant enzymes. Three mutant proteins (N113K, R114H, and N113KR114H) were constructed by the megaprimer method, as we have previously described. The mutagenic primers used were 5'-TGGCGCAAAGCTGCAAGGTT-3' for N113K, 5'-TGGCGCAAACACTGCAAGGTT-3' for R114H, and 5'-TGGCGAAAGCTGCAAGGTT-3' for N113KR114H. The mismatch is shown by underlining in the primer sequence. All mutated genes were overexpressed in P. pastoris using the expression vector pPIC9K, and the expressed proteins were purified by the same procedures as those described in our previous paper.

Peptide mapping. Digestions of reduced and carboxymethylated lysozymes with TPCK-trypsin (Cooper Biomedical Inc., USA) and the separation of tryptic peptides by reversed-phase high-pressure liquid chromatography (RP-HPLC) were accomplished as described by Araki et al. Amino acid analyses were done on a Hitachi L-8500A amino acid analyzer after hydrolysis of tryptic peptides with constant-boiling HCl containing 0.05% β-mercaptoethanol in evacuated sealed tubes at 110°C for 20 h. The N-terminal sequences of tryptic peptides were identified on an Applied Biosystems model 477A sequencer.

Circular dichroism (CD) spectroscopy. The CD spectra in the far-ultraviolet range, 200–250 nm, were recorded at 25°C with a Jasco J-820 spectropolarimeter. The enzyme concentration was 0.15 mg/ml in 10 mm sodium acetate buffer (pH 5.0) in a cell with a 0.1 cm long optical path. The data were expressed in terms of mean residue ellipticity.
Enzyme action. Lysozyme activity was measured by the method of Masaki et al.\(^{11}\) with a slight modification. Namely, the reaction mixture containing 0.1 mM lysozyme and 1 mM (GlcNAc)\(_2\), was incubated in 10 mM sodium acetate buffer (pH 5.0) at 50°C. After a given reaction time, 200 \(\mu\)L of the reaction mixture was withdrawn and rapidly chilled in a KOOL KUP (Towa Co., Japan). The reaction mixture was centrifuged with Ultrafree C3LCC (Millipore Co., USA). The filtrate was lyophilized. The dried sample was dissolved in 50 \(\mu\)L of ice-cold water, and 10 \(\mu\)L of the solution was put on TSKgel G-Oligo-PW column (7.8 \(\times\) 600 mm, Tosoh Co., Japan) using a JASCO 800 series HPLC. Elution was done with distilled water at room temperature and at flow rate of 0.3 ml/min. Each chitoooligosaccharide concentration was calculated from the peak area monitored by ultraviolet absorption at 220 nm, using the standard curve obtained from authentic saccharide solution. The relative error was defined as \(\frac{(y-x)}{x} \times 100\), where \(x\) is the concentration of initial substrate and \(y\) is the recovered concentration of all chitoooligosaccharides in (GlcNAc)\(_2\) units.

The rate equation of the lysozyme-catalyzed reaction on the initial substrate (GlcNAc)\(_2\), was numerically solved to obtain the calculated courses. A kinetic model of the lysozyme-catalyzed reactions of chitoooligosaccharides has been reported,\(^{11-13}\) and is schematically presented in Fig. 2. In the calculation, the rate equations were solved repeatedly while changing the values of each binding free energies or the rate constants, \(k_{+1}\), \(k_{-1}\), and \(k_{+2}\), so that the calculated courses fitted those experimentally obtained.

Results and Discussion

CD spectra
Since GHL carries amino acid substitutions at subsites E and F (Asn113 to Lys and Arg114 to His), this enzyme is considered to be useful for studying the role of subsites E and F in lysozyme catalysis. Our previous \(^1\)H-NMR study showed that the tertiary structure of GHL was similar to that of HEL.\(^{10}\) However, the local environments of some amino acid residues were found to be slightly different between GHL and HEL. Except for positions 113 and 114, GHL has eight amino acid substitutions outside of the substrate binding site when compared with HEL (Fig. 1). To further confirm that no large structural changes were induced by these substitutions, CD spectra of GHL and HEL were first measured before an investigation of subsites E and F by using GHL. As shown in Fig. 3, the spectra were approximately identical, suggesting that the amino acid substitutions found in GHL do not markedly change the main-chain folding of GHL. This result, together with our previous results,\(^{10}\) indicate that GHL retains almost the same global conformation as that of HEL.

Experimental courses of the reaction catalyzed by GHL
Chicken-type lysozymes have six (A-F) substrate binding sites (subsites), where the glycosidic bond
between sugar residues bound to subsites D and E is hydrolyzed through the conventional acid catalytic reaction of Glu35 and Asp52. 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 do a kinetic analysis of the experimental courses of oligomeric substrate degradation and product formation, using a kinetic model in which transglycosylation and all possible binding modes were taken into consideration. Therefore, we analyzed the reaction courses of (GlcNAc)₃ degradation catalyzed by GHL and compared them with those of HEL.

The experimental courses of HEL and GHL are shown in Fig. 4A. Under the experimental conditions used, the initial substrate (GlcNAc)₃ had disappeared almost completely in about 20 min. Although the order of the amounts of the products was (GlcNAc)₃ > (GlcNAc)₂ > (GlcNAc)₁ > (GlcNAc)₀ for both lysozymes after 20 min of reaction, the courses of GHL differed from those of HEL. The characteristic features of the courses were the increased production of (GlcNAc)₂ and the reduced production of (GlcNAc)₃. Consequently, the amounts of (GlcNAc)₂ and (GlcNAc)₃ were close, and separated from those of (GlcNAc)₀ and (GlcNAc)₁ in comparison with those of HEL. Among the amino acid substitutions found in GHL, only Lys113 and His114 are located at the substrate binding site. Thus, these two amino acids were considered to be prime candidates involved in the alteration in the courses of GHL.

**Overexpression and characterization of mutant enzymes**

To define the amino acid replacement(s) responsible for the differences in the courses between GHL and HEL, two mutant enzymes (N113K and R114H) were engineered, in which Asn113 and Arg114 in HEL were changed to the corresponding amino acid residues in GHL. Expression of the mutated cDNAs was done using the *P. pastoris* expression system, as described previously. All mutant enzymes were purified from yeast culture supernatant so as to give a single band on SDS-PAGE. The yields of protein from a 1-liter culture were 20–25 mg. The N-terminal amino acid sequences of the purified mutants were identical to that of HEL. Mutations at the Arg114 residue were confirmed by trypsin-digested peptide maps as described previously (data not shown). The integrity of the mutant enzymes was confirmed by measurements of far-ultraviolet CD. The CD spectrum of each mutant was almost indistinguishable from that of HEL, indicating that none of these mutations affected the backbone conformation (Fig. 5).

We then analyzed the experimental courses of (GlcNAc)₃ degradation catalyzed by the mutant enzymes, and compared them with those of HEL and GHL. As shown in Fig. 4A, the courses of the N113K mutant had profiles quite similar to those of HEL except that the amounts of (GlcNAc)₀ and (GlcNAc)₁ were slightly different from those of HEL. The observation that there is little difference in the reaction time-courses between HEL and the N113K mutant suggests that Asn113 in HEL contributes little, if any, to the lysozyme-catalyzed reactions. On the other hand, the courses of GHL were completely reproduced by the Arg114 to His mutation. The effect of this mutation was further corroborated by constructing a mutant protein, N113K/R114H, with two substitutions: the courses of the double mutant were almost the same as those of the R114H mutant (Fig. 4A). These results clearly indicate that the substituted His114, but not Lys113, in GHL is the critical residue that governs the characteristic courses of GHL.

**Estimation of the reaction parameters of the Arg114-mutated enzymes**

To evaluate the contribution of the substitution of Arg114 with His to the lysozyme-catalyzed reactions,
Fig. 4. Courses of Reaction of HEL, GHL, and HEL Mutants.
Panel A shows the experimental courses of HEL, GHL, and HEL mutant proteins. The initial concentrations of enzyme and substrate were 0.1 and 1 mM, respectively. Relative error indicates the recovery of the observed value at each reaction time calculated as described under "Materials and Methods." Panel B shows the calculated courses of HEL, GHL, R114H, and N113K/R114H. The parameter values used for the calculation are listed in Table 1. Numerals in the figures are the polymerization degrees of the reaction product species.

The experimental courses of GHL, R114H, and N113K/R114H were analyzed by computer simulation in order to obtain the rate constants and the binding free energy values at each subsite. The calculated courses of GHL were the same as those of the R114H and N113K/R114H mutants (Fig. 4B), and the reaction parameters thus obtained are summarized in Table 1. The binding free energy values for subsites A-D, the rate constant $k_{-1}$ for cleavage of the glycosidic linkage, and $k_{+2}$ for hydration were identical between HEL and the Arg114-mutated enzymes. On the other hand, the binding free energy values for sites E and F for the Arg114-mutated enzymes were found to be $-2.4$ kcal/mol and $-1.4$ kcal/mol, respectively, both being $-0.1$ kcal/mol lower than the respective HEL values. This indicated that the substrate binding for the Arg114-mutated enzymes is different from that for HEL at or near subsites E and F, and that the characteristic courses of these enzymes would be due to the change in the affinity of the substrate for subsites E and F from that of HEL.

On the basis of the results of X-ray crystallographic study, Arg114 is proposed to form two hydrogen bonds with the substrate: O-1 and O-5 atoms of a sugar ring in subsite F (Fig. 6), and to participate in
Guinea Hen Egg-white Lysozyme

Table 1. Estimated Reaction Parameter Values for HEL, GHL, R114H, and N113K/R114H

<table>
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<tr>
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<th>Binding free energy (kcal/mol)</th>
<th>Rate constant (s⁻¹)</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
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<tr>
<td>HEL</td>
<td>-2.0</td>
<td>-3.0</td>
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<tr>
<td>GHL</td>
<td>-2.0</td>
<td>-3.0</td>
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<tr>
<td>R114H</td>
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<td>N113K/R114H</td>
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k₊₁, k₋₁, and k₊₂ are the rate constants for cleavage of the glycosidic linkage, transglycosylation, and hydration, respectively.

Fig. 5. CD Spectra of HEL and Its Mutants in the Far-ultraviolet Region.

Spectra were measured as described in “Materials and Methods.” ⊿, ⊱, ⊳, and × indicate the CD spectra of HEL, N113K, R114H, and N113K/R114H, respectively.

The substrate binding at this site.⁹ Therefore, the decrease in substrate affinity at subsite F caused by the Arg to His mutation may be due to the partial loss of the hydrogen-bonding interactions involving the δ-guanido group of Arg114. It was thus expected that the substituted His114 in the Arg114-mutated enzymes do not form hydrogen bonds with the substrate in the same manner as those proposed in HEL. Alternatively, as suggested by Muraki et al.,¹⁰ the fine structure around subsite F may be slightly altered by the charge state of the residue at position 114, since the side chain of Arg is much more basic than that of His. Thus, the resulting conformational change around subsite F may lead to the reduction of substrate affinities at subsites E and F observed for the Arg114-mutated enzymes.

Furthermore, substitution of Arg114 with His reduced the rate constant of transglycosylation, with a k₋₁ value of 30.0 s⁻¹ compared to 40.0 s⁻¹ for HEL (Table 1). Our foregoing study by site-directed mutagenesis showed that the substitution of Asn37 in HEL with Ser, which is postulated to participate in sugar residue binding at the right-sided subsite F through hydrogen bonding, also resulted in a decrease in the transglycosylation activity of HEL.¹⁵ These results suggest that the reduction of the transglycosylation activity of HEL arising from the Arg to His mutation may be caused by a subtle rearrangement of the specific interaction between Arg114 and the sugar residue at subsite F. Since the binding of an acceptor molecule to subsites E and F should be essential for transglycosylation, the decrease in substrate affinities at subsites E and F observed for the Arg114-mutated enzymes would affect the acceptor-binding ability. Thus, substitution of the Arg residue is considered to affect the transglycosylation activity of HEL. Further studies are needed to confirm if Arg114 plays an important role in transglycosylation. This possibility is now being investigated in our laboratory.

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References


