J. Biochem. 131, 663–670 (2002)

Amino Acid Residues in Subsites E and F Responsible for the Characteristic Enzymatic Activity of Duck Egg-White Lysozyme

Shunsuke Kawamura,*[,1] Gen Toshima,* Taiji Imoto,† Tomohiro Araki,* and Takao Torikata*

Department of Bioscience, School of Agriculture, Kyushu Tokai University, Aso, Kumamoto 869-1404; and †Graduate School of Pharmaceutical Science, Kyushu University, Fukuoka 812-8582

Received January 15, 2002; accepted February 20, 2002

We analyzed the enzymatic properties of duck egg-white lysozyme II (DEL), which differs from hen egg-white lysozyme (HEL) in nineteen amino acid substitutions. A substrate binding study showed that DEL binds to the substrate analog at subsites A–C in the same manner as HEL. However, the experimental time-courses of DEL against the substrate *N*-acetylglucosamine pentamer, $(GlcNAc)_5$, revealed remarkably enhanced production of $(GlcNAc)_2$ and reduced production of $(GlcNAc)_1$ as compared to in the case of HEL. Computer simulation of the DEL-catalyzed reaction suggested that the amino acid substitutions at subsites E and F (Phe34 to Tyr and Asn37 to Ser) caused the great alteration in the time-courses of DEL. Subsequently, the enzymatic reactions of mutants, in which Phe34 and Asn37 in HEL were converted to Tyr and Ser, respectively, were characterized. The time-courses of the F34Y mutant exhibited profiles similar to those of HEL. In contrast, the characteristics of the N37S mutant were different from those of HEL and rather similar to those of DEL; the order of the amounts of $(GlcNAc)_1$ and $(GlcNAc)_2$ was reversed in comparison with in the case of HEL. Enhanced production of $(GlcNAc)_2$ was also observed for the mutant protein, F34Y/N37S, with two substitutions. These results indicated that the substitution of Asn37 with Ser can account, at least in part, for the characteristic time-courses of DEL. Moreover, replacement of Asn37 with Ser reduced the rate constant of transglycosylation. The substitution of the Asn37 residue may affect 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) 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 (*1*). The nature of the active site of HEL has been extensively studied with various methods. This enzyme includes six sugar residue binding sites called subsites A, B, C, D, E, and F. A substrate bound to subsites is cleaved between subsites D and E through the conventional acid catalytic reaction of Glu35 and Asp52 (*2, 3*).

In carbohydrate hydrolases, lysozyme exhibits high transglycosylation activity in addition to hydrolysis. However, the molecular mechanism of the highly efficient transglycosylation remains unknown. It is known that the acceptor molecule binds to subsites E and F, affecting the high efficiency of transglycosylation. Thus, more information on subsites E and F is required to elucidate the reaction mechanism of transglycosylation in lysozyme catalysis. Subsites A, B, and C have been extensively studied by means of chemical modification (*4–6*), substrate binding experiments, and kinetic analyses (*7–9*). On the contrary, information on subsites E and F is quite limited in spite of their importance in transglycosylation. This is because of some difficulties; one is the lack of characteristic amino acids at subsites E and F, and the other is the rapid hydrolysis of the GlcNAc hexamer, $(GlcNAc)_6$, through the enzyme action.

Lysozymes have been purified from various avian egg whites, and their amino acid sequences are now established (*10*). The amino acid sequences of HEL and duck egg-white lysozyme II (DEL), which were determined by Canfield (*11*) and Hermann *et al.* (*12*), respectively, are shown in Fig. 1. There are nineteen amino acid differences between the sequences of these lysozymes. Among these substitutions, it is notable that Phe34 and Asn37 at subsites E and F in HEL are replaced by Tyr and Ser, respectively, in DEL. It was thus expected that the enzymatic reaction of DEL may differ from that of HEL. From the energy minimization of HEL complexed with $(GlcNAc)_6$, it was found that two binding modes are possible for subsites E and F (*13, 14*), that is, right-sided and left-sided binding modes. In HEL, Phe34 and Asn37 are considered to be responsible for the

[1]To whom correspondence should be addressed. Tel: +81-9676-7-3918, Fax: +81-9676-7-3960, E-mail: skawa@ktmail.ktokai-u.ac.jp

Abbreviations: DEL, duck egg-white lysozyme; HEL, hen egg-white lysozyme; GlcNAc, *N*-acetylglucosamine; $(GlcNAc)_n$, β-1,4–linked oligosaccharide of GlcNAc; rHEL, recombinant HEL; CD, circular dichroism; RP-HPLC, reversed-phase high performance liquid chromatography; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; MD, minimal dextrose medium; YPD-G418, yeast extract peptone dextrose medium containing Geneticin 418.

									10									20
HEL	Lys	Val	Phe	Gly	Arg	Cys	Glu	Leu	Ala	Ala	Ala	Met	Lys	Arg	His	Gly	Leu	Asp	Asn	Tyr
DEL	···	···	Tyr	Ser	···	···	···	···	···	···	···	···	···	···	Leu	···	···	···	···	···
									30									40
HEL	Arg	Gly	Tyr	Ser	Leu	Gly	Asn	Trp	Val	Cys	Ala	Ala	Lys	Phe	Glu	Ser	Asn	Phe	Asn	Thr
DEL	···	···	···	···	···	···	···	···	···	···	···	···	Asn	Tyr	···	···	Ser	···	···	···
									50									60
HEL	Gln	Ala	Thr	Asn	Arg	Asn	Thr	Asp	Gly	Ser	Thr	Asp	Tyr	Gly	Ile	Leu	Gln	Ile	Asn	Ser
DEL	···	···	···	···	···	···	···	···	···	···	···	···	···	···	···	···	···	···	···	···
									70									80
HEL	Arg	Trp	Trp	Cys	Asn	Asp	Gly	Arg	Thr	Pro	Gly	Ser	Arg	Asn	Leu	Cys	Asn	Ile	Pro	Cys
DEL	···	···	···	···	···	···	···	Lys	···	···	···	···	Lys	···	Ala	···	Gly	···	···	···
									90									100
HEL	Ser	Ala	Leu	Leu	Ser	Ser	Asp	Ile	Thr	Ala	Ser	Val	Asn	Cys	Ala	Lys	Lys	Ile	Val	Ser
DEL	···	Val	···	···	Arg	···	···	···	···	Glu	Ala	···	Arg	···	···	···	Arg	···	···	···
									110									120
HEL	Asp	Gly	Asn	Gly	Met	Asn	Ala	Trp	Val	Ala	Trp	Arg	Asn	Arg	Cys	Lys	Gly	Thr	Asp	Val
DEL	···	···	···	···	···	···	···	···	···	···	···	···	···	···	···	Arg	···	···	···	···
									129									
HEL	Gln	Ala	Trp	Ile	Arg	Gly	Cys	Arg	Leu									
DEL	Ser	Lys	···	···	···	···	···	···	···									

Fig. 1. **Amino acid sequence alignment of HEL and DEL.** Three consecutive dots (···) indicate an amino acid residue identical to the corresponding residue in HEL. It should be noted that the amino acid residue at position 57 has been revised (Glu57 to Gln57) in this figure.

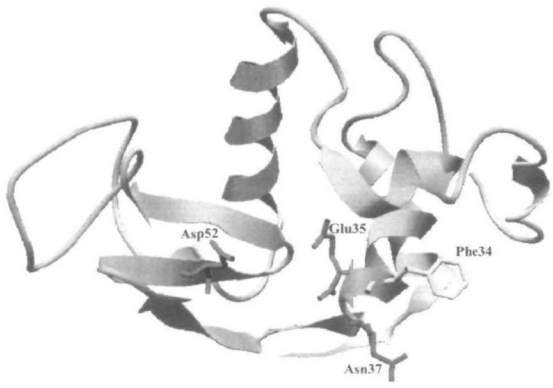

Fig. 2. **The three-dimensional structure of HEL.** The protein model is cited from Blake *et al.* (*25*). The side chains of amino acids examined in this study are shown. In addition, the catalytic residues (Glu35 and Asp52) are also shown. The figure was generated using Swiss-Pdb Viewer (v3. 7b2).

right-sided binding mode (Fig. 2). Therefore, these substitutions would be a good target for the study of subsites E and F by means of site-directed mutagenesis.

A data-fitting method for the experimental time-courses of lysozyme-catalyzed reactions with chitooligosaccharide can be directly used to estimate the binding free energy changes of the six subsites (*15–17*). Our foregoing study involving this method showed that the amino acid substitutions at subsites E and F in soft-shelled turtle lysozyme (STL) reduced the rate constant for glycosidic cleavage, and increased the binding free energy for subsites E and F (*18*).

In the present study, to better understand the roles of subsites E and F, the time-courses of the DEL-catalyzed reaction on the substrate GlcNAc pentamer, $(GlcNAc)_5$, were measured in 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 through computer simulation of the lysozyme-catalyzed reaction (*15–17*). Furthermore, mutant proteins as to subsites E and F in HEL were constructed and the effects of the mutations assessed. On the basis of the results, the differences in the enzymatic reactions between DEL and HEL are discussed in terms of the amino acid substitutions at subsites E and F.

EXPERIMENTAL PROCEDURES

Materials—Six-times recrystallized HEL was purchased from Seikagaku Kogyo. Freshly laid duck eggs were kindly donated by the Chemo-Sero-Therapeutic Research Institute, Kumamoto, and DEL was purified from the egg whites by the methods of Hermann *et al.* (*12*) with a slight modification. BCA protein assay reagent was obtained from Pierce. SP-Toyopearl 650M and CM-Toyopearl 650M were purchased from Tosoh. Lyophilized *Micrococcus luteus* cells and Geneticin 418 (G418) were from Sigma. Restriction enzymes and other DNA modification enzymes were obtained from either TaKaRa or Toyobo. The oligonucleotide primers used in this study were ordered from Hokkaido System Science. All other chemicals were of reagent grade and purchased from Nacalai Tesque or Wako Pure Chemicals.

Stains, Plasmids, and Phage—Escherichia coli strain JM109 was used for the transformation and propagation of recombinant plasmids. HA94-1, which contains HEL cDNA in a cloning vector M13 mp18 derivative, was used for site-directed mutagenesis. The *Pichia pastoris* expression system including expression vectors pPIC9 and pPIC9K was purchased from Invitrogen. *P. pastoris* GS115 (Invitrogen) was used as the host strain for expression.

Medium—E. coli cells were grown in Luria-Bertani broth. BMGY for growing *P. pastoris*, BMMY for induction of recombinant proteins, MD plates for transformation and YPD-G418 plate for selection of multicopy transformants are described in the Invitrogen manual.

Peptide Mapping—Digestion of reduced and carboxyme-

thylated lysozymes with TPCK-trypsin (Cooper Biomedical, USA) and the separation of tryptic peptides by reversed-phase high-performance liquid chromatography (RP-HPLC) were accomplished as described previously (*18*). Amino acid analyses were performed 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 determined on an Applied Biosystems model 477A sequencer.

Substrate Binding—Substrate binding was estimated by measuring the fluorescence intensity standardized as to *N*-acetyltryptophane with a Hitachi F-4500 fluorescent photometer. Namely, the GlcNAc trimer, $(GlcNAc)_3$ (0.02 mM to 0.2 mM), was incubated with lysozyme (0.02 mM) in 10 mM acetate buffer (pH 5.0) for 5 min at 30°C. The reaction mixture was measured with excitation at 291 nm and emission at 360 nm. The amount of enzyme-substrate complex was calculated from the reduction of the fluorescence intensity of the reaction mixture compared to that of lysozyme. The dissociation constant (K_d), association constant (K_a), and binding free energy were calculated by Scatchard plotting (*19*).

Enzyme Action—Lysozyme activity was measured by the method of Masaki *et al.* (*15*) with a slight modification. Namely, the reaction mixture containing 0.1 mM lysozyme and 1 mM $(GlcNAc)_5$ was incubated in 10 mM sodium acetate buffer (pH 5.0) at 50°C. After a given reaction time, 200 μl of the reaction mixture was withdrawn and rapidly chilled in the KOOL KUP (Towa). The reaction mixture was centrifuged with Ultrafree C3LCC (Millipore). The filtrate was lyophilized. The dried sample was dissolved in 50 μl of ice-cold water, and then 10 μl of the solution was applied on a TSKgel G-Oligo-PW column (7.8 × 60 mm; Tosoh) in a JASCO 800 series HPLC. Elution was performed with distilled water at room temperature and the flow rate of 0.3 ml/min. Each chitooligosaccharide concentration was calculated from the peak area monitored as to the ultraviolet absorption at 220 nm, using the standard curve obtained for authentic saccharide solutions. The relative error was defined as $(y - x)/x \times 100$, where x is the concentration of the initial substrate and y is the recovered concentration of all chitooligosaccharides in $(GlcNAc)_1$ units.

The rate equation of the lysozyme-catalyzed reaction on the initial substrate $(GlcNAc)_5$ was numerically solved to obtain the calculated time-courses. A kinetic model of the lysozyme-catalyzed reaction of chitooligosaccharides has been reported (*15–17*), and is schematically presented in Fig. 3. In the calculation, the rate equations were solved repeatedly while changing the values of the binding free energies or the rate constants, k_{+1}, k_{-1}, and k_{+2}, so that the calculated time-courses fitted those experimentally obtained.

Construction of an Expression Vector and Introduction into P. pastoris—A DNA fragment encoding mature HEL was amplified by PCR using HA94-1 as a template with primer pair 5′*Xho*I (5′-GGCCTCGAGAAAAGAAAAGTCTTTGGACGATGTGAGCTAGC-3′)/3′*Eco*RI (5′-GCCGAATTCACAGCCGGCAGCCTCTGAT-3′), as the sense and antisense primers, respectively. The PCR product was digested with *Xho*I and *Eco*RI, and then inserted into the same sites of plasmid pBluescript (Stratagene). After digestion of the resulting plasmid with *Xho*I and *Eco*RI, the DNA fragment

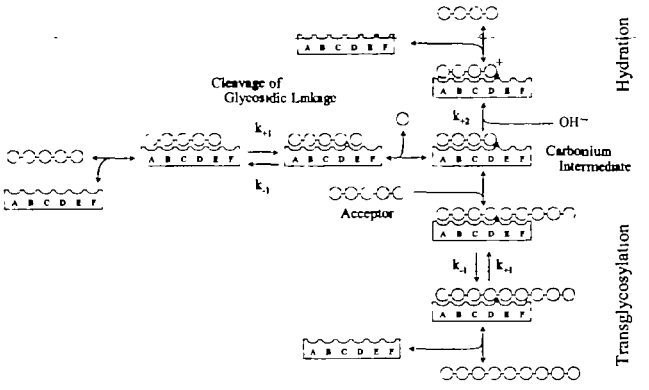

Fig. 3. **Reaction scheme for the lysozyme-catalyzed reaction of $(GlcNAc)_5$.** In this scheme, k_{+1}, k_{-1}, and k_{+2} are the rate constants for cleavage of the glycosidic linkage, transglycosylation, and hydration, respectively.

was ligated into *P. pastoris* vector pPIC9 to yield plasmid pPHEL. This cloning step resulted in translational fusion of the HEL coding region to that of the *Saccharomyces cerevisiae* α-factor prepropeptide. Subsequently, the *Bam*HI/*Eco*RI DNA fragment from pPHEL was recloned into plasmid pPIC9K, resulting in plasmid pPKHEL, which allows selection of multiple insertions of the expression cassette. Plasmid pPKHEL was digested with *Sal*I and then introduced into *P. pastoris* GS115 (*his*4) by a lithium chloride method according to the manufacturer's instructions (Invitrogen). Recombinant His^+ yeast clones obtained after transformation on MD plates were grown on YPD plates containing Geneticin 418 (0.025–0.05% G418) for selection of multiple insertions (*20*).

Site-Directed Mutagenesis—The megaprimer method for site-directed mutagenesis (*21*) was used to introduce mutations, HA94-1 serving as a template. The flanking primers used were 5′*Xho*I and 3′*Eco*RI. The mutagenic primers used were 5′-GCCGCAAAATACGAGAGTAAC-3′ for F34Y and 5′-AGAGTAGCTTCAACACCCAGG-3′ for N37S. The mutant DNA fragments were inserted into plasmid pBluescript. Mutations were confirmed by DNA sequencing with an ABI PRIME Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRIME 373A DNA Sequencer (Perkin-Elmer Applied Biosystems). Mutations were further confirmed by amino acid sequence analysis of the purified proteins after digestion with trypsin. To create the gene encoding HEL with a double mutation (F34Y/N37S), the second mutation was introduced into the gene encoding F34Y by using the primers for the N37S mutation. Cloning to expression vector pPIC9K and transformation into *P. pastoris* were performed as described above.

Protein Expression and Purification—A recombinant *P. pastoris* clone carrying plasmid pPKHEL or a derivative of it was typically inoculated into 5 ml of BMGY and then incubated at 30°C for 24 h. This seed culture was subcultured into 100 ml of the same medium for an additional 24 h. The cells were harvested and then transferred to 900 ml of BMMY to give an OD_{600} of 1.0 to induce production. The culture was maintained for 5 days, methanol being supplemented to a final concentration of 1% every 24 h.

The recombinant hen lysozyme (rHEL) and its mutants were purified by successive cation-exchange chromatogra-

phies as follows: the culture supernatant was diluted 5-fold with water and then first put on an SP-Toyopearl column (1.5 × 12.5 cm) equilibrated with 30 mM phosphate buffer (pH 6.0). The proteins were eluted with a linear gradient of 0.1 to 0.5 M NaCl in the same buffer. The lysozyme fraction was diluted 5-fold with water and then further chromatographed on a CM-Toyopearl column (1.5 × 12.5 cm) equilibrated with 30 mM acetate buffer (pH 5.0). The elution process followed a linear gradient of NaCl, from 0.2 to 0.6 M, in the buffer. The purities of enzymes were checked by SDS-PAGE. The purified enzymes were exhaustively dialyzed against distilled water and then lyophilized for storage. Enzymatic activity was monitored as to lytic activity using lyophilized cell wall of *M. luteus* as a substrate, as described previously (*18*). Protein concentrations were determined by the bicinchoninic acid method (*22*), using authentic HEL as a standard protein.

Circular Dichroism (CD) Spectra—Circular dichroism (CD) spectra were obtained at 25°C with a Jasco J-820 spectropolarimeter. Proteins were dissolved to a final concentration of 0.15 mg/ml in 10 mM sodium acetate buffer (pH 5.0). The data were expressed in terms of mean residue ellipticity. The path-length of the cells was 0.1 cm for far-ultraviolet CD spectra (200–250 nm).

RESULTS AND DISCUSSION

Amino Acid Sequence of Duck Egg-White Lysozyme II (DEL)—Cation-exchange chromatography of duck egg white yielded two active peaks, corresponding to duck egg-white lysozyme II (DEL-II) and duck egg-white lysozyme III (DEL-III), as reported previously (*12*). The former lysozyme (DEL-II) was used as DEL for all the following experiments.

The amino acid sequence of the purified DEL was reinvestigated by rapid structural analysis using peptide maps reported by Araki *et al.* (*18*). We found 19 amino acid differences between DEL and HEL, as shown in Fig. 1. Furthermore, one substitution (Glu57 to Gln57) was found when compared with the reported sequence (*12*). This may be due to the polymorphism of the lysozyme genes in domestic ducks. Based on this result, among the substitutions in DEL and HEL, Tyr34 and Ser37 are the amino acids contributing to the substrate binding; these two residues are located on the right side of subsites E and F.

Substrate Binding Property of DEL—Since DEL carries the amino acid substitutions at subsites E and F (Phe34 to Tyr and Asn37 to Ser), this enzyme would be good for studying subsites E and F in lysozyme-catalyzed reactions. However, other than positions 34 and 37, seventeen amino acid differences outside of substrate binding site were found between DEL and HEL, suggesting that the local environments in DEL may be somewhat different from those in HEL. In lysozyme, the fluorescence intensity derived from Trp residues in subsites A–C (Trp62, Trp63, and Trp108) reflects the microenvironments of these sites (*8, 23, 24*). Previously, it was reported that the substrate analog, $(GlcNAc)_3$, binds predominantly to subsites A–C in the ratio of 99% without hydrolysis (*23*). To investigate the microenvironments of subsites A–C, we analyzed the fluorescence spectrum of DEL caused by the Trp residues with and without $(GlcNAc)_3$, and compared it with that of HEL.

In Table I, the dissociation constants (K_d), association constants (K_a), and binding free energies for binding of $(GlcNAc)_3$ are summarized. No remarkable change in affinity to $(GlcNAc)_3$ was found between DEL and HEL, indicating that the amino acid substitutions found in DEL do not affect the binding of $(GlcNAc)_3$ to sites A, B, and C. Therefore, the environments of subsites A–C in DEL are similar to those in HEL.

Activity of DEL against GlcNAc Pentamer, $(GlcNAc)_5$—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. As lysozyme catalyzes not only the hydrolysis of sugar chains but also a transglycosylation reaction, the oligosaccharide product shows complicated patterns. However, analysis of the time-courses using $(GlcNAc)_5$ enabled us to evaluate the effects of different amino acids at the subsites by computer simulation analysis (*15*).

The experimental time-courses of DEL for $(GlcNAc)_5$ were analyzed and are shown in Fig. 4A. In the HEL-catalyzed reaction, the order of the amounts of the products was $(GlcNAc)_1 > (GlcNAc)_2 > (GlcNAc)_4 > (GlcNAc)_3$. The profiles of the time-courses of DEL differed greatly from those of HEL: the products formed were $(GlcNAc)_2 > (GlcNAc)_1 > (GlcNAc)_3 > (GlcNAc)_4$. The characteristic features of the time-courses were the remarkable increased production of $(GlcNAc)_2$ and the reduced production of $(GlcNAc)_1$. Among the amino acid substitutions found in DEL, only Tyr34 and Ser37 are located at the substrate binding site. Thus, the substitutions at positions 34 and 37 were considered to contribute to the difference in the product concentrations between DEL and HEL.

To confirm this, experimental time-courses were simulated by computer analysis. The calculated time-courses are shown in Fig. 4B. The binding free energy values and rate constants for calculated time-courses for these lysozymes are listed in Table II. The binding free energy values for subsites A to D and the rate constant were identical for these lysozymes. On the other hand, the binding free energy values for sites E and F for DEL were decreased by 0.2 kcal/mol and increased by 0.6 kcal/mol, respectively, compared with those of HEL. This indicated that the substrate binding for DEL is different from that for HEL at subsites E and F, and that the characteristic time-courses of DEL would be due to the difference in the binding free energy changes at subsites E and F between DEL and HEL. This observation, together with the same binding ability as to subsites A–C, strongly suggested that the substitutions of Phe34Tyr and Asn37Ser at subsites E and F caused the great alteration in the time-courses of DEL.

Expression and Characterization of Recombinant Hen Lysozyme (rHEL)—To further assess the contributions of these substitutions by means of a genetic engineering approach, we first established an expression system of rHEL

TABLE I. **Binding of DEL and HEL with $(GlcNAC)_3$.**

	K_d (M)	K_a (M)	Binding free energy (kcal/mol)
DEL	2.97×10^{-5}	3.37×10^{4}	–8.70
HEL	2.31×10^{-5}	4.33×10^{4}	–8.85

The dissociation constant (K_d), estimated from a Scatchard plot, was determined as the average of three separate experiments. (Estimated error in binding assays, ±3%.)

in yeast and used it to construct HEL mutant proteins. For this purpose, the coding region of the HEL gene was amplified by PCR and fused to the DNA encoding the *S. cerevisiae* α-factor prepropeptide in vector pPIC9K. The resulting expression plasmid, pPKHEL, was introduced into *P. pastoris* cells and recombinant clones were selected. Afterward, clones bearing multicopy insertions were screened as described previously (*20*). The protein expression was induced by the addition of methanol to the medium.

The rHEL secreted into the medium was purified by successive cation-exchange chromatographies. The purified rHEL gave a single band on analysis by SDS-PAGE (data not shown). The yield of rHEL was about 25 mg/liter induced culture. Direct sequencing of rHEL gave a single N-terminal amino acid sequence: Lys-Val-Phe, which was identical to that of authentic HEL. The trypsin-digested peptide map obtained on RP-HPLC of rHEL exhibited the same pattern as that of authentic HEL, indicating that the amino acid sequences of the two lysozymes are identical (data not shown). The time-course of rHEL with $(GlcNAc)_5$ was found to be kinetically indistinguishable from that of authentic HEL (Figs. 4A and 5A, and Table II). These results indicated that the rHEL produced by yeast was correctly folded into an active conformation, which is practically the same as that of HEL.

Preparation and Enzymatic Reactions of Mutant Proteins—Using this expression system, we generated two mutant enzymes (F34Y and N37S), in which Phe34 and Asn37 in HEL were changed to the corresponding amino acid residues in DEL. Expression and purification of the mutants were performed in the same way as for rHEL. The N-terminal amino acid sequences of the purified mutant enzymes were identical to that of HEL. The correct mutation site was confirmed for each of these mutants by digestion with trypsin; the difference was detected on comparison of peptide maps obtained on RP-HPLC, where only the retention time of the peptide containing the designed mutation was shifted (data not shown). The amino acid compositions and amino acid sequences of these peptides were completely consistent with those expected. These results indicated that the mutant proteins secreted from yeast were all mature forms having the respective designed mutations. The secondary structure of each mutant enzyme was checked by examining the CD spectrum in the 200–

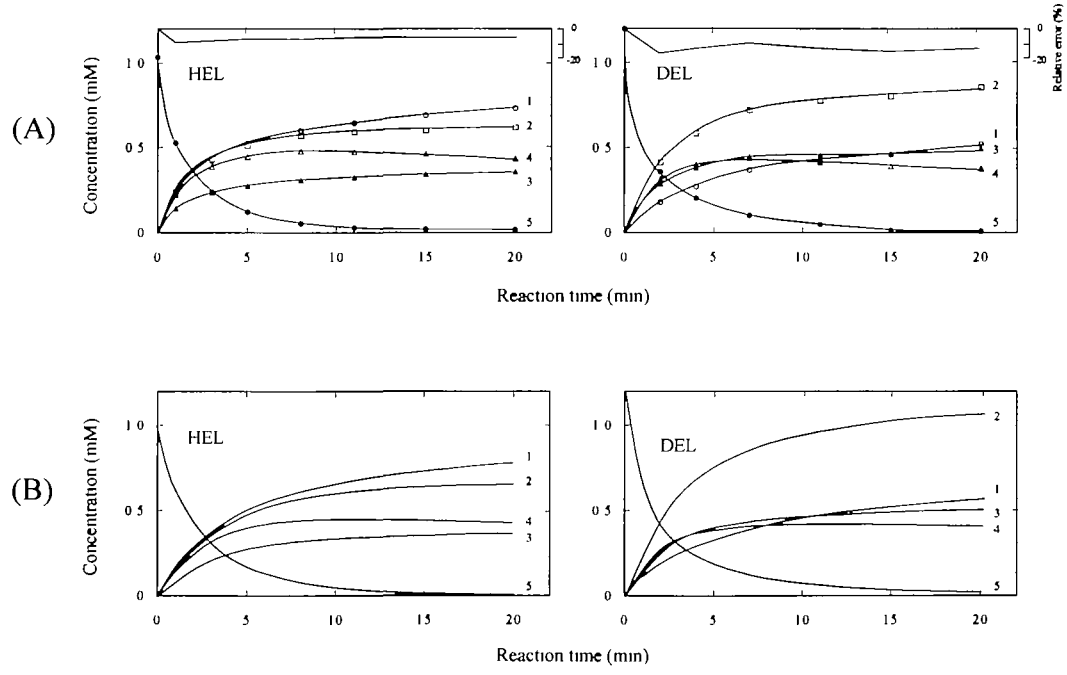

Fig. 4. **Time-courses of HEL and DEL.** Panel A shows the experimental time-courses of HEL and DEL. The initial concentrations of enzyme and substrate were 0.1 and 1 mM, respectively. Relative error indicates the recovery of the observed value at each reaction time calculated as described under "EXPERIMENTAL PROCEDURES." Panel B shows the calculated time-courses of HEL and DEL. The parameter values used for the calculation are listed in Table II. Numerals in the figures are the polymerization degrees of the reaction product species.

TABLE II. **Estimated reaction parameter values for DEL, HEL, rHEL, and HEL mutants.**

	Binding free energy (kcal/mol)						Rate constant (s^{-1})		
	A	B	C	D	E	F	k_{+1}	k_{-1}	k_{+2}
DEL	−2.0	−3.0	−5.0	4.5	−2.3	−2.1	0.93	40.0	0.30
HEL, rHEL	−2.0	−3.0	−5.0	4.5	−2.5	−1.5	0.93	40.0	0.30
F34Y	−2.0	−3.0	−5.0	4.5	−2.4	−1.4	0.93	40.0	0.30
N37S	−2.0	−3.0	−5.0	4.5	−2.5	−1.7	0.93	30.0	0.30
E34Y/N37S	−2.0	−3.0	−5.0	4.5	−2.4	−1.7	0.93	35.0	0.30

250 nm region (Fig. 6). The CD spectra of the mutants were essentially identical to that of HEL, indicating that the secondary structure was not affected by these mutations.

We then analyzed the experimental time-courses of $(GlcNAc)_5$ degradation catalyzed by the mutant enzymes, and compared them with those of HEL and DEL. As shown in Fig. 5A, the time-courses of the F34Y mutant exhibited profiles quite similar to those of HEL except that the amounts of $(GlcNAc)_1$ and $(GlcNAc)_4$ were slightly different from those in the case of HEL. In contrast, the product distribution of the N37S mutant was different from those of HEL and rather similar to those of DEL; the concentrations of $(GlcNAc)_2$ and $(GlcNAc)_1$ were in the reverse order in comparison with in the case of HEL. Thus, it is likely that the enhanced production of $(GlcNAc)_2$ in the DEL-catalyzed reaction is mainly governed by the Asn37 to Ser mutation. The enhanced production of $(GlcNAc)_2$ due to the Ser residue at position 37 was further corroborated by constructing a mutant protein, F34Y/N37S, with two substitutions: the time-courses of the double mutant were nearly the same as those of the N37S mutant (Fig. 5A). Although the time-courses of DEL, especially the reduced production of $(GlcNAc)_1$, could not be reproduced, these results indicated that the substitution of Asn37 with Ser can account, at least in part, for the origin of the characteristic time-courses of DEL.

CD Spectra—As mentioned above, the experimental time-courses of DEL showed a characteristic profile, that is, a large amount of $(GlcNAc)_2$ and a small amount of $(GlcNAc)_1$ were produced. On computer simulation, it was

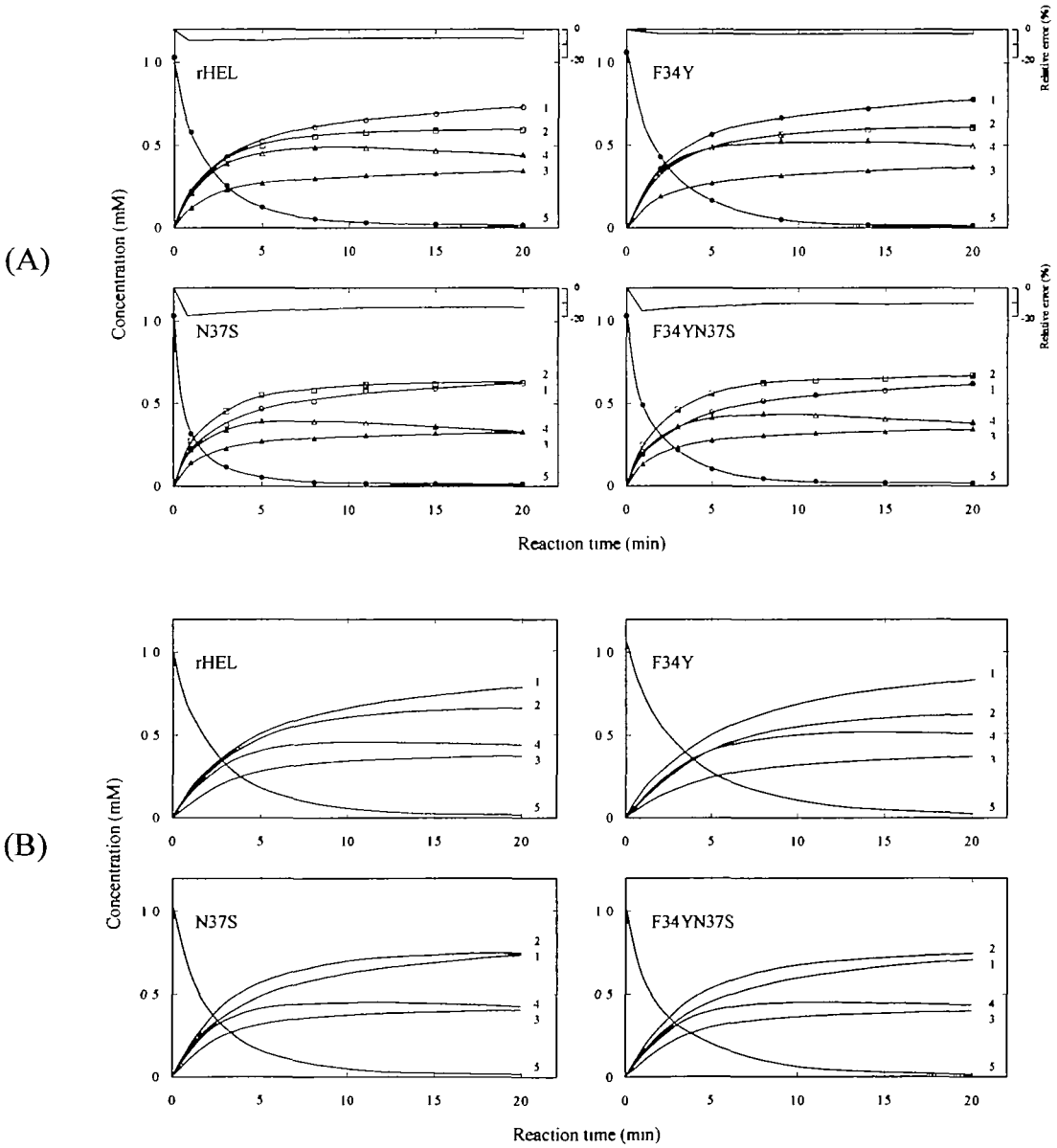

Fig. 5. **Time-courses of rHEL and its mutants.** Panel A shows the experimental time-courses of rHEL, F34Y, N37S, and F34Y/N37S. The initial concentrations of enzyme and substrate were 0.1 and 1 mM, respectively. Panel B shows the calculated time-courses of rHEL, F34Y, N37S, and F34Y/N37S. The parameter values used for the calculation are listed in Table II. Numerals in the figures are the polymerization degrees of the reaction product species.

suggested that the differences in the time-courses between DEL and HEL would be brought about by the amino-acid substitutions at subsites E and F. However, in the experimental time-courses of the double mutant, in which Phe34 and Asn37 in HEL were simultaneously mutated to the corresponding residues, Tyr and Ser, respectively, present in DEL, only increased production of $(GlcNAc)_2$ was observed and the concentration of $(GlcNAc)_1$ was still higher than that observed for DEL. Thus, the difference in the oligomer concentration between DEL and HEL was not fully accounted for by these two substitutions. This suggested that the different profiles of DEL and HEL might be caused not only by the substitutions at subsites E and F but also by other factors influencing the overall structure of the proteins. Since DEL has nineteen amino acid substitutions in its molecule when compared with HEL, it was expected that these substitutions give rise to certain structural changes in DEL which may contribute to the decreased amount of $(GlcNAc)_1$.

To study the conformational changes accompanying the amino acid substitutions, CD spectra of DEL and HEL were obtained (Fig. 7). The shapes of the two spectra were basically similar to each other, suggesting the conformational similarity of both molecules in solution. However, a small but noticeable difference in molecular ellipticity was observed, indicating that some conformational change certainly has occurred between DEL and HEL. In contrast to little change in the strength of the negative peak at 200–220 nm, the negative band in the spectrum of DEL at 220–240 nm shifted to shorter wavelengths. Therefore, the observed differences in the time-courses between DEL and mutant F34Y/N37S may be attributed to an overall conformational change in DEL due to some other amino acid substitutions in the molecule compared to HEL.

Estimation of the Reaction Parameters of Mutant Proteins—Subsequently, the experimental time-courses of the mutant proteins were analyzed by computer simulation in order to obtain the rate constants and the binding free energy values of the individual subsites. The calculated time-courses are shown in Fig. 5B and the reaction parameters thus obtained are summarized in Table II. For the F34Y mutant, the binding free energy values for sites E and F 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. On the basis of the results of the X-ray crystallographic study, the main chain carbonyl oxygen of Phe34 is proposed to form a hydrogen bond with the hydroxyl group at position 6 of a sugar ring in subsite F and to participate in the substrate binding at this site (*25*). However, the effects of the mutation on substrate binding 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 the F34Y mutant, the decreased affinities at subsites E and F most likely originate from the phenolic hydroxyl group of the substituted Tyr34.

As for the N37S mutant, the binding free energy value for subsite F was found to be –1.7 kcal/mol, which is –0.2 kcal/mol higher than that of HEL. This is consistent with the increase in the free energy value observed for DEL (Table II), although the mutant protein exhibited –0.4 kcal/mol lower affinity than that of DEL. From the results of model-building studies based on the X-ray structure, the side chain of Asn37 is postulated to interact with the hydroxyl oxygen at position 6 of a sugar ring in subsite F (*25*). The increase in affinity at subsite F in the N37S mutant suggests that Asn37 is one of the amino acids responsible for sugar binding at this site, and that a favorable interaction occurs between the serine hydroxyl group and the sugar residue at subsite F.

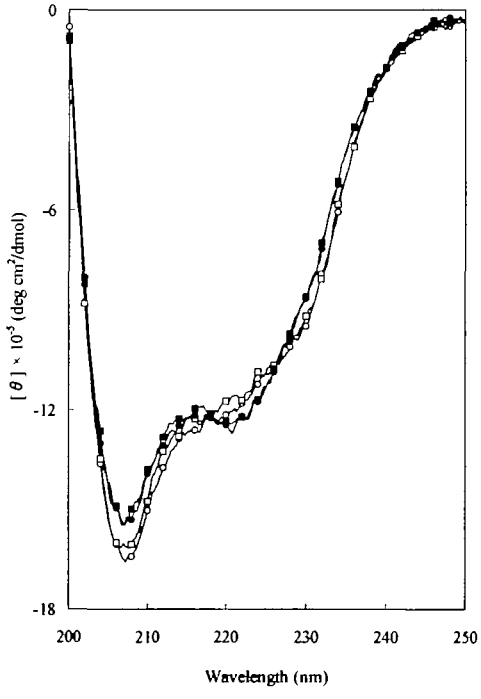

Fig. 6. **CD spectra of HEL and its mutants in the far-ultraviolet region.** Spectra were measured as described under "EXPERIMENTAL PROCEDURES". ○, □, ●, and ■ indicate the CD spectra of HEL, N37S, F34Y, and F34Y/N37S, respectively.

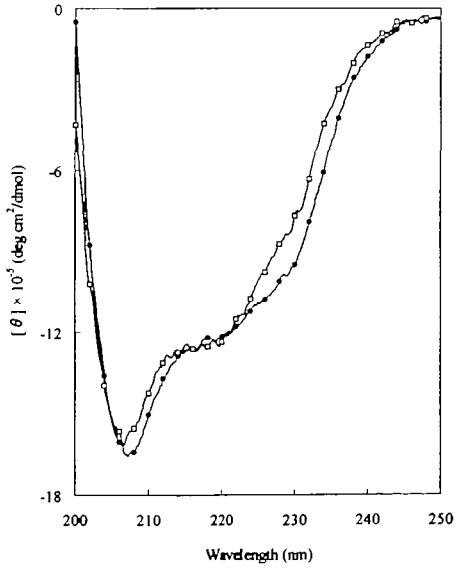

Fig. 7. **CD spectra of HEL and DEL in the far-ultraviolet region.** Spectra were measured as described under "EXPERIMENTAL PROCEDURES." ● and □ indicate the CD spectra of HEL and DEL, respectively.

Furthermore, replacement of Asn37 with Ser reduced the rate constant of transglycosylation, with a k_{-1} value of 30.0 s^{-1} compared to 40.0 s^{-1} for HEL. A reduced rate constant of transglycosylation was also observed for mutant protein F34Y/N37S: the k_{-1} value of this variant was decreased to 35.0 s^{-1} (Table II). These results suggested that the reduction of the transglycosylation activity of HEL arising from the Asn to Ser mutation might be caused by subtle rearrangement of the specific interaction between Asn37 and the sugar residue at subsite F. Therefore, substitution of the Asn37 residue may affect the transglycosylation activity of HEL. Further studies on the Asn37-mutated enzymes involving additional mutagenesis and structural analyses should provide valuable information for elucidating the molecular mechanism of the high efficiency of transglycosylation.

We wish to thank the Chemo-Sero-Therapeutic Research Institute (Aso Laboratory, Kumamoto) for supplying the eggs. We also thank Dr. Toshihiro Nakashima (Kikuchi Research Center, Chemo-Sero-Therapeutic Research Institute, Kikuchi, Kumamoto) for permission to measure the CD spectra.

REFERENCES

1. Imoto, T., Johnson, L.N., North, A.T.C., Phillips, D.C., and Rupley, J.A. (1972) Lysozyme in *Enzymes* (Boyer, P.D., ed.) Vol. 7, 3rd ed., pp. 665–868, Academic Press, New York
2. Kuroki, R., Yamada, H., Moriyama, T., and Imoto, T. (1986) Chemical mutations of the catalytic carboxyl groups in lysozyme to the corresponding amides. *J. Biol. Chem.* **261**, 13571–13574
3. Malcolm, B.A., Rosenberg, S., Corey, M.J., Allen, J.S., Baetselier, A.D., and Kirsch, J.F. (1989) Site-directed mutagenesis of the catalytic residues Asp-52 and Glu-35 of chicken egg white lysozyme. *Proc. Natl. Acad. Sci. USA* **86**, 133–137
4. Yamada, H., Imoto, T., Fujita, K., Okazaki, K., and Motomura, M. (1981) Selective modification of aspartic acid-101 in lysozyme by carbodiimide reaction. *Biochemistry* **20**, 4836–4842
5. Yamada, H., Imoto, T., and Noshida, S. (1982) Modification of catalytic groups in lysozyme with ethylenimine. *Biochemistry* **21**, 2187–2192
6. Hayashi, K., Imoto, T., and Funatsu, M. (1963) The enzyme-substrate complex in a muramidase (lysozyme) catalysed reaction. I. Difference spectrum of complex. *J. Biochem.* **54**, 381–387
7. Shrake, A. and Rupley, J.A. (1980) Oxindolealanine-62 lysozyme: equilibrium, calorimetric, and kinetic studies of the reaction with N-acetylglucosamine oligosaccharides. *Biochemistry* **19**, 4044–4051
8. Teshima, K., Kuramitsu, S., Hamaguchi, K., Sakiyama, F., Mizuno, K., and Yamasaki, N. (1980) Binding of substrate analogs to hen lysozyme in which Trp62 is modified to kynurenine. *J. Biochem.* **87**, 1015–1027
9. Fukamizo, T., Hayashi, K., and Goto, S. (1986) The role of binding subsite A in reactions catalyzed by hen egg-white lysozyme. *Eur. J. Biochem.* **158**, 463–467
10. Jolles, P. and Jolles, J. (1984) What's new in lysozyme research? Always a model system, today as yesterday. *Mol. Cell. Biochem.* **63**, 165–189
11. Canfield, R.E. (1963) The amino acid sequence of egg white lysozyme. *J. Biol. Chem.* **238**, 2698–2707
12. Hermann, J., Jolles, J., and Jolles, P. (1971) Multiple forms of duck-egg-white lysozymes. Primary structure of two duck lysozymes. *Eur. J. Biochem.* **24**, 12–17
13. Pincus, M.R. and Scheraga, H.A. (1979) Conformational energy calculations of enzyme-substrate and enzyme-inhibitor complexes of lysozyme. 2. Calculations of the structures of complexes with a flexible enzyme. *Macromolecules* **12**, 633–644
14. Pincus, M.R. and Scheraga, H.A. (1981) Prediction of the three-dimensional structures of complexes of lysozyme with cell wall substrates. *Biochemistry* **20**, 3960–3965
15. Masaki, A., Fukamizo, T., Otakara, A., Torikata, T., Hayashi, K., and Imoto, T. (1981) Estimation of rate constants in lysozyme-catalyzed reaction of chitooligosaccharides. *J. Biochem.* **90**, 1167–1175
16. Fukamizo, T., Minematsu, T., Yanase, Y., Hayashi, K., and Goto, S. (1986) Substrate size dependence of lysozyme-catalyzed reaction. *Arch. Biochem. Biophys.* **250**, 312–321
17. Fukamizo, T., Goto, S., Torikata, T., and Araki, T. (1989) Enhancement of transglycosylation activity of lysozyme by chemical modification. *Agric. Biol. Chem.* **53**, 2641–2651
18. Araki, T., Yamamoto, T., and Torikata, T. (1998) Reptile lysozyme: The complete amino acid sequence of soft-shelled turtle lysozyme and its activity. *Biosci. Biotechnol. Biochem.* **62**, 316–324
19. Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Ann. N.Y Acad. Sci.* **51**, 660–672
20. Weiss, H.M., Hasse, W., Michel, H., and Reilander, H. (1998) Comparative biochemical and pharmacological characterization of the mouse $5HT_{5A}$ 5-hydroxytryptamine receptor and the human β_2-adrenergic receptor produced in the methylotrophic yeast *Pichia pastoris*. *Biochem. J.* **330**, 1137–1147
21. Sarkar, G. and Sommer, S.S. (1990) The "megaprimer" method of site-directed mutagenesis. *Biotechniques* **8**, 404–407
22. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Garther, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of portein using bicinchroninic acid. *Anal. Biochem.* **150**, 76–85
23. Kuhara, S., Ezaki, E , Fukamizo, T., and Hayashi, K. (1982) Estimation of the free energy change of substrate binding in lysozyme-catalyzed reactions. *J. Biochem.* **92**, 121–127
24. Fukamizo, T., Kuhara, S., and Hayashi, K. (1982) Enzymatic activity of Trp 62-modified lysozyme. *J. Biochem.* **92**, 717–724
25. Blake, C.C.F., Johnson, L.N., Mair, G.A., North, A.C.T., Phillips, D.C., and Sarma, V. (1967) Crystallographic studies on the activity of hen egg white lysozyme. *Proc. Roy. Soc. Ser. B* **167**, 378–388