Multiple Role of Hydrophobicity of Tryptophan-108 in Chicken Lysozyme: Structural Stability, Saccharide Binding Ability, and Abnormal pK_a of Glutamic Acid-35†

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ABSTRACT: Trp108 of chicken lysozyme is in van der Waals contact with Glu35, one of two catalytic carboxyl groups. The role of Trp108 in lysozyme function and stability was investigated by using mutant lysozymes secreted from yeast. By the replacement of Trp108 with less hydrophobic residues, Tyr (W108Y lysozyme) and Gln (W108Q lysozyme), the activity, saccharide binding ability, stability, and pK_a of Glu35 were all decreased with a decrease in the hydrophobicity of residue 108. Namely, at pH 5.5 and 40 °C, the activities of W108Y and W108Q lysozymes against glycol chitin were 17.3 and 1.6% of that of wild-type lysozyme, and their dissociation constants for the binding of a trimer of *N*-acetyl-D-glucosamine were 7.4 and 309 times larger than that of wild-type lysozyme, respectively. For the reversible unfolding at pH 3.5 and 30 °C, W108Y and W108Q lysozymes were less stable than wild-type lysozyme by 1.4 and 3.6 kcal/mol, respectively. As for the pK_a of Glu35, the values for W108Y and W108Q lysozymes were found to be lower than that for wild-type lysozyme by 0.2 and by 0.6 pK_a unit, respectively. The pK_a of Glu35 in lysozyme was also decreased from 6.1 to 5.4 by the presence of 1–3 M guanidine hydrochloride, or to 5.5 by the substitution of Asn for Asp52, another catalytic carboxyl group. Thus, both the hydrophobicity of Trp108 and the electrostatic interaction with Asp52 are equally responsible for the abnormally high pK_a (6.1) of Glu35, compared with that (4.4) of a normal glutamic acid residue. A high pK_a of Glu35 is considered to be critical for this residue to serve as general-acid catalyst in the glycosidase activity of lysozyme at higher pHs. Therefore, it is concluded that the hydrophobicity of Trp108 plays multiple important roles in lysozyme's function and stability, such as the maintenance of the tertiary structure, effective substrate binding, and keeping the pK_a of Glu35 abnormally high.

In order to prepare an artificial protein having a structure and function designed at will by protein engineering, there are many problems to be presolved. The complete description of the roles of individual amino acid residues playing in the structures and functions of natural proteins may be one of the solutions for these problems. Since chicken lysozyme (hen egg-white lysozyme) which belongs to c-type lyszoymes is one of the best characterized proteins (Jolles & Jolles, 1984; Imoto et al., 1972), it is a good material for this purpose.

Chicken lysozyme is a carbohydrate hydrolase with an acidic pH optimum for certain substrates and catalyzes the hydrolysis of β-1,4 glycosidic bonds of polysaccharides of such as a homopolymer of *N*-acetyl-D-glucosamine (chitin) and an alternating copolymer of *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid which is the major constituent of bacterial cell walls (Imoto et al., 1972). Two carboxyl group (Glu35 and Asp52) are identified as catalytic groups, and a mechanism proposed by Blake et al. (1967b) that the carboxyl group of Glu35 in its unionized form serves as general-acid catalyst and that of Asp52 in its ionized form stabilizes, by electrostatic interaction, the oxocarbonium ion intermediate is well accepted. The pK_a is known to be abnormally high (pK_a = 6.1) (Imoto et al., 1972) compared with that of a normal glutamic acid residue (pK_a = 4.4) (Roxby & Tanford, 1971). The abnormal pK_a of Glu35 is originally attributed to the fact that this residue is located in a nonpolar region (in the vicinity of C_β of Ala110, C_γ of Gln57, and $C_{\delta 1}$ of Trp108) and in van der Waals contact with Trp108 (Blake et al., 1967a); that is, the ionized form may be destabilized by the hydrophobic environment as discussed by Rupley et al. (1967). On the other hand, Kuramitsu et al. (1974) have demonstrated that the abnormal pK_a of Glu35 is completely explained by the electrostatic interaction of two proximal carboxylate anions between Glu35 and Asp52 (7 Å) (Imoto et al., 1972). Parsons and Raftery (1972) have reported that the pK_a of Glu35 in Asp52 ethyl-esterified lysozyme, in which the electrostatic interaction between Glu35 and Asp52 is absent, is 5.2, that is, still higher than the value of a normal glutamic acid residue, suggesting that the electrostatic interaction is only partly responsible for the abnormal pK_a of Glu35. Since the abnormally high pK_a of Glu35 is considered to be critical for this carboxyl group to act as general-acid catalyst at higher pHs and since Trp108 is conserved in all of the chicken-type lysozymes in which determination of the amino acid sequences has been completed so far (Nitta & Sugai, 1989), we intended first to clarify the role of the hydrophobicity of Trp108 as well as that of the electrostatic interaction with Asp52 in the abnormal pK_a of Glu35 by utilizing mutant lysozymes secreted by yeast. However, the hydrophobicity of Trp108 was found to play a much more important role in the lysozyme structure and function than expected. That is, the role of the hydrophobicity of Trp108 is not only to keep the pK_a of Glu35 abnormally high but also to increase the structural stability and to provide the effective substrate binding ability to

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maintain the high activity of lysozyme.

Thus, in this paper, we describe the multiple roles of the hydrophobicity of Trp108 in lysozyme structure and function. The mutant lysozymes used were those in which Trp108 is replaced by Tyr (W108Y lysozyme) and by Gln (W108Q), and Asp52 is replaced by Asn (D52N) and by Glu (D52E), respectively.

Materials and Methods

Materials. Restriction enzymes, T4 polynucleotide kinase, and DNA polymerase I (Klenow fragment) were purchased from either Takara Shuzo Co. Ltd. (Kyoto, Japan) or New England Biolabs. Inc. (Beverly, MA). DNA sequencing kits (Sequenase) and radioisotopes were purchased from Amersham Japan (Tokyo). CM-Toyopearl 650M, a cation-exchange resin for purification of secreted lysozymes, was obtained from Tosoh (Tokyo). A column of Wakopak 5C18 (mesh 200) was from Wako Pure Chemicals Institute (Osaka). *Micrococcus luteus*, a substrate of lysozyme, was from Sigma. Glycol chitin, a synthetic substrate of lysozyme (Yamada & Imoto, 1981), and chitin-coated Celite, an affinity adsorbent for lysozyme (Yamada et al., 1985a), were prepared as described previously. A β(1–4)-linked trimer of *N*-acetyl-D-glucosamine [$(NAG)_3$][1] was prepared according to the method of Rupley (1964). All other chemicals were of analytical grade for biochemical use.

Strains, Plasmids, and Media. Escherichia coli strain RR1 [F^-, *hsdS20* (r_B^-, m_B^-), *supE44*, *ara-14*, *galK2*, *rpsL20* (Sm^r), *xyl-5*, *mtl-1*, lambda$^-$] (Boliver et al., 1977) was used for cloning. Yeast *Saccharomyces cerevisiae* strain AH22 (*a*, *leu2*, *his4*, *can1*, *cir*$^+$) (Hinnen et al., 1978), which was kindly supplied by Dr. F. Hamada (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), was used for expression and secretion of chicken lysozyme. *E. coli* cells were grown in LB broth as described previously (Miki et al., 1987). Yeast cells were grown in a modified Burkholder minimal medium (Toh-e et al., 1973) supplemented with 8% sucrose, 5 g/L asparagine, 100 mg/L tryptophan, 100 mg/L histidine, and 0.6 mM KH_2PO_4 (pH 5.3) at 30 °C.

Plasmid pKLZ58, which contains lysozyme cDNA in the pUC9 derivative, was the previous construct (Imoto et al., 1987). Plasmid pAM82 (PHO5 promoter, *Leu2*, 2μori, *ars1*, Ap^r; yeast–*E. coli* shuttle vector) (Miyanohara et al., 1983) was also kindly supplied by Dr. Hamada, and used as a vector for expression of chicken lysozyme in yeast.

Construction of pAM82 Derivatives for Expression and Secretion of Wild-Type and Mutant Chicken Lysozymes from Yeast. Oligonucleotides used as mutagenic primers for site-directed mutagenesis and as desired DNA fragments for insertion or replacement were synthesized and purified as described previously (Miki et al., 1987). Site-directed mutagenesis was carried out by the method described previously (Miki et al., 1987). The structures of the mutagenic primers used for replacements of Trp108 with Tyr (W108Y) and Gln (W108Q), and those of Asp52 with Asn (D52N) and Glu (D52E), were 5′-GAACGCCTACGTCGCC-3′ (W108Y), 5′-GAACGCGCAAGTCGCCTG-3′ (W108Q), 5′-GAGTACCAACTACGG-3′ (D52N), and 5′-GAGTACCGAGTACGGAATC-3′ (D52E). Isolation and manipulation of plasmid DNA using *E. coli*, and determination of nucleotide sequences, were performed as described previously (Miki et al., 1987).

[1] Abbreviations: $(NAG)_3$, a β(1–4)-linked trimer of *N*-acetyl-D-glucosamine; GdnHCl, guanidine hydrochloride; GC, glycol chitin; HPLC, high-performance liquid chromatography.

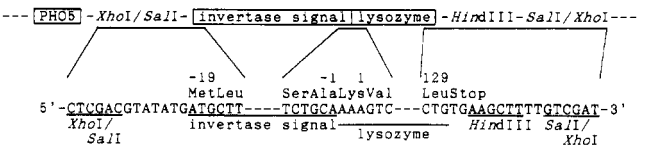

FIGURE 1: Schematic representation of the DNA region coding for the fused protein between the yeast invertase signal peptide and the mature form of chicken lysozyme inserted downstream from the yeast PHO5 promoter in the *Xho*I site of pAM82. Size is arbitrary.

In order to express and secrete wild-type and mutant chicken lysozymes from yeast as mature forms, we selected plasmid pAM82 as an expression vector and the yeast invertase signal peptide as a secretion-promoting signal (Taussig et al., 1983; Chang et al., 1986). Thus, on the basis of plasmid pKLZ58, and using the synthetic yeast invertase signal peptide DNA fragment, the pUC9 derivative, in which the multicloning site between the *Eco*RI and *Hin*dIII sites was replaced with the sequence of *Sal*I-(yeast invertase signal peptide DNA)-(mature form of wild-type or mutant lysozyme DNA)-*Hin*dIII-*Sal*I, was constructed. Details of this plasmid construction will be reported separately. Each pUC9 derivative thus prepared was digested with *Sal*I, and the resulting small DNA fragment coding for the fused protein between the yeast invertase signal peptide and the mature form of wild-type or mutant lysozyme was then inserted in the *Xho*I site of pAM82 in a direction under control of the repressible acid phosphatase promoter (PHO5) of yeast. The properly oriented plasmids were selected by size analysis of the fragments formed from digestion with *Hin*dIII. Figure 1 shows the schematic representation of the DNA region coding for the fused protein between the invertase signal peptide and the mature form of chicken lysozyme inserted in the *Xho*I site of pAM82.

Transformation of yeast *S. cerevisiae* AH22 with pAM82 derivative was carried out by the method of Hinnen et al. (1978). Leu^+ transformants were selected on Burkholder minimal medium containing 2% agar.

Analytical Methods. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer after hydrolysis of samples in 6 N HCl under vacuum at 110 °C for 20 h. The NH_2-terminal sequences of protein and peptide samples were determined with an Applied Biosystems Model 470A/120A gas-phase protein sequencer. Digestions of reduced and S-carboxymethylated lysozymes with TPCK-trypsin (Worthington) and separation of the resulting peptides on reversed-phase HPLC were accomplished as described by Yamada et al. (1985b).

Affinity HPLC of lysozymes for the determination of their substrate binding abilities was performed on a chitin-coated Celite column (4 × 100 mm) which was eluted with a gradient of 20 mL of 0.1 M acetate buffer (pH 5.5) and 20 mL of 1 M acetic acid, both containing 0.25 M NaCl at a flow rate of 0.5 mL/min and 0 °C (Yamada et al., 1985a).

Activities of lysozymes against *M. luteus* were determined turbidimetrically at 450 nm in 0.05 M potassium phosphate buffer at pH 7.0 and 30 °C. Activities against glycol chitin were measured in 0.1 M acetate buffer at pH 5.5 and 40 °C as described elsewhere (Yamada & Imoto, 1981).

The dissociation constants of lysozymes for binding to $(NAG)_3$ in 0.1 M acetate buffer at pH 5.5 and 40 °C were determined by the method of UV difference spectroscopy (Dahlquist et al., 1966) with a Hitachi 150-20 double-beam spectrophotometer.

Circular dichroism (CD) spectra were measured with a Jasco J-720 spectropolarimeter at 20 °C using 1.3×10^{-5} M protein in 0.01 M potassium phosphate buffer (pH 7).

Spectroscopic Titration of Glu35 of Lysozyme. The pH dependence of the tryptophyl absorbance of lysozyme at 30 °C was measured at 301 nm with a Hitachi U-2000 spectrophotometer. Namely, lysozyme (final concentration to be 8.4×10^{-5} M) was dissolved in 20 mL of 0.1 M KCl solution containing 1 mM acetic acid and 1 mM phosphoric acid. The pH of the solution was lowered to about 2 with HCl, and the absorbance at 301 nm was measured. Then the pH was increased little by little by addition of concentrated NaOH using a capillary in order to minimize the change of volume of the solution, and at each pH, the absorbance at 301 nm was recorded. This procedure was repeated until the pH of the solution was raised to about 8.

The pH dependence of the tryptophyl fluorescence of lysozyme in the presence of $(NAG)_3$ was measured with a Hitachi F-2000 fluorescence spectrophotometer thermostated at 30 °C. The fluorescence at 340 nm was measured by using 280-nm light for excitation. A protein (final concentration to be 1.5×10^{-6} M) and $(NAG)_3$ (final concentration to be 1.2×10^{-4} M) were dissolved in 20 mL of the same solution as in the case of absorbance measurements, and the relative fluorescence intensity at each pH was recorded in a similar way.

Unfolding Equilibrium. Unfolding equilibria of lysozymes by guanidine hydrochloride (GdnHCl) were measured at various pHs (pH 3.5–5.5) and 35 ± 0.2 °C by the fluoresence at 360 nm (excited at 280 nm). The protein concentration was 9.3×10^{-7} M. The buffers used were 0.1 M sodium acetate adjusted to the respective pHs (pH 3.5–5.5) with HCl.

Purification and Identification of Lysozymes Secreted from Yeast. Each yeast *S. cerevisiae* AH22 transformant was cultivated at 30 °C for 125 h to express and secrete each lysozyme from yeast. The lysozyme secreted in the culture supernatant was isolated by cation-exchange chromatography on a column (1.3 × 40 cm) of CM-Toyopearl 650M which was eluted with a gradient of 500 mL of 0.05 M phosphate buffer and 500 mL of the same buffer containing 0.5 M NaCl at pH 7 and 4 °C. The lysozyme thus eluted was collected, dialyzed against distilled water, and then lyophilized.

Results

Expression and Secretion of Wild-Type, W108Y, W108Q, D52N, and D52E Lysozymes from Yeast. Expression and secretion of wild-type or mutant lysozyme as a mature form from yeast were carried out by cultivation of yeast *S. cerevisiae* AH22 harboring pAM82 derivative, that carries DNA coding for a fused protein between an yeast invertase signal peptide and a mature form of lysozyme (wild-type or mutant lysozyme) inserted in the *Xho*I site in the direction under control of the repressible acid phosphatase promoter (PHO5) of yeast, at 30 °C for 125 h in modified Burkholder minimal media (Toh-e et al., 1973) in the presence of a limited amount of phosphate (0.6 mM KH_2PO_4). The lysozymes secreted in the culture media were purified by cation-exchange chromatography, respectively. The amounts of lysozymes secreted in the media were found to be 5.0, 0.9, 0.3, 1.3, and 1.5 mg/L for wild-type, W108Y, W108Q, D52N, and D52E lysozymes, respectively. We also estimated the amounts of some lysozymes retained within cells by Western blotting of the harvested cells to be 1.0, 1.2, and 3.4 mg/mL for wild-type, W108Y, and W108Q lysozymes, respectively. Interestingly, the W108Q lysozyme was mostly retained in cells.

The NH_2-terminal amino acid sequences of the purified enzymes were all single and identical to that of native lysozyme obtained from hen egg white, indicating that the yeast invertase signal peptide in a fused protein was correctly processed off

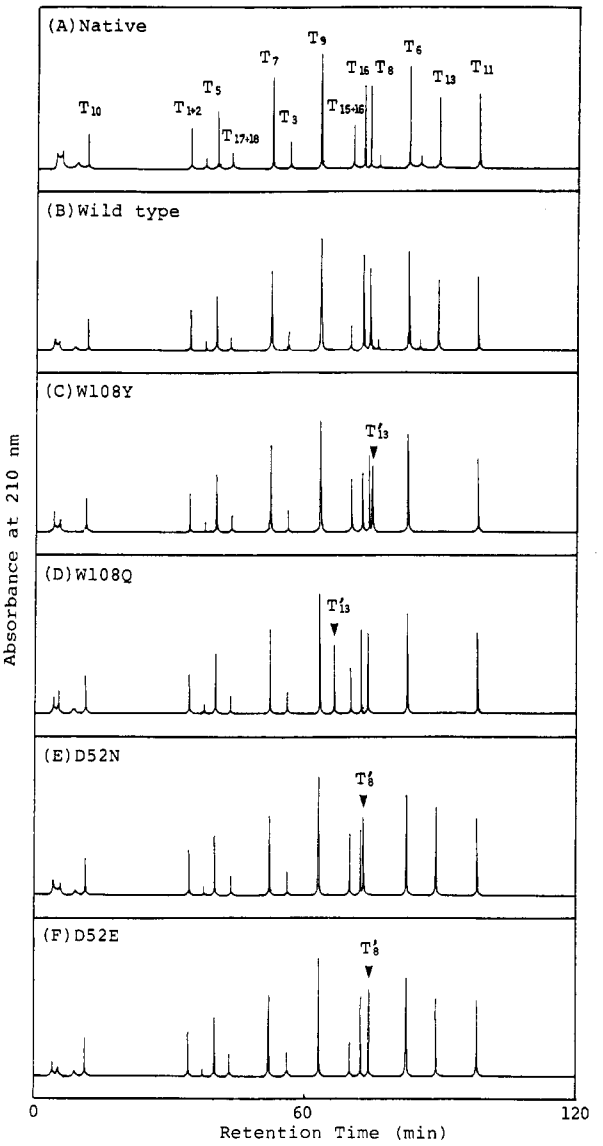

FIGURE 2: Reversed-phase HPLC of tryptic peptides obtained from reduced and S-carboxymethylated lysozymes on a column (4 × 250 mm) of Wakopak 5C18. The column was eluted with a gradient formed from 50 mL of 1% acetonitrile and 50 mL of 50% acetonitrile, both containing 0.1% concentrated HCl at a flow rate of 0.6 mL/min. (A) From native lysozyme; (B) from wild-type lysozyme; (C) from W108Y lysozyme; (D) from W108Q lysozyme; (E) from D52N lysozyme; (F) from D52E lysozyme. T refers to the tryptic peptides, and peptide numbering is from the N-terminal peptide. The arrows represent peptides containing a mutation.

in every case. Amino acid compositions of these lysozymes were all consistent with those expected from the mutations introduced (data not shown). In order to confirm further the mutations in the respective mutant lysozymes, they were reduced, S-carboxymethylated, and digested with TPCK-trypsin, and the peptides in question were separated by reversed-phase HPLC (Figure 2). The amino acid compositions and amino acid sequences of these peptides were also consistent with those expected. Thus, we concluded that lysozyme secreted from yeast here were all mature forms having the respective mutations as designed.

To study conformational changes of secreted lysozymes in solution, circular dichroism spectra were measured. As shown in Figure 3, the spectra of native and wild-type lysozymes were essentially identical. On the other hand, the spectra of W108Y and W108Q lysozymes were slightly different from that of native (or wild-type) lysozyme. However, in these lysozymes,

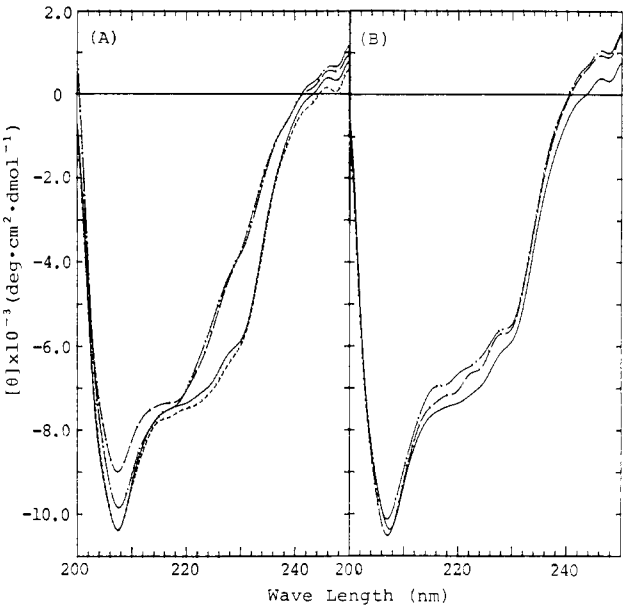

FIGURE 3: Circular dichroism spectra of lysozymes in 0.01 M potassium phosphate buffer, pH 7 at 20 °C. (A) Native lysozyme (---); wild-type lysozyme (—); W108Y lysozyme (-·-); W108Q lysozyme (--). (B) Wild-type lysozyme (—); D52N lysozyme (--); D52E lysozyme (-·-).

Table I: Comparisons of Some Properties of Wild-Type and Mutant Chicken Lysozymes

	act. (%)		substrate binding	
lysozyme	lysis[a]	GC[b]	K_d (10^{-5} M) for $(NAG)_3$[c]	rel retention time on chitin-coated column[d]
wild type	100	100	1.4	1
W108Y	37.1	17.3	10.4	0.75
W108Q	25.4	1.6	432.2	0.09
D52N	5.3	4.6	1.5	0.95
D52E	3.7	0.8	2.2	0.70

[a] Against *M. luteus* in 0.05 M phosphate buffer at pH 7.0 and 30 °C. [b] Against glycol chitin in 0.1 M acetate buffer at pH 5.5 and 40 °C. [c] Dissociation constant of the complex between lysozyme and $(NAG)_3$ in 0.1 M acetate buffer at pH 5.5 and 40 °C. [d] Relative retention time in the affinity HPLC on chitin-coated Celite at 0 °C. Details are given in the text.

Trp, an intense chromophore, is replaced with Tyr and Gln, respectively, which should affect the spectra. Therefore, these results would suggest that the native lysozyme and secreted lysozymes have almost the same conformation.

Activities and Saccharide Binding Abilities of Mutant Lysozymes. As shown in Table I, the enzymatic activity of lysozyme against glycol chitin at pH 5.5 and 40 °C (GC activity) was dramatically decreased when Trp108 was replaced with less hydrophobic Tyr or Gln. The lytic activity of lysozyme against *M. luteus* at pH 7 and 30 °C was also decreased by these replacements, although the extents of decrease were less eminent. As for the mutation of Asp52, both GC and lytic activities were greatly decreased by the replacement of this residue with either Asn or Glu, being consistent with the fact that Asp52 is a catalytic group (Imoto et al., 1972).

The dissociation constant (K_d) of lysozyme for the binding to $(NAG)_3$ at pH 5.5 and 40 °C was increased by the replacement of Trp108 with Tyr (7.4-fold) or Gln (309-fold), while it was little affected by the replacement of Asp52 with either Asn or Glu (Table I), indicating that Trp108 participates in the binding of $(NAG)_3$ which is known to bind to A, B, and C sites of the six saccharide binding subsites (A–F sites) (Blake et al., 1967b).

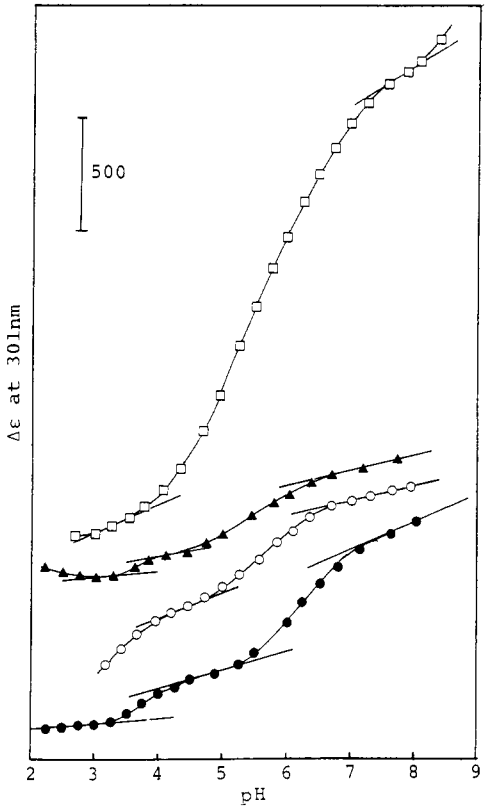

FIGURE 4: pH dependence of the absorbance at 301 nm of lysozymes in 0.1 M KCl, 1 mM acetic acid, and 1 mM phosphoric acid at 30 °C. (●) Wild-type lysozyme in the absence of additive; (○) wild-type lysozyme in the presence of 1 M NaCl; (▲) wild-type lysozyme in the presence of 1 M GdnHCl; (□) D52N lysozyme in the absence of additive.

Table I also indicates the relative retention times of lysozymes in affinity chromatography on a column of chitin-coated Celite at 0 °C. Clearly, the affinity of lysozyme for chitin was decreased by replacements of Trp108 with Tyr and Gln, as well as by those of Asp52 with Asn and Glu. The relative retention times in this affinity chromatography are considered to reflect the substrate binding abilities (through A–F sites) of respective lysozymes.

Titration of Glu35 by Tryptophyl Absorbance and Fluorescence. It is known that the pK_a of Glu35 in lysozyme can be determined by the pH dependence of the tryptophyl absorbance at 301 nm because Trp108 is in van der Waals contact with Glu35 (Imoto et al., 1972) and its absorbance is sensitive to the ionizations of Glu35 and Asp52 (Itani et al., 1975). Similarly, the pK_a of Glu35 in the lysozyme–$(NAG)_3$ complex can be determined by the pH dependence of the intensity of the tryptophyl fluorescence of lysozyme in the presence of $(NAG)_3$, although the pK_a of Glu35 in the absence of $(NAG)_3$ cannot be determined by the latter method because the fluorescence intensity of free lysozyme is insensitive to the ionization of Glu35 (Lehrer & Fasman, 1967).

Since these methods are convenient for the determination of the pK_a's of Glu35 in lysozymes possessing Trp108, we employed them to investigate the effect of the electrostatic interaction between Asp52 and Glu35 on the pK_a of Glu35 in 0.1 M KCl at 30 °C. Figure 4 shows the pH dependences of the absorbance at 301 nm of wild-type lysozyme in the absence and in the presence of a high concentration of salt (1 M NaCl or 1 M GdnHCl) and that of D52N lysozyme in the absence of the additive. Transitions observed at around pH

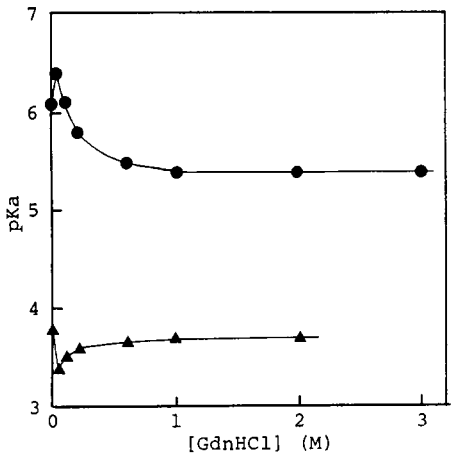

FIGURE 5: Effect of [GdnHCl] on the pK_a's of Glu35 (●) and Asp52 (▲) in wild-type lysozyme in 0.1 M KCl, 1 mM acetic acid, and 1 mM phosphoric acid at 30 °C.

Table II: pK_a's of Glu35 in Wild-Type and Mutant Lysozymes

	in 0.1 M KCl at 30 °C				from GdnHCl denaturation at 35 °C[c]
lysozyme	no additive[a]	1 M NaCl[a]	1–3 M GdnHCl[a]	1.4×10^{-4} M $(NAG)_3$[b]	
wild type	6.1	5.8	5.4	6.4	5.4
W108Y					5.2
W108Q					4.8
D52N	5.5			5.0	

[a] Determined by the pH dependence of the absorbance at 301 nm. [b] Determined by the pH dependence of the fluorescence intensity at 340 nm. [c] See the text.

4 and 6 are of the ionizations of Asp52 and Glu35, respectively (Itani et al., 1975). Clearly, the pK_a of Glu35 was lowered from 6.1 to 5.8 by 1 M NaCl and to 5.4 by 1 M GdnHCl or to 5.5 by the replacement of Asp52 with Asn. The decrease of the pK_a of Glu35 in the presence of GdnHCl has been also reported by Ogasahara and Hamaguchi (1967).

In order to examine the effect of GdnHCl on the pK_a of Glu35 in more detail, the UV titration at 301 nm of wild-type lysozyme was carried out in 0.1 M KCl containing various concentrations of GdnHCl less than the concentration causing denaturation at 30 °C. As shown in Figure 5, the pK_a of Glu35 changed with three phases; that is, with an increase of the concentration of GdnHCl, it increased first from 6.1 to 6.4 (at 0.04 M), then gradually decreased to 5.4 up to 1 M, and did not change further up to 3 M. At more than 3 M, denaturation occurred at pH less than 4.5, which prevented the titration of Glu35. The pK_a of Asp52 also changed with three phases identical to those observed for the pK_a of Glu35, although the pK_a change was opposite and small. The values of the pK_a of Asp52 were 3.8 in the absence of the denaturant and 3.4 and 3.7 in the presence of 0.04 and 1–2 M GdnHCl, respectively. The pK_a of Glu35 in the presence of more than 1 M NaCl could not be measured because of the precipitation of lysozyme.

Figure 6 shows the pH dependence of the intensities of tryptophyl fluoresence (emission at 340 nm excited at 280 nm) for wild-type and D52N lysozyme in the presence of 1.2×10^{-4} M $(NAG)_3$, respectively. From these titration curves, the pK_a's of Glu35 in $(NAG)_3$ complexes of wild-type and D52N lysozymes were determined to be 6.4 and 5.0, respectively. The values of the pK_a of Glu35 determined above are summarized in Table II.

Conformational Stabilities of Wild-Type, W108Y, and W108Q Lysozymes Determined by GdnHCl Denaturation.

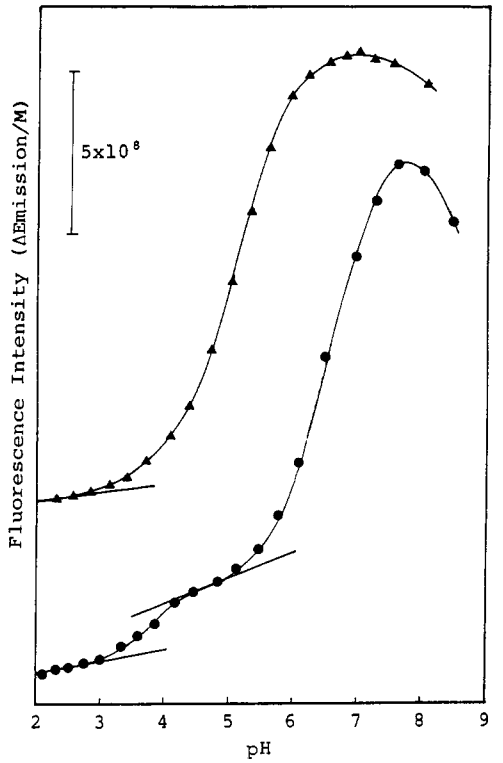

FIGURE 6: pH dependence of the fluorescence intensities at 340 nm of wild-type (●) and D52N (▲) lysozymes in 0.1 M KCl, 1 mM acetic acid, and 1 mM phosphoric acid containing 1.2×10^{-4} M $(NAG)_3$ at 30 °C. For excitation, 280-nm light was used.

The unfolding transitions of wild-type, W108Y, and W108Q lysozymes induced by GdnHCl were followed by observing the changes in the tryptophyl fluorescence (emission at 360 nm excited at 280 nm) as a function of the denaturant concentration at acidic pHs (pH 3.5–5.5) and 35 °C. By assuming a two-state transition for unfolding (Tanford et al., 1966), the equilibrium constant between the folded (N) and unfolded (D) states, $K_D = D/N$, and the free energy of unfolding, $\Delta G_D = -RT \ln K_D$, at a given concentration of GdnHCl were calculated from each unfolding curve. For all results reported here, ΔG_D was found to vary linearly with GdnHCl concentration, and a least-squares analysis was used to fit the data to the equation:

$$\Delta G_D = \Delta G_D(H_2O) - m[\text{GdnHCl}] \quad (1)$$

where $\Delta G_D(H_2O)$ is the value of ΔG_D in the absence of GdnHCl and m is a measure of the dependence of ΔG_D on GdnHCl concentration (Pace, 1975). The midpoint of GdnHCl denaturation, $C_{1/2} = \Delta G_D(H_2O)/m$, since $\Delta G_D = 0$ at $C_{1/2}$.

Since m seemed independent of pH but dependent on the mutation, we used the average value of m for each lysozyme to calculate $\Delta G_D(H_2O)$ of each lysozyme at each pH. In Table III, the values of $C_{1/2}$ and $\Delta G_D(H_2O)$ of the respective lysozymes at various pHs as well as the average m values of them are summarized. It is evident that the conformational stabilities decreased in the order of wild-type, W108Y, and W108Q lysozymes.

Estimation of pK_a's of Glu35 in Wild-Type, W108Y, and W108Q Lysozymes from the pH Dependence of $\Delta G_D(H_2O)$'s. Figure 7 shows the pH dependence of $\Delta G_D(H_2O)$ for wild-type, W108Y, and W108Q lysozymes, respectively. Besides the differences in intrinsic stability, clear differences in the shape of the pH dependence around pH 4.5–5.5 were observed among these lysozymes. Aune and Tanford (1969) have shown

Table III: Parameters Characterizing the GdnHCl Denaturation of Wild-Type, W108Y, and W108Q Lysozymes at 35 °C

	wild-type lysozyme			W108Y lysozyme			W108Q lysozyme		
pH	m^a (kcal mol^{-1} M^{-1})	$C_{1/2}$ (M)	$\Delta G_D(H_2O)$ (kcal/mol)	m^a (kcal mol^{-1} M^{-1})	$C_{1/2}$ (M)	$\Delta G_D(H_2O)$ (kcal/mol)	m^a (kcal mol^{-1} M^{-1})	$C_{1/2}$ (M)	$\Delta G_D(H_2O)$ (kcal/mol)
3.5	2.59 ± 0.18	2.71	7.02	3.19 ± 0.21	1.76	5.61	2.91 ± 0.28	1.18	3.43
4.0		3.26	8.44		2.20	7.02		1.66	4.83
4.5		3.69	9.56		2.57	8.20		1.91	5.56
5.0		3.63	9.40		2.55	8.13		2.01	5.85
5.5		3.61	9.35		2.52	8.04		2.03	5.91

[a] Average value.

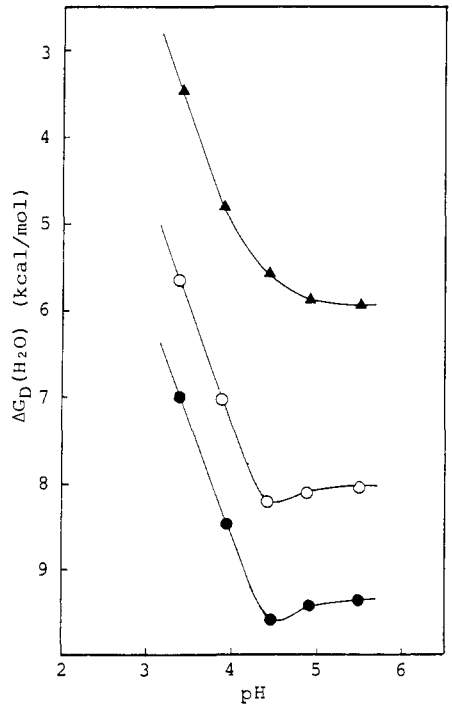

FIGURE 7: pH dependence of the free energies for unfolding of wild-type (●), W108Y (○), and W108Q (▲) lysozymes in the absence of GdnHCl at 35 °C. Details are given in the text.

that the free energy of unfolding of lysozyme as a function of $[H^+]$ $(=10^{-pH})$ can be expressed by the equations:

$$\Delta G_D(H_2O) = \Delta G_D(H_2O)° - RT \ln F([H^+]) \quad (2)$$

$$F([H^+]) = \prod_i (1 + K_{i,D}/[H^+])/\prod_i (1 + K_{i,N}/[H^+]) \quad (3)$$

where $\Delta G_D(H_2O)°$ is the free energy of unfolding at low pH in which all dissociable groups are protonated and $K_{i,D}$ and $K_{i,N}$ are the dissociation constants of residues i in the unfolded and folded states, respectively. Assuming the involvement of Glu35 ($pK_{Glu35,D} = 4.35$) and of six other dissociable groups [$pK_{1,N} = 1.9$, $pK_{1,D} = 3.4$ (α-COOH); $pK_{2,N} = 1.9$, $pK_{2,D} = 3.9$ (Asp66); $pK_{3,N} = 3.0$, $pK_{3,D} = 3.9$ (Asp119); $pK_{4,N} = 2.5$, $pK_{4,D} = 3.9$ (Asp18); and $pK_{5,N} = 5.8$, $pK_{5,D} = 6.5$ (His15)], curve-fitting analysis for wild-type lysozyme was carried out as a function of the pK_a of Glu35 ($pK_{Glu35,N}$). The values of $pK_{i,D}$ in 6 M GdnHCl are taken from the values of Roxby and Tanford (1971). The results indicated that the experimental data did not fit to $pK_{Glu35,N}$ of 6.1 but to that of 5.4. Since the values of $\Delta G_D(H_2O)$ were extrapolated from the GdnHCl denaturation experiments and since the pK_a of Glu35 in the presence of 1–3 M GdnHCl was 5.4 (Table II), these results indicate that the pK_a of Glu35 determined by this method is not that in the absence of the denaturant but that in the presence of high concentrations of GdnHCl.

Assuming the pK_a values of the other five residues to be little affected by the mutation of Trp108, the values of $pK_{Glu35,N}$ in W108Y and W108Q lysozymes were then calculated by a least-squares curve-fitting analysis. The results are indicated in the last column of Table II. The pK_a of Glu35 of lysozyme was found to be reduced by 0.2 and 0.6 pK_a unit by the replacement of Trp108 with Tyr and Gln, respectively.

Discussion

Origins of the Abnormal pK_a of Glu35 in Lysozyme. Our initial attempt was to determine the contributions of the electrostatic interaction between Asp52 and Glu35 and of the hydrophobic effect of Trp108 on the abnormal pK_a (=6.1) of Glu35 in lysozyme by using Asp52 and Trp108 mutant lysozymes. We first evaluated the contribution of the electrostatic interaction with Asp52 to the abnormal pK_a of Glu35. D52N lysozyme which lacks the electrostatic interaction had a pK_a of 5.5 (Table II), which is in good agreement with the value of 5.2 in Asp52 ethyl-esterified lysozyme (Parsons & Raftery, 1972), indicating that the electrostatic interaction with Asp52 surely contributes to the abnormal pK_a of Glu35.

An electrostatic interaction is expected to be weakened in high ionic strength media. In fact, the pK_a of Glu35 of wild-type lysozyme was reduced to 5.8 and 5.4 in 1 M NaCl and in 1–3 M GdnHCl, respectively (Table II). The facts that GdnHCl was much more effective than NaCl in reducing the abnormality in the pK_a of Glu35, that GdnHCl affected both the pK_a's of Glu35 and Asp52, and that the effect of GdnHCl seemed saturated less than 1 M (Figure 5) suggest the specific binding of GdnHCl (probably two molecules per lysozyme molecule, see Figure 5) at around Asp52 and Glu35. Furthermore, the pK_a of 5.4 in the presence of 1–3 M GdnHCl is close to the value of 5.5 in D52N lysozyme, suggesting that GdnHCl specifically and completely abolishes the electrostatic interaction between Asp52 and Glu35 at a concentration of 1–3 M. An X-ray crystallographic study of lysozyme indicates that Asp52 is not so close to Glu35 (7 Å apart) (Imoto et al., 1972) but they are connected with two tightly bound water molecules through hydrogen bonds (Figure 8). Thus, we suppose that the electrostatic interactions between Asp52 and Glu35 is the one through the hydrogen bonds of these bound water molecules rather than the direct one and that GdnHCl concentration less than 1 M specifically breaks this hydrogen bond network to result in the elimination of this electrostatic interaction.

Since the pK_a of 5.4–5.5 is still higher than the value of the normal glutamic acid residue ($pK_a = 4.4$), we examined next the contribution of the hydrophobicity of Trp108 to the abnormal pK_a of Glu35 by using W108Y and W108Q lysozymes. Because these mutant lysozymes lack Trp108, the spectroscopic titration of Glu35 using the absorbance or fluorescence change of Trp108 was not possible. Thus, we estimated these values by analysis of the pH dependence of the structural stabilities of these lysozymes in the absence of GdnHCl [$\Delta G_D(H_2O)$'s] which were determined by extrapolation from the GdnHCl denaturation experiments (Figure 7 and Table II). Interestingly, this method did not give the pK_a values of Glu35 in

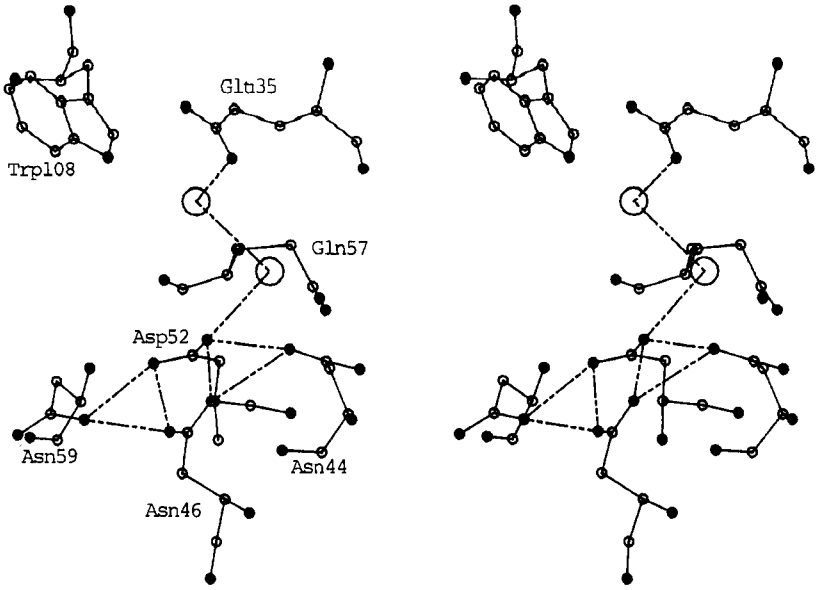

FIGURE 8: Stereoview of the structure around Glu35 and Asp52 in the crystal of chicken lysozyme. Coordinates (2LZT) are cited from the Protein Data Bank. Carbon atoms, heteroatoms, and water molecules are indicated by small open circles, small closed circles, and large open circles, respectively.

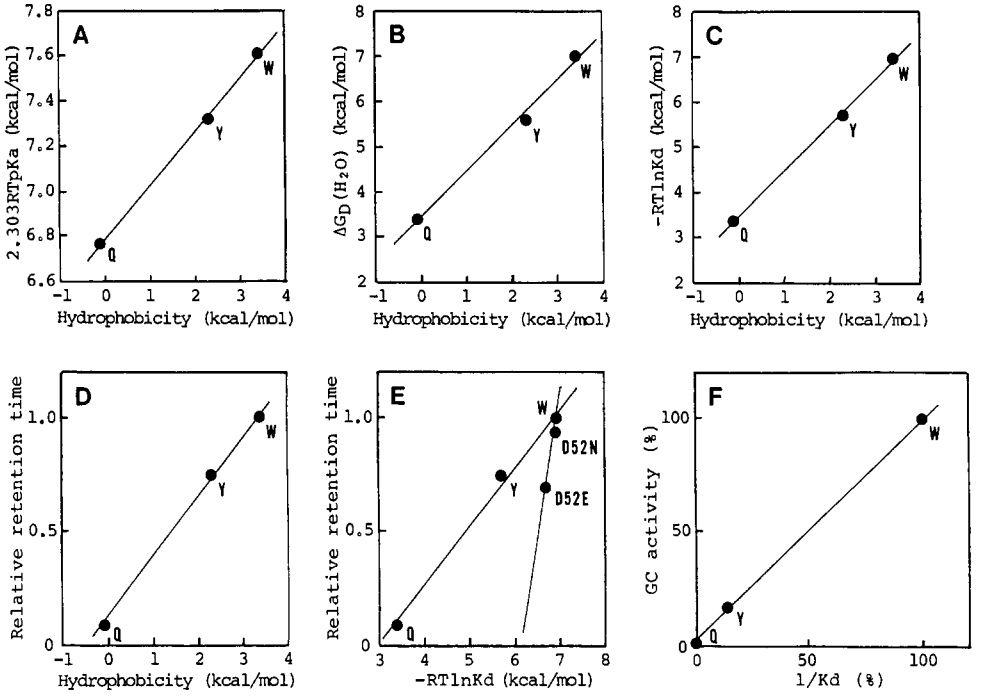

FIGURE 9: Correlations between two sets among the properties of lysozymes and hydrophobicities of residue 108. (A) Dissociation free energy of Glu35 vs hydrophobicity of residue 108; (B) free energy for unfolding at pH 3.5 vs hydrophobicity of residue 108; (C) binding energy to $(NAG)_3$ vs hydrophobicity of residue 108; (D) relative retention time on a chitin-coated Celite column vs hydrophobicity of residue 108; (E) relative retention time on a chitin-coated Celite column vs binding energy to $(NAG)_3$; (F) activity against glycol chitin vs binding constant for $(NAG)_3$ expressed as a percentage of that of wild-type lysozyme. Details are given in the text.

the absence of GdnHCl but those in the presence of GdnHCl. Thus, the values determined by this method were regarded as those in the absence of the electrostatic interaction between Asp52 and Glu35.

As shown in Figure 9A, a plot of $2.303RTpK_a$ as a function of the hydrophobicity of the side chain of residue 108 (3.4, 2.3, and −0.1 kcal/mol for Trp, Tyr, and Gln) (Tanford, 1962; Nozaki & Tanford, 1971) gave a straight line with a slope of 0.24. These results suggest not only that the hydrophobicity of Trp108 contributes to the abnormal pK_a of Glu35 in lysozymes but also that 24% of the hydrophobic free energy of Trp108 (0.82 kcal/mol) is reflected on the abnormality of the pK_a of Glu35 (0.6 pK_a unit).

As shown above, the electrostatic interaction with Asp52 and the hydrophobic effect of Trp108 were found to contribute almost equally to the abnormal pK_a of Glu35. However, it should be noted that the increase of about 0.4 pK_a unit of Glu35 remains to be explained by other factors. Although we have no experimental evidence for these factors, one could be a pole–dipole interaction between the negative charge of Glu35 and the C-terminus of the α-helix 25–35 macrodipole as discussed by Spassov et al. (1989).

Being consistent with the report of Kuramitsu et al. (1974), the increase in the pK_a of Glu35 of wild-type lysozyme by a complexation with $(NAG)_3$ was observed (from 6.1 to 6.4, Table II). Interestingly, in the case of D52N lysozyme which

lacks the electrostatic interaction, the pK_a of Glu35 decreased from 5.5 to 5.0 with the complexation. These observations may suggest that the electrostatic interaction between Asp52 and Glu35 in wild-type lysozyme is greatly increased and the hydrophobic effect of Trp108 on the pK_a of Glu35 is decreased by the complexation with $(NAG)_3$, and that the increase of the electrostatic interaction overcomes the decrease of the hydrophobic effect.

Effects of the Hydrophobicity of Trp108 on the Structural Stability and Enzymatic Function of Lysozyme. Since at low pHs where Glu35 is almost completely protonated, the values of $\Delta G_D(H_2O)$ of wild-type, W108Y, and W108Q lysozymes are considered to be proportional to the intrinsic free energies of unfolding of these lysozymes (see eq 2 and 3, the values at pH 3.5 are plotted against the respective hydrophobicities of residue 108 in Figure 9B. Clearly, a good straight line with a slope of 1.0 is obtained, indicating that the hydrophobic free energy of Trp108 (3.4 kcal/mol) entirely contributes to the stabilization of lysozyme structure. A similar hydrophobic stabilization effect has also been reported in T4 lysozyme (Ile3) (Matsumura et al., 1988) and tryptophan synthase α subunit (Glu49) (Yutani et al., 1987). At pHs where Glu35 is deprotonated, lysozyme having an abnormal p$K_{Glu35,N}$ of 6.1 should be less stable by about 2.4 kcal/mol (at around 35 °C) than a hypothetical lysozyme having a normal p$K_{Glu35,N}$ of 4.4 (see eq 2 and 3). The hydrophobic stabilization energy of 3.4 kcal/mol due to Trp108 thus overcomes the destabilization energy due to the abnormal pK_a of Glu35. In other words, the hydrophobicity of Trp108 contributes to the stabilization of lysozyme structure, and some of this energy is used for the maintenance of the abnormal pK_a of Glu35.

$(NAG)_3$, an inhibitor, is known to form a complex with lysozyme occupying A, B, and C sites of lysozyme out of six saccharide binding subsites named A–F sites (Blake et al., 1967b). On the other hand, chitin, a substrate, is considered to occupy whole subsites. Therefore, the dissociation constant, K_d, for the binding to $(NAG)_3$ and the relative retention time on a chitin-coated Celite column should reflect the substrate binding abilities of A–C and A–F sites of each lysozyme, respectively. A plot of $-RT \ln K_d$ against the hydrophobicity of residue 108 again gave a straight line with a slope of 1.0 (Figure 9C), indicating that the hydrophobic free energy of Trp108 entirely contributes to the substrate binding ability at A–C sites. Interestingly, the retention times of wild-type, W108Y, and W108Q lysozymes on the chitin-coated Celite column were also well correlated with the hydrophobic free energies of residue 108 (Figure 9D), suggesting that the retention time on this column is somehow associated linearly with the *free energy* for substrate binding. In Figure 9E, the relative retention times of all lysozymes on this column are plotted against the values of $-RT \ln K_d$, binding energy through A–C sites. Clearly, Trp108 mutant lysozymes and Asp52 mutant lysozymes seemed to constitute two classes different from each other. As for Trp108 mutant lysozymes, the retention times on the chitin-coated Celite column decreased along with a considerable decrease of $-RT \ln K_d$, suggesting that Trp108 is involved in substrate binding at A–C sites. On the other hand, as for Asp52 mutant lysozymes, the retention time on the chitin column greatly decreased with a marginal decrease of $-RT \ln K_d$, suggesting that Asp52 would be involved in substrate binding at other than A–C sites, namely, at D–F sites. Probably Trp108 and Asp52 may participate in substrate binding at C and D sites, respectively.

Enzymatic activities of lysozyme against glycol chitin at pH 5.5 and 40 °C (GC activities) also decreased with a decrease in the hydrophobicity of residue 108 (Table I). If a catalytic constant of lysozyme, k_{cat}, is not much affected by the replacement of Trp108, and if a Michaelis constant, K_m, is relatively large compared with the concentration of the substrate (glycol chitin of 0.5 mg/mL), the activity would primarily be proportional to $1/K_m$. Although the values of K_m are not known, it may be reasonable to assume that they are proportional to the values of K_d, dissociation constants for binding to $(NAG)_3$. Thus, in Figure 9F, the GC activities are plotted against the values of $1/K_d$ expressed as percentages of that of wild-type lysozyme. A good straight line with a slope of 0.98 suggests that the GC activities of Trp108 mutant lysozymes are primarily determined by their substrate binding abilities.

The lytic activity against *M. luteus* at pH 7.0 and 30 °C was also decreased by the replacement of Trp108 with a less hydrophobic amino acid residue, but it was not so sensitive as the GC activity. Interestingly, there seems a linear correlation between both activities as expressed as a equation of lytic activity = 0.759 × GC activity + 24.09. Probably, some factors besides saccharide binding ability would operate to increase the lytic activities of Trp108 mutant lysozymes.

Conclusions

The hydrophobicity of Trp108 of chicken lysozyme was found to contribute (i) to increase the pK_a of Glu35 by 0.6 unit, corresponding to 24% of the hydrophobic free energy of Trp108, (ii) to stabilize the folding structure of lysozyme by a free energy corresponding to full hydrophobicity of Trp, and (iii) to increase the substrate binding ability of lysozyme by a free energy corresponding to full hydrophobicity of Trp. There may exist a hydrophobic interaction between Trp108 and a saccharide unit of substrate at the C site of lysozyme. Thus, it is concluded that the hydrophobicity of Trp108 is critical for the structural stability and function of lysozyme.

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A Hybrid Ribulosebisphosphate Carboxylase/Oxygenase Enzyme Exhibiting a Substantial Increase in Substrate Specificity Factor†

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ABSTRACT: Two hybrid ribulose-1,5 bisphosphate carboxylase/oxygenase (RubisCO) enzymes were constructed using RubisCO small subunit genes (*rbcS*) from two eucaryotic marine organisms, *Cylindrotheca* sp. N1 and *Olisthodiscus luteus*, cloned downstream of the RubisCO large subunit gene (*rbcL*) of the cyanobacterium *Synechococcus* PCC 6301. The expression products synthesized by *Escherichia coli* JM107 (pVTAC223 and pANOLI) were purified and examined by polyacrylamide gel electrophoresis and compared to the purified products generated by *E. coli* MV1190 (pBGL710), containing cyanobacterial *rbcL* and *rbcS* genes. Both *Cylindrotheca* and *Olisthodiscus* small subunits were able to assemble in vivo with the *Synechococcus* large subunit octamer to form heterologous hexadecameric L_8S_8 enzymes, the pVTAC223 and pANOLI hybrid enzymes, respectively. Like the *Synechococcus* RubisCO, the hybrid enzymes were rapidly activated by Mg^{2+} plus HCO_3^-, even in the presence of RuBP. The hybrid enzymes, however, were considerably more sensitive to the competitive inhibitor 6-phosphogluconate. Detailed kinetic analysis indicated that while the carboxylase activity of both chimeric enzymes was severely reduced, in the case of the pVTAC223 hybrid enzyme, the degree of partitioning between carboxylation and oxygenation was increased nearly 60% relative to the *Synechococcus* RubisCO. Other kinetic properties, including the Michaelis constants for the gaseous substrates and RuBP, were altered in the hybrid proteins. These studies also led to the finding that the substrate specificity factor of the *Cylindrotheca* RubisCO is unusually high.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)[1] is a bifunctional enzyme that is fundamental to the metabolism of all plants, algae, and most photosynthetic bacteria. The enzyme assumes a pivotal role in catalyzing the initial reaction in two competing metabolic pathways: (i) photosynthetic carbon fixation and (ii) photorespiration. Because oxygen inhibits carboxylation and photorespiration leads to the oxidation of reduced carbon with no apparent selective advantage to the plant, it is thought that photosynthetic efficiency and net productivity might be enhanced by

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[1] Abbreviations: RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; *rbcS*, small subunit gene; *rbcL*, large subunit gene; IPTG, isopropyl β-D-thiogalactopyranoside; TEM, 15 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; TEMMB, 25 mM TEM, pH 8.0, 10 mM $MgCl_2$, and 50 mM $NaHCO_3$; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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