Stabilization of a Protein by Removal of Unfavorable Abnormal pK_a: Substitution of Undissociable Residue for Glutamic Acid-35 in Chicken Lysozyme†

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ABSTRACT: Glu35 in chicken lysozyme has an abnormally high pK_a (6.1) partly due to the hydrophobic environment provided by Trp108. The relationship between protein stability and abnormal pK_a was investigated in detail by using mutant lysozymes in which Glu35 was replaced by undissociable residues and an oppositely ionizable residue. It was found that lysozyme was stabilized at alkaline pH range by the replacement of Glu35 with an undissociable residue, Gln (E35Q lysozyme) or Al (E35A lysozyme). On the other hand, when Glu35 was replaced by His (E35H lysozyme), which could have an opposite charge to Glu by ionization, the introduced His35 was found to have an abnormally low pK_a (3.6), leading to the destabilization of lysozyme at acidic pH. These observations are completely consistent with the situation that the environment around Glu35 is highly hydrophobic and therefore the placement of either a positive or negative charge in such an environment leads to destabilization of lysozyme. These observations also indicate that the replacement of an acidic residue having abnormally high pK_a or a basic residue having abnormally low pK_a by an undissociable residue is a very efficient and general method for stabilization of a protein.

Many proteins contain dissociable residues with abnormal pK_a's. Wyman's theory (Wyman, 1964) predicts that the presence of an acidic group having abnormally high pK_a and a basic group having abnormally low pK_a should destabilize a protein. Although many approaches such as introduction of salt bridges, hydrophobic interactions, and cross-linkages have been successfully carried out to stabilize proteins (Beasty et al., 1987), removal of the unfavorable dissociable residues with abnormal pK_a's has never been attempted.

Since chicken lysozyme has an abnormally high pK_a carboxyl group, Glu35 (Imoto et al., 1972), this protein would be a good model to examine the efficiency of such a method for stabilization of a protein. Thus, in this study, we prepared mutant lysozymes in which Glu35 was replaced by undissociable residues (E35Q and E35A lysozymes) and an oppositely dissociable residue (E35H lysozyme) and evaluated the effects of the mutations on the stability of lysozyme in a pH range of 3.5–5.5. We found that this new method is very efficient at stabilizing a protein.

MATERIALS AND METHODS

Materials. Wild-type chicken lysozyme was prepared as described previously (Inoue et al., 1992) by expression and secretion from yeast transformants. CM-Toyopearl 650M, a cation-exchange resin, was obtained from Tosoh (Tokyo, Japan). A column of Wakosil 5C18-200 (4.6 × 250 mm) was obtained from Wako Pure Chemicals Institute (Osaka, Japan). *Micrococcus luteus*, a substrate of lysozyme, was from Sigma (St. Louis, MO). 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.

Mutant Lysozymes. Three mutant lysozymes in which Glu35 is replaced by Gln (E35Q lysozyme), Ala (E35A lysozyme), and His (E35H lysozyme) were prepared according to the methods described previously (Inoue et al., 1992). The structures of the mutagenic primers used for site-directed mutagenesis to replace Glu35 with Gln (E35Q), Ala (E35A), and His (E35H) were 5′-CGCAAAATTCCAGAGTAACTTC-3′, 5′-CGCAAAATTCGCTAGTAACTTC-3′, and 5′-CGCAAAATTCCACAGTAACTTCAAC-3′, respectively.

Each yeast *Saccharomyces cerevisiae* AH22 transformant thus prepared was cultivated at 30 °C for 125 h to express and secrete each lysozyme from yeast as described previously (Inoue et al., 1992). 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.

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 473A 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).

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

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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 lysozyme 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 (Yamada & Imoto, 1981).

The dissociation constants of lysozymes for the binding to $(NAG)_3$ in 0.1 M acetate buffer at pH 5.5 and 40 °C were determined by a 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 His35 in E35H Lysozyme. The pH dependence of tryptophyl fluorescence of wild-type or E35H lysozyme in 0.1 M KCl at 30 °C in the presence of $(NAG)_3$ was measured with a Hitachi F-2000 fluorescence spectrophotometer as described previously (Inoue et al., 1992) by measuring the fluorescence at 330 nm with the excitation at 280 nm. Concentrations of the protein and $(NAG)_3$ employed were 1.5×10^{-6} and 1.2×10^{-4} M, respectively.

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

Differential Scanning Calorimetry (DSC). The determination of the transition temperatures for the thermal denaturation of wild-type and E35A lysozymes was carried out by use of Seiko DSC-10 differential scanning calorimeter equipped with a Seiko SSC-580 thermal controller (Seiko Instrument and Electrics, Tokyo, Japan). A 55-μL aliquot of a lysozyme solution in 0.1 M acetate buffer at pH 3.5 or 5.5 (30 mg/mL) was sealed in a silver vessel and heated from 10 to 140 °C at the rate of 1.0 °C/min. The transition temperature was obtained as the temperature showing the maximal excess heat capacity.

RESULTS

Expression and Secretion of E35Q, E35A, and E35H Lysozymes from Yeast. Expression and secretion of mutant lysozymes from yeast was carried out in the same manner as described previously (Inoue et al., 1992). The lysozymes secreted in the culture media were purified by cation-exchange chromatography. The amounts of lysozymes secreted in the media were found to be 1.1, 3.5, and 1.5 mg/L for E35Q, E35A, and E35H lysozymes, respectively, while wild-type lysozyme was secreted in the amount of 5.0 mg/L under the same conditions.

The NH_2-terminal amino acid sequences of purified mutant lysozymes were all single and identical to that of native lysozyme obtained from hen egg white, as in the case of wild-type lysozyme (Inoue et al., 1992). The amino acid compositions of these lysozymes were all consistent with those expected from the mutations introduced (data not shown). The mutant lysozymes were reduced, S-carboxymethylated, and digested with TPCK-trypsin, and the peptides in question were separated by reversed-phase HPLC (peaks indicated by arrows in Figure 1). The amino acid compositions of these

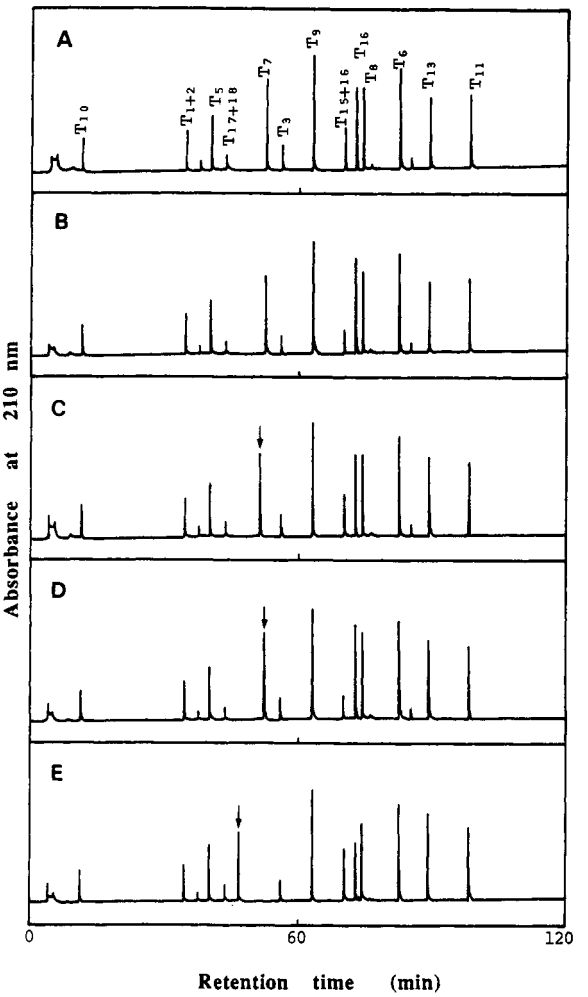

FIGURE 1: Reversed-phase HPLC of tryptic peptides obtained from reduced and S-carboxymethylated lysozymes on a column (4 × 250 mm) of Wakopak 5C18-200. The column was eluted with a gradient formed from 50 mL of 1% acetonitrile and 50 mL of 50% acetonitrile, both containing 0.1% concentrated HCl at a flow rate of 0.6 mL/min: (A) from native lysozyme; (B) from wild-type lysozyme; (C) from E35Q lysozyme; (D), from E35A lysozyme; (E), from E35H lysozyme. T refers to the tryptic peptides, and peptide numbering is from the N-terminal peptide. The arrows represent the peptides containing the mutation.

peptides (Phe34–Arg45) were consistent with those expected. The amino acid sequence analyses of these peptides also supported the respective mutations (Table I). Thus, we concluded that the lysozymes secreted from yeast were all mature forms having only the respective mutations as designed.

In order to confirm that the structures of these mutant lysozymes were almost identical to that of wild-type lysozyme, their CD spectra in the far-ultraviolet region, reflecting the content of secondary structures, were measured at pH 7 and 20 °C. As shown in Figure 2, no appreciable differences were observed among wild-type and mutant lysozymes in the spectra, suggesting that the wild-type and mutant lysozymes have essentially the same tertiary structures.

Activities and Saccharide Binding Abilities of Mutant Lysozymes. Table II shows activities and saccharide binding abilities of the mutant lysozymes as well as those of wild-type lysozyme.

Enzymatic activity of lysozyme was completely lost by the mutation of Glu35 either against *M. luteus* at pH 7 and 30 °C (lytic activity) or against glycol chitin at pH 5.5 and 40 °C (GC activity), consistent with this residue being essential for catalysis (Imoto et al., 1972; Malcolm et al., 1989).

The dissociation constant (K_d) of lysozyme for binding to $(NAG)_3$ at pH 5.5 and 40 °C, which reflects the saccharide

Table I: Amino Acid Sequence Analyses of Tryptic Peptide T7's Derived from Mutant Lysozymes[a]

	lysozyme											
	wild			E35Q			E35A			E35H		
cycle	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
	PTH-Amino Acid (pmol)											
1st largest	Phe	Glu	Ser	Phe	Gln	Ser	Phe	Ala	Ser	Phe	His	Ser
	504	317	121	981	698	147	271	160	28	421	77	88
2nd largest	Val	Phe	Glu	Gly	Glu	Asp	Gly	Phe	Asp	Asp	Asp	Gly
	35	29	50	26	223	36	18	6	19	60	31	20
3rd largest	Gly	Ala	Leu	Ala	Gly	Gln	Ala	Asp	Ala	Gly	Gly	Asp
	20	24	8	18	21	25	14	6	16	25	24	19

[a] Peptides indicated by arrows in Figure 1. Only first three cycles are shown.

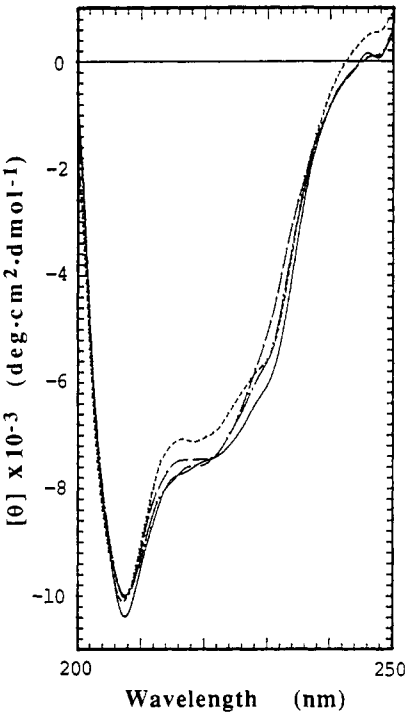

FIGURE 2: Circular dichroism spectra of wild-type (—), E35Q (– –), E35A (– · –), and E35H lysozyme (- - -) in 0.01 M phosphate buffer (pH 7) at 20 °C.

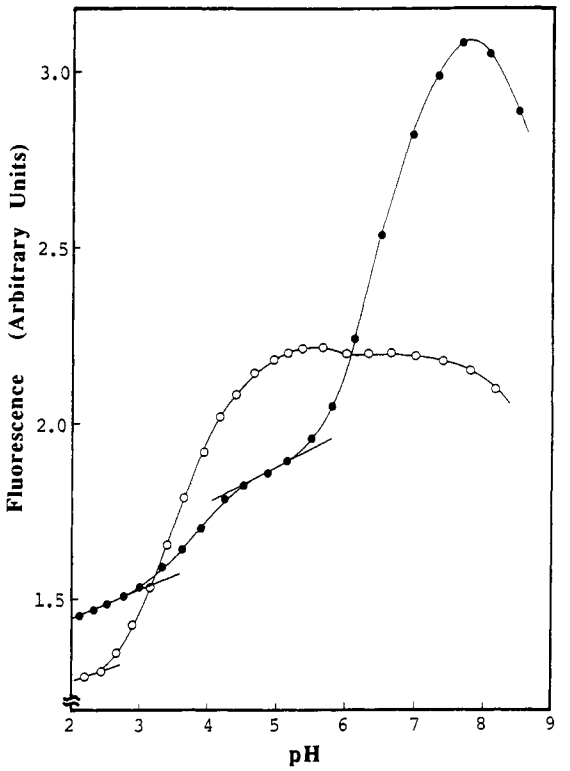

FIGURE 3: pH dependences of the fluorescence intensities at 330 nm of wild-type (●) and E35H (○) lysozymes in 0.1 M KCl, 1 mM acetic acid, and 1 mM phosphoric acid containing 1.2×10^{-4} M $(NAG)_3$ at 30 °C. For excitation, 280-nm light was used.

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

	activity (%)		substrate binding	
lysozyme	lysis[a]	GC[b]	K_d (10^{-5} M) for $(NAG)_3$[c]	rel retentn time[d]
wild type	100	100	1.4	1
E35Q	0	0	3.5	0.85
E35A	0	0	1.6	0.90
E35H	0	0	1.0	0.76

[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.

binding ability of A–C sites out of six binding subsites [(A–F sites (Blake et al., 1967a)], was not significantly changed by the replacement of Glu35 with Ala or His but was slightly increased by the replacement of Glu35 with Gln. The value of 3.5×10^{-5} M for E35Q lysozyme was in good agreement with the value of 4.4×10^{-5} M for the same mutant lysozyme prepared by chemical derivatization (Kuroki et al., 1986).

The relative retention times of mutant lysozymes in affinity chromatography on a column of chitin-coated Celite at 0 °C, which reflect the relative saccharide binding abilities of all six binding subsites, indicated that the affinity toward chitin was decreased in the order of wild-type, E35A, E35Q, and E35H lysozyme.

Titration of His35 in E35H Lysozyme by Tryptophyl Fluorescence. 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 $(NAG)_3$ (Itani et al., 1975) because Trp108 is in van der Waals contact with Glu35 (Imoto et al., 1972) and its fluorescence is sensitive to the ionizations of Glu35 and Asp52 in the presence of $(NAG)_3$ (Lehrer & Fasman, 1967; Inoue et al., 1992). Since it has been reported that a histidine residue quenches the tryptophyl fluorescence and this quenching is more effective in a protonated form (Loewenthal et al., 1991), we tried to determine the pK_a of His35 in E35H isozyme by measuring its pH dependence of fluorescence intensity.

Figure 3 shows the pH dependence of the intensity of tryptophyl fluorescence (emission at 330 nm, excited at 280 nm) of E35H lysozyme as well as that of wild-type lysozyme in the presence of 1.2×10^{-4} M $(NAG)_3$. While wild-type lysozyme showed two transitions, that is a large intensity change due to the ionization of Glu35 with pK_a of 6.4 and a small one due

Table III: Parameters Characterizing the GdnHCl Denaturation of Wild-Type, E35Q, E35A and E35H Lysozymes at 35 °C

	wild type			E35Q			E35A			E35H		
pH	m^a	$C_{1/2}{}^b$	ΔG_D (H_2O)^c	m^a	$C_{1/2}{}^b$	ΔG_D (H_2O)^c	m^a	$C_{1/2}{}^b$	ΔG_D (H_2O)^c	m^a	$C_{1/2}{}^b$	ΔG_D (H_2O)^c
3.5	2.59	2.71	7.02	3.21	2.02	6.49	2.80	3.03	8.48	3.24	1.11	3.59
4.0	(±0.18)	3.26	8.44	(±0.16)	2.48	7.97	(±0.40)	3.42	9.58	(±0.31)	1.64	5.31
4.5		3.69	9.56		2.84	9.13		3.72	10.42		2.10	6.80
5.0		3.63	9.40		2.97	9.55		3.91	10.95		2.42	7.84
5.5		3.61	9.35		3.06	9.83		3.97	11.12		2.70	8.74

^{a} Average values, in kcal mol^{-1} M^{-1}. ^{b} Values in M. ^{c} Values in kcal/mol.

to that of Asp52 with pK_a of 3.8, E35H lysozyme showed only one transition with pK_a of 3.6. Careful examination of Figure 3 leads us to the conclusion that the transition at around pH 2.5–5 in E35H lysozyme reflects the pK_a of His35 in E35H lysozyme–$(NAG)_3$ complex. Clearly, the pK_a of His35 in E35H lysozyme is abnormally low compared with that of normal His residue (pK_a = 6.5) (Roxby & Tanford, 1971). Probably, a transition showing a small intensity change due to the ionization of Asp52 is hidden underneath the large one. Because the intensity of tryptophyl fluorescence of wild-type lysozyme in the absence of $(NAG)_3$ is insensitive to the ionization of Glu35 (Lehrer & Fasman, 1967), the pK_a of Glu35 in free lysozyme cannot be determined by the titration of tryptophyl fluorescence. However, in E35H lysozyme, the transition of tryptophyl fluorescence reflecting the pK_a of His35 was also observed in the absence of $(NAG)_3$, although the transition was about half of that in the presence of $(NAG)_3$ (data not shown). The pK_a of His35 in free E35H lysosome was thus determined to be 3.6, which is the same as that in the E35H lysozyme–$(NAG)_3$ complex.

Conformational Stabilities of Wild-Type and Mutant Lysozymes Determined by GdnHCl Denaturation. The unfolding transitions of mutant lysozymes as well as that of wild-type lysozyme induced by GdnHCl were followed by observing changes in the tryptophyl fluorescence (emission at 360 nm, excited at 280 nm) as a function of denaturant concentration at acidic pH's (pH 3.5–5.5) and 35 ± 0.2 °C. Details for analysis were the same as described previously (Inoue et al., 1992). That is, by assuming a two-state transition for unfolding, 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, and a least squares analysis was used to fit the data to the equation

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

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 et al., 1975). The midpoint of GdnHCl denaturation is $C_{1/2} = \Delta G_D(H_2O)/m$, since $\Delta G_D = 0$ at $C_{1/2}$. m seemed to be independent of pH but dependent on the mutation, so we used the average value of m for each of the lysozymes. In Table III, the values of $C_{1/2}$ and $\Delta G_D(H_2O)$ of the respective lysozymes at various pH's as well as their average m values are summarized. In Figure 4A, the values $\Delta G_D(H_2O)$ are plotted against pH. To make clear the differences among these lysozymes, the differences of the values at the respective pH's from that at pH 4.5, $\Delta\Delta G_D(H_2O)$'s, are replotted in Figure 4B. It is evident that the transition due to the ionization of Glu35 observed in wild-type lysozyme in a pH range of 4.5–5.5 is completely absent in the mutant lysozymes. As a result, E35Q and E35A lysozymes become more stable by 0.70 kcal/mol on going from pH 4.5 to pH 5.5 while wild-type lysozyme becomes less stable by 0.21 kcal/mol. On the other hand, E35H lysozyme showed a steeper pH dependence and becomes more stable by 1.94 kcal/mol on going from pH 4.5 to 5.5, although the absolute stability of E35H lysozyme at pH 5.5 is still lower by 0.61 kcal/mol than wild-type lysozyme.

Transition Temperatures for the Thermal Denaturation of Wild-Type and E35A Lysozymes. It was shown previously that the presence of GdnHCl (1–3 M) decreases the pK_a of Glu35 from 6.1 to 5.4, and the pK_a of Glu35 estimated from GdnHCl denaturation experiments was 5.4 even if ΔG_D was extrapolated to the absence of GdnHCl (Inoue et al., 1992). Therefore the pH dependence of $\Delta G_D(H_2O)$ for wild-type lysozyme extrapolated from the GdnHCl denaturation experiments does not correctly reflect the true pH dependence of the stability of wild-type lysozyme in the absence of GdnHCl, because the abnormality of Glu35 was partly depressed. This means that the stabilization effect of the replacement of Glu35 with an undissociable residue in the absence of the denaturant should be larger than estimated from the GdnHCl denaturation experiments. In order to confirm this, the melting temperatures for the thermal unfolding of wild-type and E35A lysozymes in the absence of GdnHCl were directly measured by use of differential scanning calorimetry (DSC) at pH 3.5 and 5.5, respectively. The melting temperatures thus determined were 73.0 and 76.4 °C at pH 3.5 and 75.6 and 81.7 °C at pH 5.5 for wild-type and E35A lysozymes, respectively.

The difference in ΔG_D between mutant and native proteins ($\Delta\Delta G_D$) at the transition temperature of the native protein (T_m) can be often calculated by

$$\Delta\Delta G_D = \Delta T \Delta S_D$$

where ΔT is the difference in the transition temperature between mutant and native proteins and ΔS_D is the entropy of unfolding for the native protein at T_m (Becktel & Schellman, 1987). Thus, we also calculated the values of $\Delta\Delta G_D$ of lysozyme at pH 3.5 and 5.5 due to the mutation of Glu35 to Ala by using this equation.

From the temperature dependence of ΔH_D, the enthalpy of unfolding, for wild-type lysozyme (Sturtevant, 1977), the values of ΔH_D for wild-type lysozyme at 73.0 and 75.6 °C, T_m's at pH 3.5 and 5.5, were calculated to be 132.4 and 136.5 kcal/mol, respectively. By use of these values and the equation $\Delta G_D = \Delta H_D - T_m \Delta S_D = 0$, the values of ΔS_D for wild-type lysozyme at pH 3.5 and 5.5 were calculated to be 382.5 and 391.4 cal deg^{-1} mol^{-1}, respectively. Thus, the values of $\Delta\Delta G_D$ of lysozyme due to the mutation of Glu35 to Ala were calculated to be 1.38 kcal/mol at 73.0 °C and pH 3.5 and 2.39 kcal/mol at 75.6 °C and pH 5.5. As shown in Table III, the corresponding values estimated from the GdnHCl experiments at 35 °C were 1.46 and 1.77 kcal/mol at pH 3.5 and 5.5, respectively. Clearly, at pH 3.5 where Glu35 is undissociated, the value of $\Delta\Delta G_D$ determined by DSC in the absence of GdnHCl (1.38 kcal/mol) was in good agreement with the value estimated from GdnHCl experiments (1.46 kcal/mol), while at pH 5.5 where Glu35 is partly dissociated, the former (2.39 kcal/mol) was considerably larger than the

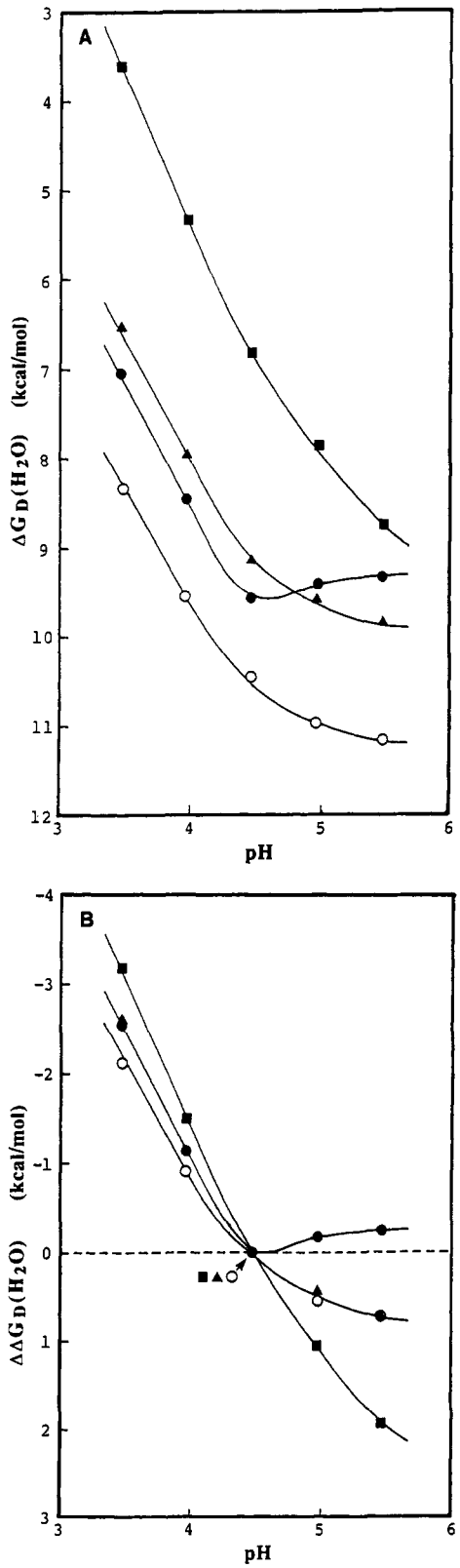

FIGURE 4: (A) pH dependences of the free energies for unfolding of wild-type (●), E35Q (▲), E35A (○), and E35H (■) lysozymes in the absence of GdnHCl at 35 °C. (B) The same, but the values of the respective lysozymes at pH 4.5 are adjusted to zero. Details are given in the text.

latter (1.77 kcal/mol). Although the temperatures in these experiments were not the same, these results indicate that the effect of the replacement of abnormal Glu35 with an undissociable residue, Ala or Gln, on the stabilization of lysozyme at relatively neutral pH's in the absence of GdnHCl is much larger than that estimated by the extrapolation from the GdnHCl denaturation experiments.

DISCUSSION

In this paper, we employed three Glu35 mutant lysozymes prepared by using the yeast expression and secretion system described previously (Inoue et al., 1992). All mutant lysozymes have correct NH_2-terminals and were supposed to have essentially the same conformations as wild-type lysozyme (Figure 2). The following discussion is based on this assumption.

Properties of Glu35 Mutant Lysozymes. Both lytic activity at pH 7 and GC activity at pH 5.5 were completely lost by the mutation of Glu35 to Gln, Ala, or His (Table II). These results are consistent with the fact that Glu35 is essential for lysozyme catalysis (Blake et al., 1967b; Imoto et al., 1972; Kuroki et al., 1986; Malcolm et al., 1989). According to a well-accepted catalytic mechanism proposed by Blake et al. (1967b), the carboxyl group of Glu35 in its unionized form serves as a general-acid catalyst to donate a proton to the substrate. Since Gln and Ala cannot act as a proton donor, the lack of the activity in E35Q and E35A lysozymes is reasonable. However, E35H lysozyme is also inactive even though protonated His residue can act as a proton donor in many enzymes. As the pK_a of His35 in E35H lysozyme is abnormally low (pK_a = 3.6), this residue cannot exist as a protonated form under the conditions where activities were measured (pH 5.5 and 7). However, the preliminary experiments at pH 3.0 and 40 °C suggested that E35H lysozyme had very small GC activity (1.5% the activity of native lysozyme). Although more experiments at lower pH's are apparently required to determine whether the small activity observed in E35H lysozyme at pH 3.0 is real or not, it can be safely mentioned that the His35 residue in E35H lysozyme does not act as a good proton donor for lysozyme activity.

As shown in Table II, the $(NAG)_3$ binding ability of lysozyme (K_d) was marginally affected by the mutation of Glu35, but the affinity for chitin (relative retention time in the affinity chromatography on chitin-coated Celite column) was significantly decreased, as observed in the cases of Asp52 mutant lysozymes (Inoue et al., 1992). Thus, we suppose that Glu35 like Asp52 in lysozyme participates in saccharide binding at the D site in the active site cleft (Blake et al., 1967b; Strynadka & James, 1991).

Stabilization of a Protein by the Substitution of a Residue Having Unfavorable Abnormal pK_a. Our main purpose in this paper is to show the removal of an unfavorably abnormal pK_a to be a very efficient and general method for stabilization of a protein by using Glu35 mutant lysozymes in which Glu35 with an abnormally high pK_a value (6.1) is replaced by an undissociable residue (Gln or Ala) or oppositely ionizable residue (His).

E35Q and E35A lysozymes showed titration shapes considerably different from wild-type lysozyme in the pH dependence of the stability at around pH 4.5–5.5 (Figure 4). Aune and Tanford (1969) have shown that the free energy of unfolding of lysozyme as a function of $[H^+]$ ($=10^{-pH}$) can be expressed by the equations

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

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

where $\Delta G_D(H_2O)^\circ$ 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 residue i in the unfolded and folded states, respectively. As shown previously (Inoue et al., 1992), six dissociable groups ($pK_{1,N}$ = 1.9, $pK_{1,D}$ = 3.4

(α-COOH); p$K_{2,N}$ = 1.9, p$K_{2,D}$ = 3.9 (Asp66); p$K_{3,N}$ = 3.0, p$K_{3,D}$ = 3.9 (Asp119); p$K_{4,N}$ = 2.5, p$K_{4,D}$ = 3.9 (Asp18); p$K_{5,N}$ = 5.8, p$K_{5,D}$ = 6.5 (His15); and p$K_{Glu35,N}$ = 5.4, p$K_{Glu35,D}$ = 4.35) can explain the pH dependence of $\Delta G_D(H_2O)$ of wild-type lysozyme extrapolated from the GdnHCl denaturation experiments, in which the value of pK_a for Glu35 in the folded state of lysozyme is that in the presence of GdnHCl (5.4) instead of that in the absence of the denaturant (6.1). This predicts that Glu35 contributes to the destabilization of lysozyme by 0.86 kcal/mol on going from pH 4.5 to 5.5 and elimination and abnormal Glu35 would lead to the stabilization of lysozyme by 0.86 kcal/mol. As mentioned above, E35Q and E35A lysozymes become more stable by 0.70 kcal/mol and wild-type lysozyme becomes less stable by 0.21 kcal/mol on going from pH 4.5 to pH 5.5, indicating that the destabilization energy of 0.91 kcal/mol is eliminated by the replacement of Glu35 by undissociable Gln or Ala. The value of 0.91 kcal/mol is in good agreement with the value of 0.86 kcal/mol predicted above. Thus, we concluded that the destabilization caused by the abnormally high pK_a of Glu35 in lysozyme was successfully eliminated by the mutation of Glu35 to Gln or Ala.

Since in the absence of GdnHCl the true pK_a of Glu35 is more abnormal (pK_a = 6.1) than that estimated from the GdnHCl denaturation experiments (pK_a = 5.4) (Inoue et al., 1992), the stabilization effect by removing this abnormal pK_a in the absence of GdnHCl should be much larger than predicted above. The fact that the unfolding free energy changes due to the mutation of Glu35 to Ala calculated from the melting temperatures for the thermal unfolding of wild-type and E35A lysozymes in the absence of GdnHCl determined by DSC were almost identical at pH 3.5 and larger at pH 5.5 than those estimated from GdnHCl experiments (1.38 vs 1.46 kcal/mol at pH 3.5 and 2.39 vs 1.77 kcal/mol at pH 5.5) supports this idea.

In E35H lysozyme, the pH dependence of $\Delta G_D(H_2O)$ was much steeper than those of E35Q and E35A lysozymes (Figure 4). Since the pK_a of His35 is shown to be abnormally low (pK_a = 3.6), the curve-fitting analysis of $\Delta G_D(H_2O)$ was carried out by eliminating the terms of Glu35 and by adding the terms of His35 instead. p$K_{His35,D}$ was set as 6.5 as in the case of p$K_{His15,D}$ and the other five dissociable groups were assumed to be the same pK_i's as those in wild-type lysozyme, and then p$K_{His35,N}$ was treated as unknown. The pK_a of His35 thus determined was 3.6, which is abnormally low and consistent with the value determined by the titration of tryptophyl fluorescence in the presence of $(NAG)_3$ (Figure 3). Thus, the reason for the steeper slope in the pH dependence of $\Delta G_D(H_2O)$ for E35H lysozyme than for other lysozymes in an acidic region is shown to be attributed to the abnormally low pK_a of His35, which contributes by destabilizing lysozyme in acidic pH range.

We have shown that the abnormally high pK_a of Glu35 is caused by a hydrophobic environment around Glu35 provided by Trp108 and by the electrostatic interaction between Glu35 and Asp52 through a hydrogen-bonding network formed via two tightly bound water molecules (Inoue et al., 1992). The result that His35 in E35H lysozyme shows an abnormally low pK_a and the fact that Glu35 in wild-type lysozyme shows an abnormally high pK_a indicate that the placement of a charged group, either positive or negative, at position 35 is energetically costly. Such a phenomenon would arise if the environment around Glu35 is highly hydrophobic. If the electrostatic interaction between His35 and Asp52 in E35H lysozyme was as strong as that between Glu35 and Asp52 in wild-type lysozyme, it would mostly cancel the decrease in pK_a of His35 due to the hydrophobic environment. However, this was not the case. Probably, water molecules in native lysozyme which tightly bridge Glu35 and Asp52 are no longer available in E35H lysozyme to bridge His35 and Asp52.

CONCLUSIONS

Chicken lysozyme was stabilized successfully by the mutation of Glu35, which has an abnormally high pK_a value, to undissociable residues (Gln or Ala) but not by the mutation of Glu35 to an oppositely ionizable residue (His). These observations are completely consistent with the fact that the environment around Glu35 is highly hydrophobic and therefore that the placement of either a positive or negative charge in such an environment leads to destabilization of lysozyme. These observations also indicate that the replacement of an acidic residue having an abnormally high pK_a or a basic residue having an abnormally low pK_a with an undissociable residue is a very efficient and general method for stabilization of a protein.

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Registry No. Glu, 56-86-0; His, 71-00-1; Gln, 56-85-9; Ala, 56-41-7; lysozyme, 9001-63-2.