Redesign of the Substrate-binding Site of Hen Egg White Lysozyme Based on the Molecular Evolution of C-type Lysozymes*

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On the basis of the molecular evolution of hen egg white, human, and turkey lysozymes, three replacements (Trp62 with Tyr, Asn77 with Gly, and Asp101 with Gly) were introduced into the active-site cleft of hen egg white lysozyme by site-directed mutagenesis. The replacement of Trp62 with Tyr led to enhanced bacteriolytic activity at pH 6.2 and a lower binding constant for chitotriose. The fluorescence spectral properties of this mutant hen egg white lysozyme were found to be similar to those of human lysozyme, which contains Tyr at position 62. The replacement of Asn77 with Gly had little effect on the enzymatic activity and binding constant for chitotriose. However, the combination of Asn77 → Gly (N37G) replacement with Asp101 → Gly (D101G) and Trp62 → Tyr (W62Y) conversions enhanced bacteriolytic activity much more than each single mutation and restored hydrolytic activity toward glycol chitin. Consequently, the mutant lysozyme containing triple replacements (N37G, W62Y, and D101G) showed about 3-fold higher bacteriolytic activity than the wild-type hen lysozyme at pH 6.2, which is close to the optimum pH of the wild-type enzyme.

* c-type lysozymes from many vertebrates and some invertebrates have been identified and isolated (Jollès and Jollès, 1984). They exhibit hydrolytic activity on β-1,4 glycosidic bonds of polysaccharides, comprising the pentagonal layer of bacterial cell walls, and polymers of N-acetyl-D-glucosamine (GlcNAc), chitin. Their primary and tertiary structures display a high degree of homology, especially at their active sites. Among them, hen egg white lysozyme, an enzyme consisting of 129 amino acid residues, was the first enzyme whose three-dimensional structure was analyzed by x-ray crystallography (Blake et al., 1965). The active site of the lysozyme is believed to consist of six subsites, named A–F, which are able to bind six sugar residues. The technique of x-ray crystallography was able to show that a trimer of GlcNAc bound to the A, B, and C subsites, and that GlcNAc bound selectively to subsite C (Blake et al., 1967).

There is conservation of the amino acid residues comprising the substrate-binding region among c-type lysozymes. However, a number of substitutions that may modulate enzymatic activity have been observed (Jollès and Jollès, 1984). For example, Trp62 of hen egg white lysozyme is replaced with Tyr in human and rat lysozymes, which exhibit 2–4-fold enhanced bacteriolytic activity (Mulvey et al., 1973, 1974). We converted Trp62 of hen egg white lysozyme to Tyr by site-directed mutagenesis and found that the mutated lysozyme showed enhanced bacteriolytic activity (Kumagai et al., 1987; Kumagai and Miura, 1989).

Although human lysozyme shows 4-fold higher lytic activity than does hen egg white enzyme, the mutant hen lysozyme in which Trp62 was replaced with Tyr showed at best twice the lytic activity of the wild-type hen enzyme. Another difference in substrate-binding sites between human and hen enzymes is the replacement of Asp101 in the hen enzyme with Gly. Asp101 is a conserved amino acid residue in c-type lysozymes. In turkey lysozyme, however, Asp101 is replaced with Gly, and the lysozyme also exhibits 1.5-fold higher lytic activity (LaRue and Speck, 1970). Fig. 1 shows amino acid substitutions that are found in substrate-binding sites of the three c-type lysozymes.

It is believed that Asp101 interacts with the A and B sugar residues at the A and B subsites by hydrogen bonding and that Trp62 directly interacts with GlcNAc residues bound to subsites B and C. On the other hand, Asn77 is thought to contribute to the binding of sugar residues at the F subsite, on the basis of model-building studies (Blake et al., 1967).

We then introduced these three replacements found in nature either alone or in combination into the active site of hen egg white lysozyme by site-directed mutagenesis. It was found that the mutant lysozyme in which all three replacements (N37G, W62Y, and D101G) had been introduced showed the highest lytic activity. We report the findings in this paper, together with the fluorescence spectroscopic properties of the mutated lysozymes, and the effects of these replacements on their ligand-binding activities.

**EXPERIMENTAL PROCEDURES**

*Mats—Restriction endonucleases, DNA-modifying enzymes, and DNA-sequencing kits were purchased from Takara Shuzo (Kyoto) and Toyobo (Osaka). Micrococcus lysoedenticus cells were from Sigma. Hen egg white lysozyme, glycol chitin, and N-acetylchitoooligosaccharides were from Seikagaku Kogyo (Tokyo). S-Sepharose (fast flow) was obtained from Pharmacia. CM-Tyopearl 650M and TSK gel ODS-120A columns were products of Tosoh (Tokyo). Other chemicals were of reagent grade.

Site-directed Mutagenesis—A 20-base and two 19-base mutation primers synthesized by phosphoramidite chemistry were used to replace the Asn77 codon, AAC, with the Gly codon, GGT; the Trp62 codon, TGG, with the Tyr codon, TAT; and the Asp101 codon, CAT, with the Gly codon, GGT. Site-directed mutagenesis was carried out by the methods described by Morinaga et al. (1984) using a double-stranded plasmid, pKK-1, which contains a hen egg white lysozyme

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Expression of the Mutant Hen Egg White Lysozymes—For construction of the expression plasmids, the mutated hen lysozyme cDNAs were inserted into the SaI site of pYG-100, as previously described (Kumagai and Miura, 1989). The expression plasmids were introduced into Saccharomyces cerevisiae AH22 strain (MAT a, leu2, his4, cir) and the Leu⁺ transformatants were grown in yeast minimal medium supplemented with histidine (20 μg/ml). After cultivation of the transformants at 30 °C for 120 h, the mutant lysozymes secreted into the growth medium were purified by cation-exchange chromatography on S-Sepharose (fast flow) followed by a CM-Toyopearl 650M column. The size and homogeneity of the purified enzymes were confirmed by DNA sequencing analysis.

Confirmation of Replacement of Trp with Tyr in the Mutant Hen Lysozyme by Peptide Mapping Analysis—The wild-type hen lysozyme and the W62Y mutant lysozyme were denatured with 6 M guanidine hydrochloride and reduced with β-mercaptoethanol. After S-carboxymethylation, the derivatives were digested with 1:1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (1% lysozyme by weight) at 37 °C for 10 h. The tryptic peptides were separated by reverse-phase high performance liquid chromatography (TSK gel ODS-120A 4.6 × 250 mm, Tosoh). The elution was performed by applying a linear gradient of 0-100% acetonitrile in 0.1% concentrated hydrochloric acid for 100 min at a flow rate of 0.8 ml/min. Peptide peaks were detected at both 210 and 280 nm. The peptides containing Trp and Tyr were collected and subjected to amino acid sequence analysis on an Applied Biosystems 473A Protein Sequencer.

The high performance liquid chromatography analysis patterns for the wild-type and the W62Y mutant enzymes appeared to be exactly the same, except that one peak of the mutant detected at 280 nm was eluted at an earlier retention time than the corresponding peak of the wild-type. Sequence analysis of this peptide peak clearly showed the replacement of Trp with Tyr at position 62 of the W62Y mutant enzyme (data not shown).

Enzymatic Assays—Bacteriolytic activity of lysozyme was assayed by the method of Parry et al. (1965) with slight modification. Lysozyme was added to 1 ml of a suspension of M. lysodeikticus cells in 50 mM sodium phosphate buffer (pH 6.2) or in 0.1 M NaCl and 50 mM Tris-HCl (pH 7.5) at 25 °C. Absorbance at 540 nm was monitored on a Hitachi U-3210 spectrophotometer against a water blank. The decrease in absorbance at 540 nm after 1 min was measured and compared with the wild-type and mutant lysozymes. Hydrolytic activity toward glycol chitin was measured following the reducing group procedure (Imoto and Yagishita, 1971). Glycol chitin (0.05% (w/v)) was incubated with or without lysozyme at 25 °C for 1 h in 20 mM sodium acetate buffer (pH 5.0), whose ionic strength was adjusted to 0.1 with NaCl, or in 0.1 M NaCl and 50 mM Tris-HCl (pH 7.5).

Fluorescence Spectra—A Hitachi 850 fluorescence spectrophotometer was used for the measurement of fluorescence spectra. The excitation wavelength was 280 nm, and the protein concentration was 0.012 mg/ml. Relative quantum yields were determined in 0.1 M NaCl and 50 mM Tris-HCl (pH 7.5) at 25 °C by comparing the protein emission spectrum with that of tryptophan solution in the same buffer (Mulvey et al., 1973). Because the quantum efficiencies of tryptophan in water and the above assay buffer were within 2%, we assumed the quantum efficiency of tryptophan to be 0.2 (Teale and Weber, 1967).

Binding Constants of N-Acetylchitooligosaccharides—The fluorescence spectra of a series of enzyme-inhibitor complex solutions were measured with (GlcNAC)₃, (GlcNAC)₄, and GlcNAc in the assay buffer at 25 °C. Five concentrations of GlcNAc were used from 0 to 0.13 × 10⁻⁶ M. The six of (GlcNAC)₃ were from 0 to 7 × 10⁻⁶ M. The seven of (GlcNAC)₄ were from 0 to 1 × 10⁻⁵ M. The data are analyzed by the derived Scatchard plot of Mulvey et al. (1973), \((F - F_e)/[I] = K_e\)

RESULTS

Fluorescence Spectra of the W62Y Mutant Lysozyme—The fluorescence emission spectra of the W62Y mutant lysozyme and its complex with the oligomer of N-acetylglucosamine are shown in Fig. 2. The emission spectrum of the wild-type lysozyme is included for comparison. The emission maximum for the mutant lysozyme was found to be 336 nm, which is at a slightly shorter wavelength than that of the wild-type enzyme (341 nm). When (GlcNAC)₃ is bound to the wild-type lysozymes at pH 7.5, the fluorescence emission is enhanced and its maximum is blue-shifted from 341 to 335 nm. On the other hand, the quantum yield of the W62Y mutant was found to be 1.03 ± 0.03 at pH 7.5.
protein concentration was 0.012 mg/ml in 0.1 M Tris-HCl (pH 7.5) at 25 °C. The excitation wavelength was 280 nm. The emission maximum was scarcely blue-shifted. These spectral features seem to be closely related to those of human lysozyme, which contains a Tyr residue at position 197. It was shown by X-ray crystallography that GlcNAc binds to the B subsite, (GlcNAc)\(_b\) to the A and C subsites in the substrate-binding sites of the wild-type lysozyme. If it is assumed that the binding mode of these GlcNAc residues to the mutant lysozymes is the same as that of the wild-type enzyme, the binding of GlcNAc monomer to the C subsite seems to be unchanged upon conversion of Trp\(_{62}\) to Tyr. However, since the free energy change on the binding of (GlcNAc)\(_b\) to the mutant lysozyme was found to be very close to that of (GlcNAc)\(_b\) (Table I), the replacement of Trp\(_{62}\) may mainly affect the binding of GlcNAc to the B subsite.

**Enzymatic Activities of the Single and Multiple Mutated Lysozymes**—We have already reported the enhanced bacteriolytic activity of the W62Y mutant lysozyme (Kumagai et al., 1987). Fig. 3 summarizes the bacteriolytic activities of wild-type, three single mutants (N37G, W62Y, and D101G), three double mutants (N37G W62Y, W62Y D101G, and N37G D101G), one triple mutant (N37G W62Y D101G), and human lysozyme. At pH 6.2, replacement of Trp\(_{62}\) with Tyr (Kumagai et al., 1987) and Asp\(_{101}\) with Gly enhanced the bacteriolytic activity. The double mutants exhibited much more enhanced activities. Although a single replacement of Aaa\(_{37}\) with Gly alone did not increase the activity, the combination of the N37G mutation with either W62Y or D101G gave remarkably enhanced activity. The mutant N37G W62Y contains the same set of amino acid residues involved in substrate binding by human lysozyme. The triple mutant (N37G W62Y D101G) showed about 3-fold higher lytic activity. However, this was still lower than that of human lysozyme.

The lytic activities of these mutants at pH 7.5 were lower than that of the wild-type enzyme at pH 7.5, except for one double mutant (N37G D101G). It is noteworthy that the activities of mutants containing W62Y were greatly reduced at pH 7.5.

Fig. 4 compares the hydrolytic activities toward glycol chitin of wild-type, the mutant lysozymes described above, and human lysozyme. At pH 5.0, which is the optimum pH for the wild-type enzyme, introduction of single mutations reduced the activities of the mutant enzymes. On the other hand, double mutations restored the hydrolytic activities on glycol chitin. In particular, the replacements of Aaa\(_{37}\) with Gly and Asp\(_{101}\) with Gly enhanced the bacteriolytic activities of the W62Y mutant lysozyme. In addition, the triple mutant (N37G W62G D101G) exhibited about 90% of the wild-type lysozyme activity. As observed with bacteriolytic activities, most mutants of the enzymes showed reduced activities toward glycol chitin at pH 7.5.

**Binding of (GlcNAc)\(_b\) to the Mutant Lysozymes**—The fluorescence emission spectra of the mutant enzymes containing conversions of Aaa\(_{37}\) to Gly and Asp\(_{101}\) to Gly were essentially unchanged. The bacteriolytic activities were measured in 50 mM sodium phosphate buffer (pH 6.2) or in 0.1 M NaCl and 50 mM Tris-HCl (pH 7.5), as described under “Experimental Procedures.” Relative activities are expressed by taking the activity of the wild-type hen lysozyme at pH 6.2 to be 100.
FIG. 4. Hydrolytic activities of the mutant hen lysozymes toward glycol chitin. The activities were measured in 20 mM sodium acetate buffer (pH 5.0), whose ionic strength was adjusted to 0.1 with NaCl, or in 0.1 M NaCl and Tris-HCl (pH 7.5), as described under "Experimental Procedures." Relative activities are expressed by taking the activity of the wild-type hen lysozyme at pH 5.0 to be 100.

### Table II
Comparison of binding constants of (GlcNAc)_2 to the wild-type hen, the mutant hen, and human lysozymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>Enzyme</th>
<th>$K_a$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>$7.5 \times 10^4$</td>
<td>N37G</td>
<td>$7.0 \times 10^4$</td>
</tr>
<tr>
<td>W62Y</td>
<td>$1.2 \times 10^4$</td>
<td>N37G W62Y</td>
<td>$1.2 \times 10^4$</td>
</tr>
<tr>
<td>D101G</td>
<td>$7.5 \times 10^4$</td>
<td>N37G D101G</td>
<td>$5.8 \times 10^3$</td>
</tr>
<tr>
<td>W62Y D101G</td>
<td>$2.6 \times 10^4$</td>
<td>N37G W62Y D101G</td>
<td>$3.1 \times 10^3$</td>
</tr>
<tr>
<td>Human</td>
<td>$2.6 \times 10^4$</td>
<td>Human</td>
<td>$2.6 \times 10^4$</td>
</tr>
</tbody>
</table>

The replacement of Trp$^{62}$ with Tyr led to a reduction of the quantum yield and blue shift of the emission maximum. Since the contribution of the Tyr residue to fluorescence is probably very small, Trp$^{106}$ may dominate the fluorescence spectrum of the Tyr$^{62}$ mutant. This is further supported by the fact that the fluorescence of the W62Y mutant was found to be more similar to that of human lysozyme, in which Trp$^{62}$ is replaced with Tyr, and Trp$^{63}$ and Trp$^{106}$ are conserved. The quantum yield of the W62Y mutant was between those of the wild-type hen and human lysozymes. The mutant showed fluorescence emission maximum at the same wavelength as human lysozyme, which is shorter than that of the wild-type hen enzyme.

Studies of the binding of ligands to the W62Y mutant showed that the mutant binds (GlcNAc)$_2$ and (GlcNAc)$_3$ less strongly than does the wild-type lysozyme. On the other hand, the GlcNAc binding constant to the mutant was almost identical with that of the wild-type enzyme. Analysis by x-ray crystallography suggests that Trp$^{62}$ interacts with the sugar residue at subsite B by van der Waals contact and with the sugar C-6 hydroxyl group at subsite C through hydrogen bonding. However, studies on the lysozyme-GlcNAc complex by NMR (Cassels et al., 1978) and the binding capability of 6-deoxy-GlcNAc to the enzyme have shown that such a hydrogen bond would be too weak for complex formation. The observation that there is little difference between interactions of GlcNAc with the wild-type and the mutant lysozyme suggests a small contribution of hydrogen bonding between Trp$^{62}$ and GlcNAc residue for the inhibitor binding. Alternatively, the reduced binding constants for (GlcNAc)$_2$ and (GlcNAc)$_3$ to the W62Y mutant may be due to the difference in nonpolar interactions with Tyr and Trp residues. Of course, the possibility that the binding mode of these inhibitors and the topographies of their binding sites could be altered by the replacement of Trp with Tyr cannot be ruled out.

On the basis of molecular evolution of three c-type lysozymes, multiple mutations were introduced into the substrate-binding region of hen egg white lysozyme, and unique features of the mutant enzymes were found. The replacement of Asn$^{37}$ with Gly did not affect the binding of (GlcNAc)$_2$ to the enzymes, which is consistent with the observation that (GlcNAc)$_3$ binds to the A, B, and C subsites. On the other hand, mutations of Asp$^{62}$ and Trp$^{62}$, which comprise the A, B, and C subsites, led to large reductions in the binding constants. Analysis of changes in free energy on binding to single and double mutant enzymes revealed that the effects of conversion of Asp$^{62}$ and Trp$^{62}$ on ligand binding are interdependent. The estimated free energy change on replacement of Asp$^{62}$ with Gly was almost consistent with that reported by Kirsch et al. (1989).

**DISCUSSION**

Hen egg white lysozyme contains six tryptophanyl residues. Of these, three tryptophans (Trp$^{62}$, Trp$^{63}$, and Trp$^{106}$) are located in the substrate-binding cleft and are believed to interact with sugar rings placed in subsites B, C, and D. Conversion of Trp$^{62}$ to Tyr by site-directed mutagenesis markedly altered the fluorescence properties of the protein, which may be explained in terms of the spectroscopic features of Trp$^{62}$ and Trp$^{106}$.

Trp$^{62}$ is the tryptophan most exposed to solvent and is very susceptible to chemical reagents (Hayashi et al., 1965). A chemical modification study suggested that Trp$^{62}$ provides 35–38% of the fluorescence of the lysozyme (Imoto et al., 1971). Trp$^{62}$ is expected to emit at a longer wavelength. The other major fluorophore of the enzyme is Trp$^{106}$, which is partially buried and may emit at a shorter wavelength. The slight blue shift of the emission maximum upon (GlcNAc)$_2$ binding is due to the removal of Trp$^{62}$ from its exposed, solvent-accessible region to a less polar environment.

The replacement of Trp$^{62}$ with Tyr led to a reduction of the quantum yield and blue shift of the emission maximum. Since the contribution of the Tyr residue to fluorescence is probably very small, Trp$^{106}$ may dominate the fluorescence spectrum of the Tyr$^{62}$ mutant. This is further supported by the fact that the fluorescence of the W62Y mutant was found to be more similar to that of human lysozyme, in which Trp$^{62}$ is replaced with Tyr, and Trp$^{63}$ and Trp$^{106}$ are conserved. The quantum yield of the W62Y mutant was between those of the wild-type hen and human lysozymes. The mutant showed fluorescence emission maximum at the same wavelength as human lysozyme, which is shorter than that of the wild-type hen enzyme.

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Redesign of the Active Site of Hen Lysozyme

deikticus cells is highly negatively charged, and lysozyme is a very basic protein. On binding of the lysozyme to the cells, electrostatic interaction between them may be dominant, and the efficiency of hydrolysis of the -GlcNAc\(^\beta\)1–4MurNAc– linkage may directly reflect the lytic process. Alternatively, about 40% of -GlcNAc\(^\beta\)1–4MurNAc– linkages are anchored to the peptidoglycan layer through short peptides linked to lactic acid groups of MurNAc residues in the M. lysodeikticus cell wall. Therefore, the conformation of the -GlcNAc\(^\beta\)1–4MurNAc– polymer may be more rigid than the free chitin or glycol chitin polymers. Slightly altered topographies of substrate-binding sites of the mutant lysozymes may more readily accommodate the rigid structure of -GlcNAc\(^\beta\)1–4-MurNAc– regions in the M. lysodeikticus cell wall.

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