Relationships between Conformational Stabilities and Surface Functional Properties of Mutant Hen Egg-White Lysozymes Constructed by Genetic Engineering

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A genetic engineering approach was introduced to elucidate the relationships between conformational stabilities and surface functional properties of hen egg-white lysozyme (HEWL). To obtain the mutants having different stabilities, amino acid replacements were carried out by site-directed mutagenesis in the cDNA of HEWL to remove the S-S bond between Cys 76 and Cys 94 (C94A) and to delete the salt linkage between Lys13 and Leu129 (K13D). The cDNAs of mutant lysozymes C94A and K13D were inserted into a yeast expression vector (PYG-100) and expressed in *Saccharomyces cerevisiae*. The mutant lysozymes were secreted in yeast medium in correctly processed forms. The lytic activities of mutant lysozyme C94A and K13D were 86% and 70% that of the wild-type enzyme with a slight shift to the acidic side of optimal pH. The conformational stabilities (estimated by ΔG) of lysozyme were significantly decreased by removing the S-S bond and deleting the salt linkage. The surface properties (foaming and emulsifying properties) of lysozyme were significantly enhanced by the removal of the S-S bond and the deletion of the salt linkage. These results indicate the importance of the conformational stability in the surface functional properties of proteins.

INTRODUCTION

We have reported that surface hydrophobicity (Kato and Nakai, 1980), conformational stability (Kato and Yutani, 1988), flexibility (Kato et al., 1985), and protein-protein interaction (Kato, 1991) are the structural factors governing the surface functional properties of proteins. Nevertheless, a clear-cut explanation of the relationship between the structural and functional properties of proteins remains to be found. To estimate the role of conformational parameters in the surface properties, the same protein should be used where only the conformational factors differ from one another. In this way, the influence of the structural factor on the surface properties may be precisely addressed. For this purpose, genetic engineering is one of the most promising approaches. Thus, attempts to elucidate the relationships between structural and functional properties of proteins have been done by using the genetic approach (Kato and Yutani, 1988; Utsumi et al., 1993).

Since it was reported that the correctly processed mature lysozyme was expressed in the culture medium from yeast carrying the lysozyme expression plasmid, HEWL has been studied extensively to assess the role of specific residues in the lytic activity (Kumagai and Miura, 1989) and to enhance the conformational stability by using protein engineering (Wilson et al., 1992). The construction of various mutants having different structural parameters, such as hydrophobicity, stability, and flexibility, is essential to elucidate the relationships between structural and functional properties of proteins. We have reported in a previous paper (Kato et al., 1992) that the surface functional properties of deamidated lysozyme constructed by genetic modification were significantly improved with the increases in the flexibility estimated by protease digestion, regardless of their conformational stabilities. Although the importance of conformational flexibility was suggested in the surface properties of lysozyme, the significance of the conformational stability also should be investigated. Thus, the construction of unstable lysozyme is desirable for evaluating the role of the stability in the surface properties. However, it is difficult to obtain unstable protein without the destruction of conformation. It was reported that human lysozyme was converted to unstable protein without loss of lytic activity by deleting the S-S bond between positions 77 and 95, although the same attempt was unsuccessful for three other S-S bonds (Taniyama et al., 1988). Therefore, the mutant protein with the S-S bond deleted between positions 76 and 94 in HEWL seemed to be promising for the experiments of structural and functional relationships. In addition, we found that the deletion of the salt linkage between the ϵ-amino group in Lys13 and the carboxyl group in C-terminal Leu129 greatly decreased the stability with a slight loss of activity. These mutant proteins were secreted in the culture medium of yeast in sufficient amounts to examine the conformational stability and surface properties. Therefore, it became possible to evaluate the relationships between conformational stability and surface functional properties of HEWL by using these mutant proteins.

MATERIALS AND METHODS

Materials. T4DNA ligase, alkaline phosphatase and restriction enzymes were purchased from Takara Shuzo Co. (Kyoto). The Takara 7-DEAZA sequence kit for sequencing and the Takara blunting kit for blunting were also purchased from Takara Shuzo Co. (Kyoto). The oligonucleotide-directed in vitro mutagenesis system (version 2) for site-directed mutagenesis and α-^{32}P dCTP (800 Ci/mmol) were purchased from Amersham. *Micrococcus lysodeikticus* cells for lysozyme assay were from Sigma, and CM-Toyopearl resin was a product of Toso (Tokyo). All other chemicals were of analytical grade of biochemical use.

Plasmids. The yeast expression plasmid, pYG-100, was supplied by Dr. K. Matsubara, Osaka University. The recombinant plasmid (pKK-1) containing 16 bp fo the 5'-noncoding region, 440 bp of the coding region, and about 120 bp of the 3'-noncoding region of hen prelysozyme cDNA in the same orientation as lacZ in pUC18 was supplied by Dr. I. Kumagai, University of Tokyo.

Oligonucleotide-Directed Mutagenesis of HEWL cDNA. The conversion of Cys94 and Lys13 codon to Ala and Asp, respectively, were carried out by site-directed mutagenesis with bacteriophage vector M13mp19. The *ECo*RI/*Hin*dIII fragment of the pKK-1 plasmid containing almost full-length cDNA encoding HEWL was subcloned into the *ECo*RI/*Hin*dIII site of

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bacteriophage vector M13mp19. The mutant HEWL cDNAs were constructed in the recombinant M13mp19 vector by the Amersham oligonucleotide-directed mutagenesis system (version 2). For the S–S bond deletion mutant C94A, a mutagenic oligonucleotide primer, 5′-AGCGTGAACGCCGCGAAGAAG-3′, was used to convert Cys94 (TGC) to the Ala (GCC) codon. For the salk linkage deletion mutant K13D, a mutagenic oligonucleotide primer, 5′-GCAGCTATGGACCGTCACGG-3′, was used to convert Lys13 (AAG) to Asp (GAC) codon. The DNA sequence of each mutant was confirmed by the dideoxy sequencing method (Sanger et al., 1977).

Construction of Expression Vector for HEWL in Yeast. The wild-type or mutant prelysozyme cDNA fragment was isolated by double digestion of M13mp19 with *Eco*RI/*Sal*I. The cDNA fragments were ligated to pYG-100, which had been digested with *Sal*I. The nonligated *Eco*RI/*Sal*I site in cDNA fragments and *Sal*I site in pYG-100 were blunt-ended by the blunt-end kit and self-ligated. The recombinant plasmid was propagated in *E. coli* JM 107 (*rec*A) in LB medium (1% Bacto tyrpton, 5% yeast extract, 0.5% NaCl). The expression plasmid, which had the cDNA insert located downstream of the GPD promoter in the correct orientation, was identified by a digestion analysis with restriction endonucleases, *Hin*dIII and *Bam*HI.

Yeast Transformation and Cultivation. The expression plasmid of HEWL was introduced in *S. cerevisiae* AH22 (*a, Leu2, His4, Cir*$^{+}$) according to the lithium acetate procedure (Itoh et al., 1983). Leu^{+} transformants were selected in yeast minimum medium plates supplemented with histidine (20 μg/mL) at 30 °C. Cultivation of the Leu^{+} transformants was then carried out. *S. cerevisiae* AH22 (*a, Leu2, His4, Cir*$^{+}$) carrying the lysozyme expression plasmid was inoculated into 3 mL of yeast minimum medium supplemented with histidine (20 μg/mL) in a 15-cm test tube and incubated with vigorous shaking for 2 days at 30 °C. This seed culture (2 mL) was subcultured into 100 mL of the same medium in a reciprocal shaking flask (500 mL) for another 2 days at 30 °C. A second seed culture (20 mL) was inoculated with 1 L of yeast minimum medium in a reciprocal shaking flask (3000 mL) for 2 days at 30 °C.

Purification of Wild and Mutant HEWL. Wild-type and mutant lysozymes secreted in the growth medium were purified in two steps by cation-exchange chromatography on CM-Toyopearl (Kumagai et al., 1987). The growth medium of *S. cerevisiae* AH22 (*a, Leu2, His4, Cir*$^{+}$) carrying the lysozyme expression plasmid cultivated for 2 days was centrifuged at 5000 rpm for 20 min at 4 °C and diluted with deionized water at least two times. The solution was directly applied to a CM-Toyopearl 650M opened column (12 × 90 mm) in 50 mM Tris-HCl buffer (pH 7.5), and the column was washed with the buffer until the washing solution was free from proteins. The adsorbed lysozyme was eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl. The protein solution was diluted with deionized water at least five times and again applied to the regenerated CM-Toyopearl 650M opened column (12 × 90 mm). The pure HEWL was eluted with a linear gradient of 0–0.5 M NaCl in 50 mM Tris-HCl buffer at pH 7.5.

Measurement of Lysozyme Activity. The lysozyme activity was measured by a turbidimetric assay. Suspensions of *M. lysodeikticus* cell (OD_{450} = 0.7) were prepared in 0.05 M citric acid–0.1 M sodium phosphate buffer at pH 4.0–8.0. A 100-μL lysozyme solution (37 μg/mL) was added to 2.4 mL of the cell suspension in each buffer. The initial decrease in the absorbance at 450 nm of the mixture caused by lysis of *M. lysodeikticus* cells was measured at 20 °C for 1 min. A decrease in the absorbance at 450 nm of 0.001/min is defined as one unit. The lytic activity was represented as the percentage to that of the wild-type lysozyme at pH 6.0.

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was conducted in 15% gels according to the method of Laemmli (1970). After electrophoresis, the gels were stained with 0.2% Coomassie brilliant blue-R250 and destained with 10% acetic acid containing 20% methanol.

CD Analysis. Thermal denaturation curves were drawn by monitoring the changes in ellipticity at 222 nm of the CD spectrum (Yutani et al., 1984). CD measurements were carried out with a JASCO J-500 recording spectropolarimeter at a protein concentration of 0.05 mg/mL in a 40 mM glycine–HCl buffer (pH 3.0) containing 0.05 M sodium chloride at 20 °C in a cell with a 10-mm path.

Determination of ΔG. The Gibbs free energy change (ΔG) was determined from the denaturation curves which were drawn by following the changes in the ellipticity at 222 nm during heating according to the method of Yutani et al. (1984). The temperature was controlled during all the measurements by circulating water in the cell holder from a thermostated bath with a heating rate of 1 °C/min from 30 to 82 °C. By using the data of thermal denaturation curves, the apparent fraction (f_{app}) of unfolding was represented as a function of temperature to show clearly the denaturation curves. Since the thermal denaturation of lysozymes at pH 3 were completely reversible, we can calculate the equilibrium constant between the native and the denatured forms by determining the apparent fraction of unfolding, f_{app}, from the denaturation curves by the eq 1. The unfolding enthalpy change

$$K = f_{app}/(1 - f_{app}) \quad (1)$$

(ΔH) was calculated by van't Hoff eq 2 as a function of the temperature near T_m, transition point of denaturation

$$\ln K_1/K_2 = -\Delta H^*/R(1/T_1 - 1/T_2) \quad (2)$$

where ΔH^* is enthalpy change at T_m, T_1 or T_2 is the temperature near T_m, K_1 or K_2 is the equilibrium constant at T_1 or T_2, respectively, and R is the gas constant. Entropy (ΔS) can be calculated from

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

Since ΔG is 0 at T_m, $\Delta S^* = \Delta H^*/T_m$, where ΔS^* is the entropy change at T_m. The Gibbs energy change (ΔG) of unfolding can be calculated by

$$\Delta G = \Delta H^* - T\Delta S^* + \Delta C_p[T - T_m - T \ln (T/T_m)] \quad (4)$$

where ΔC_p is denaturational increment of heat capcity and the value of lysozyme is 1.594 kcal/mol.

Measurement of Foaming Properties. The foaming properties of proteins were determined by measuring the electrical conductivity of the foams. Air was introduced through a glass filter (G-4) into 2 mL of a 0.1% protein solution in 0.05 M sodium phosphate buffer containing 0.05 M sodium chloride at pH 7.4 at a constant flow rate of 90 cm^3/min (Kato et al., 1983). When the air had been introduced into the protein solution for 15–30 s, the conductivity of foam reached the maximal value and the aeration was stopped. The change in the conductivity of foam was measured on a time course. The foaming power of proteins is expressed as the maximal conductivity of the foam produced during passing air through the solution for 15–30 s.

Measurement of Emulsifying Properties. The emulsifying activity of HEWL was determined by the method of Pearce and Kinsella (1978). To prepare the emulsion, 0.33 mL of corn oil and 1 mL of a 0.1% HEWL solution in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.05 M sodium chloride were homogenized in an Ultra Turrax homogenizer (Hansen and Co., Germany) at 12 000 rpm for 1 min at 20 °C. A 0.1-mL portion of the emulsion was taken from the bottom of the container after different time intervals and diluted with 5 mL of a 0.1% sodium dodecyl sulfate solution. The turbidity of the diluted emulsion was then measured at 500 nm. The emulsifying activity was determined from the absorbance measured immediately after emulsion formation.

RESULTS AND DISCUSSION

Although various mutant lysozymes were constructed, it could not obtain sufficient amounts of proteins for the experiments. Therefore, only two mutants C94A and K13D were used for the estimation of the role of conformational stability in the surface properties.

Figure 1 shows the SDS–PAGE patterns of wild-type and mutant lysozymes. The patterns revealed that the secreted mutant had the same molecular size as the authentic egg-white lysozyme. From the amino acid sequence analysis of the N-terminal region in the wild-type and mutant lysozymes, it was confirmed that they were correctly processed in yeast.

Table 1 shows the secreted amounts and lytic activities of mutant lysozymes C94A and K13D. The secreted

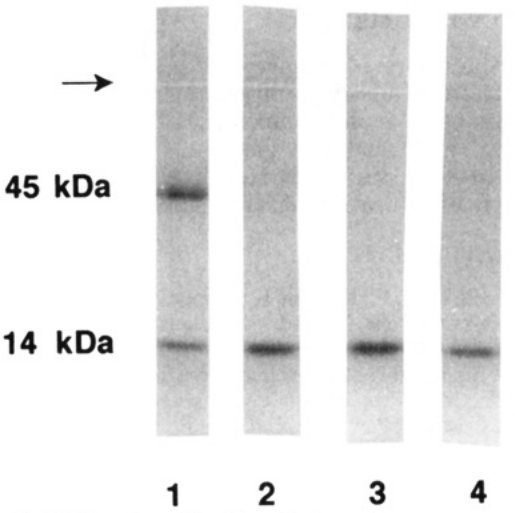

Figure 1. SDS–polyacrylamide gel electrophoretic patterns of wild-type and mutant lysozymes expressed in *S. cerevisiae*. Lane 1: ovalbumin (45 kDa) and authentic lysozyme (14 kDa). Lane 2: wild-type lysozymes. Lane 3: C94A. Lane 4: K13D.

Table 1. Secreted Amounts and Lytic Activities of Mutant Lysozymes

	secreted amounts (mg/L)	rel activity[a] (%)
wild	1.14	100
C94A	0.18	86
K13D	0.20	70

[a] The lytic activities of the mutant lysozymes at each optimal pH are represented as relative to that of wild-type lysozyme at pH 6.

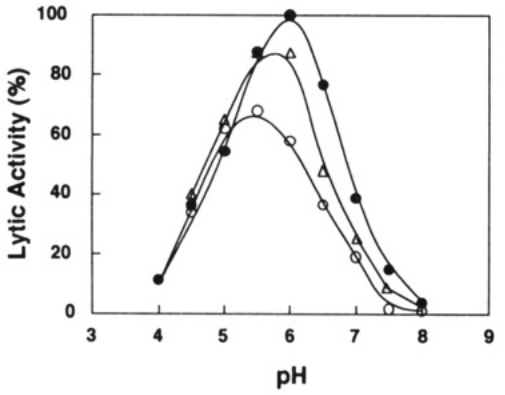

Figure 2. Dependence of lytic activity of wild-type (●), mutant C94A (△), and K13D (○) lysozymes on pH.

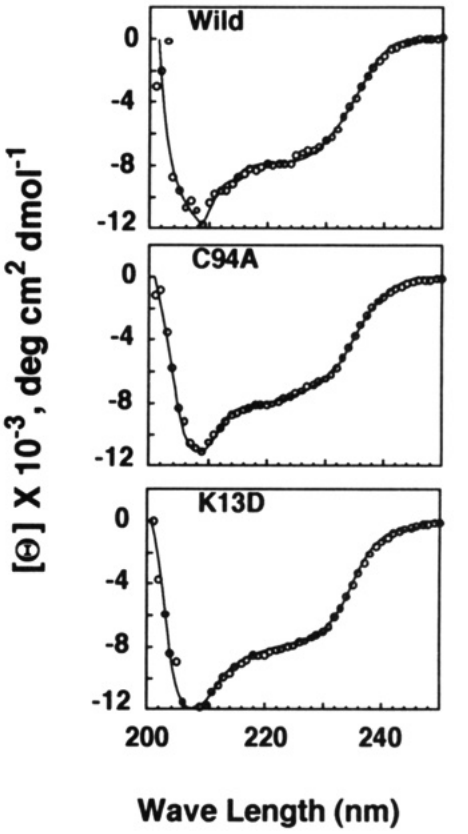

Figure 3. CD spectra of wild-type and mutant lysozymes.

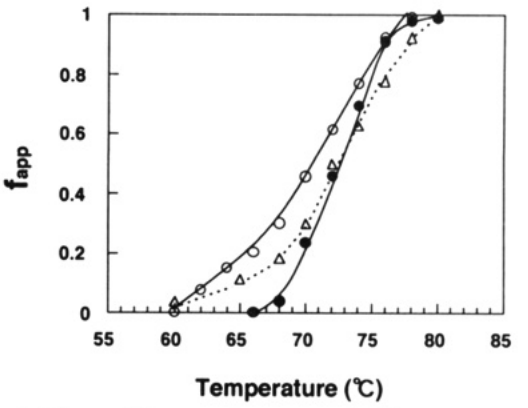

Figure 4. Thermal denaturation curves of wild-type (●), mutant C94A (△), and K13D (○) lysozymes.

amount of mutant lysozymes was lower than that of wild-type lysozyme. The wild-type lysozyme secreted from yeast showed the same lytic activity as the enzyme from hen egg-white. The lytic activities of C94A and K13D lowered to 86 and 70% of that of wild-type lysozyme, respectively, suggesting slight changes in tertiary structure.

Figure 2 shows the pH–activity profile of wild type and mutant lysozymes. The optimal pH of mutant lysozyme K13D was shifted to a lower side. The shift in K13D mutant may be attributed to the increase in the negative charge on the molecular surface. The electrostatic interaction between positively charged lysozyme and negatively charged *M. lysodeikticus* may be shifted to the acidic side because of the change of isoelectric point in the mutant lysozyme. The result is consistent with that of the deamidated lysozyme reported in a previous paper (Kato et al., 1992). On the other hand, the optimal pH of C94A mutant lysozyme was almost the same as that of wild-type lysozyme.

As shown in Figure 3, CD spectra of mutant lysozymes were the same as wild type protein, indicating that there are no significant changes in the secondary structure by these single amino acid substitutions. However, the conformational stability of the lysozyme was significantly decreased by the deletion of the S–S bond and salt linkage, as described below. The conformational stability was estimated by the denaturation curves which were drawn by following the changes in the ellipticity at 222 nm during heating, as shown in Figure 4. The mutant C96A and K13A become sensitive to heat denaturation, and the transition points of denaturation shift to lower temperature. To obtain a quantitative measure of the conformational stability of proteins, we estimated the Gibbs energy change (ΔG) of denaturation at pH 3 where the denaturation was reversible. The thermodynamic parameters were calculated from the curves using the equations mentioned above (Table 2). The ΔG for the S–S removed and salt linkage–deleted mutants greatly decreased at the room temperature. It is reasonable that the deletion of one bond of four S–S bonds in lysozyme causes unstable conformation. It is interesting that the deletion of the salt linkage between ϵ-amino group in Lys

Table 2. Thermodynamic Parameters of Mutant Lysozymes[a]

	T_m (°C)	ΔH_m (kcal)	ΔS_m (cal)	ΔG (kcal)
wild	72.2	127	368	12.6
C94A	71.8	93	270	7.8
K13D	70.5	81	237	5.9

[a] The transition temperature (T_m) was determined from the denaturation curves in Figure 4. ΔH_m and ΔS_m indicate the enthalpy and entropy changes at T_m, respectively. ΔG represents the Gibbs free energy change of unfolding at 20 °C.

Table 3. Surface Properties of Wild and Mutant Lysozymes

	foaming power (μv/cm)	emulsifying activity (OD_{500})	emulsion stability (min)
wild	250	0.157	0.5
C94A	540	0.180	2
K13D	790	0.190	3

13 and carboxyl group in C-terminal Leu 129 resulted in a more unstable conformation than the removal of the S–S bond between positions 94 and 76. It is probable that the stability of the helix region along positions 5–15 of the amino acid residues may be affected by the substitution of Lys 13 with Glu, in addition to the cleavage of the salt linakge, because the dipole moment of the helix region (5–15) is decreased by the negative charge at position 13. The thermal denaturation curve of wild-type lysozyme showed monophasic transition and sharp cooperativity, while that of C94A and K13A mutants revealed biphasic transition and broad cooperativity. This may be attributed to the destabilization of tertiary structure due to the removal (or deletion) of S–S bond and salt linkage, thereby resulting the domain sensitive to thermal denaturation at a lower temperature.

Further, the thermal denaturation curves monitored by lytic activity were also drawn (data not shown). Although the lytic activity was used as an indicator of the conformational changes, the thermal denaturation curves were almost the same as Figure 4. This suggests that the thermal denaturation curves drawn by following changes in CD at 222 nm reflect the changes not only in the secondary structure but also in the tertiary structure.

Thus, relationships between structural and surface functional properties of lysozyme were investigated by using these mutants. The foaming and emulsifying properties of mutant lysozymes are shown in Table 3. The foaming power of the mutant C94A and K13D was greatly enhanced, and their emulsion stability was also significantly increased. These surface properties of the mutant lysozymes were well related with the decrease in their ΔG. It is apparent that the more unstable the lysozymes, the greater the increase in the foaming and emulsifying properties. Thus, the relationships between the structural and surface functional properties of lysozyme can be precisely estimated by using a genetic engineering approach. The exact correlation should be estimated by using a number of samples. Therefore, we are further constructing other mutant lysozymes to further investigate on the relationship between structural and functional properties.

In conclusion, a genetic engineering approach was useful to elucidate the relationships between structural and surface functional properties. The surface functional properties of lysozyme were significantly increased in proportion to decreases in the conformational stabilities by the deletion of the S–S bond (C94A) and salt linkage (K13A). Thus, it was confirmed that the conformational stability was an important structural factor for the surface functional properties of proteins.

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