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

(Received for publication, May 3, 1991)

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On the basis of the molecular evolution of hen egg white, human, and turkey lysozymes, three replacements (Trp with Tyr, Asn with Gly, and Asp with Gly) were introduced into the active-site cleft of hen egg white lysozyme by site-directed mutagenesis. The replacement of Trp 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 Asn with Gly had little effect on the enzymatic activity and binding constant for chitotriose. However, the combination of Asn with Gly (N37G) replacement with Asp replacement with Gly (D101G) and Trp replacement with Tyr (W62Y) conversions enhanced bacteriolytic activity much more than each single mutation and restored hydrolytic activity toward glycol chitin. Consequently, the mutant lysozyme containing three 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 C-type lysozymes, which contains Tyr at position 62. The replacement of Asn with Gly had little effect on the enzymatic activity and binding constant for chitotriose. However, the combination of Asn with Gly (N37G) replacement with Asp replacement with Gly (D101G) and Trp replacement with Tyr (W62Y) conversions enhanced bacteriolytic activity much more than each single mutation and restored hydrolytic activity toward glycol chitin. Consequently, the mutant lysozyme containing three 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.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, DNA-modifying enzymes, and DNA-sequencing kits were purchased from Takara Shuzo (Kyoto) and Toyobo (Osaka). Micrococcus lysodeikticus cells were from Sigma. Hen egg white lysozyme, glycol chitin, and N-acetylchitosan were from Seikagaku Kogyo (Tokyo). S-Sepharose (fast flow) was obtained from Pharmacia, CM-Txopearl 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 phosphoramidate chemistry were used to replace the Asn codon, AAC, with the Gly codon, GGT; the Trp codon, TGG, with the Tyr codon, TAT; and the Asp codon, GAT, 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

* This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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5 The abbreviations used are: GlcNAc, N-acetyl-D-glucosamine; MurNAc, N-acetylmuramic acid.
Expression of the Mutant Hen Egg White Lysozymes—For construction of the expression plasmids, the mutant hen lysozyme cDNAs were inserted into the SalI site of pYG-100, as previously described (Kumagai and Miura, 1989). The cDNAs were inserted into Saccharomyces cerevisiae AH22 strain (MAT a, leu2, his3, cir) and the Leu+ transformants 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. The purified enzymes were adjusted by a factor of 0.89 (Kumagai et al., 1987). 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 1-40% guanidine hydrochloride 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.

Confirmation of Replacement of Trp62 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 1-40% 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.

Results

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, 1957).

Binding Constants of N-Acetylchitooligosaccharides—The fluorescence spectra of a series of enzyme-inhibitor complex solutions were measured with (GlcNAc)2, (GlcNAc)3, 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)2 were from 0 to 7 × 10−6 M. The seven of (GlcNAc)3 were from 0 to 1 × 10−6 M. The data are analyzed by the derived Scatchard plot of Mulvey et al. (1973), (F − F0)/[I] = Kd (Fmax − F0) − Kd (F − F0), where F0 and Fmax are the fluorescence intensities of the free enzyme and the saturated complex, respectively, and F is the fluorescence intensity in the presence of I mol of the added inhibitors. The binding constants of Kd were evaluated by the slopes in the plots of (F − F0)/[I] versus F − F0.

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)2 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 0.89 (Kumagai et al., 1987). The expression plasmids were introduced into Saccharomyces cerevisiae AH22 strain (MAT a, leu2, his3, cir) and the Leu+ transformants 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. The purified enzymes were adjusted by a factor of 0.89 (Kumagai et al., 1987). 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 1-40% 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.

Confirmation of Replacement of Trp62 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 1-40% 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. The seven peaks between 10 and 40% acetonitrile were collected and subjected to amino acid sequence analysis on an Applied Biosystems 473A Protein Sequencer.
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<sup>62</sup> to Tyr. However, since the free energy change on the binding of (GlcNAc)<sub>3</sub> to the mutant lysozyme was found to be very close to that of (GlcNAc)<sub>3</sub> (Table I), the replacement of Trp<sup>62</sup> 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<sup>62</sup> with Tyr (Kumagai et al., 1987) and Asp<sup>101</sup> with Gly enhanced the bacteriolytic activity. The double mutants exhibited much more enhanced activities. Although a single replacement of Aan<sup>37</sup> 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 replacement of Aan<sup>37</sup> with Gly of the single mutant enzymes seemed to be very effective in recovering their activities. As a consequence, two double mutants involving conversion of Aan<sup>37</sup> to Gly showed almost the same activity as the wild-type enzyme. 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)<sub>3</sub> to the Mutant Lysozymes**—The fluorescence emission spectra of the mutant enzymes containing conversions of Aan<sup>37</sup> to Gly and Asp<sup>101</sup> to Gly were essentially unchanged at pH 6.2.

![Fluorescence spectra of the wild-type hen lysozyme](image)

**Table 1**

| Enzyme          | Inhibitor | K<sub>i</sub> (M) | ΔG<sup>B</sup> (kcal/mol) | ΔG<sup>A</sup> (kcal/mol) |
|-----------------|-----------|----------------|--------------------------|--------------------------|
| Wild-type       | GlcNAc    | 21 (22°)      | 1.8                      | 4.2 (4.2°)               |
|                 | (GlcNAc)<sub>2</sub> | 3.0 x 10<sup>-3</sup> (2.0 x 10<sup>-3</sup>) | 4.7                      | 7.1 (6.9°)               |
|                 | (GlcNAc)<sub>3</sub> | 7.5 x 10<sup>-3</sup> (8.2 x 10<sup>-3</sup>) | 6.6                      | 9.0 (9.1°)               |
| W62Y mutant     | GlcNAc    | 37            | 2.1                      | 4.5                      |
|                 | (GlcNAc)<sub>2</sub> | 6.8 x 10<sup>-3</sup> | 3.9                      | 6.3                      |
|                 | (GlcNAc)<sub>3</sub> | 1.2 x 10<sup>-3</sup> | 5.6                      | 8.0                      |

*See Kuramitsu et al. (1975).*
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)_{n} to the wild-type hen, the mutant hen, and human lysozymes

| Enzyme          | $K_{a}$ M$^{-1}$ | $K_{m}$ M$^{-1}$ |
|-----------------|-----------------|-----------------|
| Wild-type       | 7.5 x 10^{6}    | 7.0 x 10^{4}    |
| N37G            | 7.5 x 10^{6}    | 7.0 x 10^{4}    |
| W62Y            | 1.2 x 10^{6}    | 1.2 x 10^{4}    |
| D101G           | 7.5 x 10^{5}    | 5.8 x 10^{3}    |
| W62Y D101G      | 2.6 x 10^{5}    | 3.1 x 10^{3}    |
| Human           | 2.6 x 10^{4}    | 2.6 x 10^{4}    |

the same as that of the wild-type enzyme. On the other hand, double or triple mutants containing Tyr at position 62 showed the same emission spectra as the W62Y mutant. Therefore, conversion of Asp$^{37}$ and Asn$^{37}$ seem not to influence fluorescence.

Table II summarizes the binding constants of (GlcNAc)$_{n}$ to the mutant lysozymes measured by changes in the fluorescence spectra. As clearly shown in the table, the conversion of Asn$^{37}$, which is probably located near the F subsite, did not affect the binding of (GlcNAc)$_{n}$ to sites A, B, and C. On the other hand, conversion of Asp$^{37}$ and Trp$^{64}$, 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$^{37}$ and Trp$^{64}$ on ligand binding are interdependent. The estimated free energy change on replacement of Asp$^{37}$ 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$^{22}$, Trp$^{64}$, and Trp$^{108}$) 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$^{64}$ 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$^{64}$ and Trp$^{108}$.

Trp$^{64}$ 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$^{64}$ provides 35-38% of the fluorescence of the lysozyme (Imoto et al., 1971). Trp$^{64}$ is expected to emit at a longer wavelength. The other major fluorophore of the enzyme is Trp$^{108}$, which is partially buried and may emit at a shorter wavelength. The slight blue shift of the emission maximum upon (GlcNAc)$_{n}$ binding is due to the removal of Trp$^{64}$ from its exposed, solvent-accessible region to a less polar environment.

The replacement of Trp$^{64}$ 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$^{108}$ may dominate the fluorescence spectrum of the Tyr$^{64}$ 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$^{64}$ is replaced with Tyr, and Trp$^{64}$ and Trp$^{108}$ 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$^{64}$ 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$^{64}$ 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)$_{n}$ to the enzymes, which is consistent with the observation that (GlcNAc)$_{n}$ binds to the A, B, and C subsites. On the other hand, mutations of Asp$^{37}$ and Trp$^{64}$ led to a significant reduction in the binding constant. Judging from the analysis of Asp$^{37}$ and Trp$^{64}$ double mutations, the effects of these mutations on (GlcNAc)$_{n}$ binding were not independent, probably due to subtle structural changes in the A, B, and C subsites. With the exception of Asn$^{37}$, mutations enhanced bacteriolytic activity but reduced hydrolytic activity on glycol chitin and the binding constant of (GlcNAc)$_{n}$. However, it should be noted that replacement of Asn$^{37}$ with Gly in combination with the Asp$^{37}$ and Trp$^{64}$ mutations gave an even greater enhancement of bacteriolytic activity and restored the hydrolytic activity on glycol chitin. This observation will provide some insight into the contribution of the F subsite to substrate binding, which has previously been possible only by model building (Blake et al., 1967) or energy calculation (Pincus and Scheraga, 1979).

Most of the mutant lysozymes showed enhanced bacteriolytic activity and reduced hydrolytic activity on glycol chitin. In preliminary kinetic experiments using small synthetic substrates, we have found a reduced $K_{m}$ but increased $k_{cat}$ of the Tyr$^{64}$ mutant (data not shown). The cell surface of M. lyso-
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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 -GlcNAcβ1-4MurNAc-linkage may directly reflect the lytic process. Alternatively, about 40% of -GlcNAcβ1-4MurNAc-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 -GlcNAcβ1-4MurNAc-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 -GlcNAcβ1-4-MurNAc-regions in the M. lysodeikticus cell wall.

Acknowledgment—We thank Prof. K. Hamaguchi of Osaka University for kindly providing the human lysozyme.

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