Enthalpic Destabilization of a Mutant Human Lysozyme Lacking a Disulfide Bridge between Cysteine-77 and Cysteine-95

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ABSTRACT: To understand the role of disulfide bridges in protein stability, the thermodynamic changes in the denaturation of two mutant human lysozymes lacking a disulfide bridge between Cys-77 and Cys-95 (C77A and C77/95A) were analyzed using differential scanning calorimetry (DSC). At pH 3.0 and 57 °C, the stabilities of both the C77A and C77/95A mutants were decreased about 4.6 kcal·mol^{-1} in Gibbs free energy change. Under the same conditions, the enthalpy changes (ΔH) were 94.8 and 90.8 kcal·mol^{-1}, respectively, which were smaller than that of the wild type (100.8 kcal·mol^{-1}). The destabilization of the mutants was caused by enthalpic factors. Although X-ray crystallography indicated that the mutants preserve the wild-type tertiary structure, removal of the disulfide bridge increased the flexibility of the native state of the mutants. This was indicated both by an increase in the crystallographic thermal factors (*B*-factors) and by a decrease in the affinity of *N*-acetylglucosamine trimer [$(NAG)_3$] observed using isothermal titration calorimetry (DTC) due to entropic effects. Thus, the effect of cross-linking on the stability of a protein is not solely explained by the entropy change in denaturation.

It is known that the disulfide bridge is a natural cross-link observed in proteins and is considered to stabilize a protein by reducing the entropy of the denatured state (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965). Does the introduction or removal of a cross-link really affect the stability of a protein due to the change in chain entropy in the denatured state? In spite of the importance of this theme in questions of protein stability, it seems that there is no direct experimental proof for it. Although there are many reports describing the introduction of disulfide bridges in proteins (Perry & Wetzel, 1984, 1986; Villafranca et al., 1987; Wetzel et al., 1988; Matsumura et al., 1989a,b; Michinson & Wells, 1989), the introduction of disulfide cross-links did not always effectively stabilize proteins. The only way to clarify the effect of cross-linking on the stability of a protein is to investigate the change of thermodynamic parameters accompanied with the denaturation of a protein by using a high-sensitivity differential scanning calorimeter (DSC).[1] Differential scanning calorimetry enables us to obtain directly accurate estimates of the parameters of thermodynamic change, i.e., the denaturation temperature (T_d), the enthalpy change (ΔH), and the heat capacity change (ΔC_p) for the denaturation of a protein (Privalov & Khechinashvili, 1974). From these values, all of the thermodynamic parameters in the denaturation of a protein at a given temperature (T, °C) can be calculated using the following equations, which in turn allow one to compare the stability of proteins from the thermodynamic point of view:

$$\Delta H(T) = \Delta H(T_d) - \Delta C_p(T_d - T) \quad (1)$$

$$\Delta S(T) = \Delta H(T_d)/T_d - \Delta C_p \ln (T_d/T) \quad (2)$$

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

In the above, T_d is the denaturation temperature, and the ΔC_p values are assumed not to depend on temperature (Privalov & Khechinashvili, 1974).

For a variety of reasons, we have chosen to study the effect of the removal of the disulfide bridge between Cys-77 and Cys-95 upon the stability of human lysozyme. First, the mutants lacking this disulfide bridge have already been found to be expressed and secreted effectively (Taniyama et al., 1988, 1990). Second, the tertiary structures of the wild-type, C77/95A (Inaka et al., 1991a), and C77A mutants (reported in this paper) are available for detailed study. Third, lysozyme is one of the most thoroughly investigated proteins (via calorimetry) because of its high reversibility against heat denaturation (Privalov & Khechinashvili, 1974; Segawa et al., 1989). In this paper, we show that the destabilization due to the removal of the disulfide bridge cannot always be explained by the entropic effect alone. Some disulfide bridges, like a disulfide between Cys-77 and Cys-95 in human lysozyme, act to keep a protein molecule folded tightly. The removal of these disulfide bridges might make the protein conformation more flexible and affect the thermodynamic character in the native state in addition to affecting the denatured state.

MATERIALS AND METHODS

Materials. Human lysozyme from urine was purchased from Green Cross Corp. (Osaka, Japan). The mutant human lysozymes (C77A and C77/95A) lacking a disulfide bridge

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[1] Abbreviations: C77/95A, mutant human lysozyme in which both Cys-77 and Cys-95 were replaced with alanines; C77A, mutant human lysozyme in which Cys-77 was replaced with an alanine; DSC, differential scanning calorimetry; $(NAG)_3$, β-1,4-linked trimer of *N*-acetyl-D-glucosamine.

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between Cys-77 and Cys-95 were obtained as described previously (Taniyama et al., 1988, 1990), in which the mutant (C77A) was described as C77A-b. Trimer of *N*-acetyl-D-glucosamine [$(NAG)_3$] was purchased from Seikagaku Kogyo (Tokyo, Japan). All other chemicals were of reagent-grade quality.

Protein Concentrations. Concentrations of the protein solution were determined spectrophotometrically using $E^{1\%}_{280nm}$ = 25.6 for human lysozyme (Parry et al., 1969) and its mutants.

Differential Scanning Calorimetry (DSC). DSC measurements were carried out with a DASM-4 microcalorimeter at a scan rate of 1.0 K/min. This system is the same as that reported previously (Yutani et al., 1991). The wild-type and mutant human lysozymes were dialyzed against distilled water exhaustively, and lyophilized for storage. Sample solutions for DSC measurements between pH 4.0 and 4.5 were prepared by dissolving the lysozyme in 0.05 M sodium acetate buffer; solutions between pH 2.0 and 3.5 were prepared using 0.05 M glycine hydrochloride. The pH of the sample solution was confirmed both before and after the measurement. Lysozyme concentrations used were 1.0–1.5 mg/mL. Calorimetric and van't Hoff enthalpies (ΔH_{cal} and ΔH_{vH}) were calculated by a computer program developed with Kidokoro and Wada (1987).

Binding Isothermal Calorimetry. Isothermal calorimetry for the determination of the enthalpy change in the binding of $(NAG)_3$ to the wild-type and mutant human lysozymes was performed using a Micro Cal OMEGA titration calorimeter (Wiseman et al., 1989). One milligram of sample was dissolved in 2 mL of 0.05 M sodium acetate buffer (pH 4.5), and 1.7 mL of this solution was injected into the cell and titrated with the same buffer containing 5 mM $(NAG)_3$ at 40 °C. Calorimetric enthalpies (ΔH_b), the binding constant (K_b), and the number of bound $(NAG)_3$ were calculated using the computer program ORIGIN (Micro Cal Inc.).

X-ray Crystallography. Crystallization, X-ray diffraction data collection, and structure refinements were performed as described previously (Inaka et al., 1991a,b). Crystals of C77A lysozyme were grown from 30 mM phosphate buffer (pH 6.0) containing 20 mg/mL protein and 2.5 M NaCl. After a month, the crystals grew up to 0.5 mm on an edge.

RESULTS

Thermal Denaturation of the Wild-Type and Mutant Human Lysozymes. Differential scanning calorimetry was performed to investigate the temperature dependencies of the thermodynamic parameters in the denaturation of the mutant human lysozymes. The particular pH region used was chosen because a high reversibility of the denaturation of the mutant human lysozyme (more than 90%) can be observed over that range (from pH 2.3 to 4.5 for the wild-type and C77/95A lysozymes and from pH 2.3 to 3.0 for C77A lysozyme). In the case of the C77A lysozyme, measurement over pH 3.0 could not be performed because the reversibility decreased due to the free cysteine remaining at position 95. For comparison with these newer results, previous data on the thermal stability of the wild-type human lysozyme (Kuroki et al., 1992) were also used in this paper. The typical excess heat capacity curves of the wild-type, C77A, and C77/95A lysozymes at pH 3.0 are shown in Figure 1. The calorimetric enthalpies (ΔH_{cal}) and van't Hoff enthalpies (ΔH_{vH}) and the heat capacity changes (ΔC_p) in the denaturation were obtained directly from analysis of these curves. In each case, the denaturation transition gave a single peak, the top of which

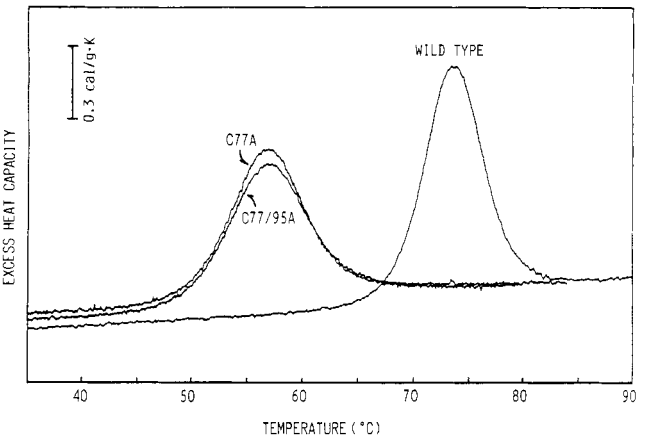

FIGURE 1: Typical excess heat capacity curves of the two mutant lysozymes, C77A and C77/95A, and the wild-type human lysozyme at pH 3.0.

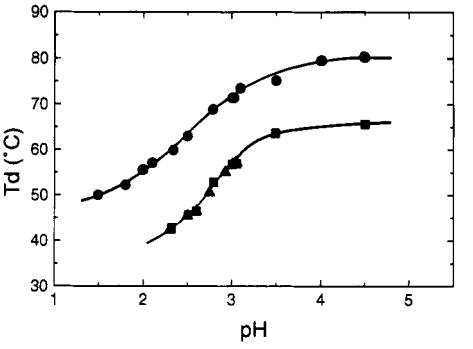

FIGURE 2: Denaturation temperatures of the wild-type (●), C77A (▲), and C77/95A (■) lysozymes at various pHs.

defines the denatured temperature (T_d). This definition of T_d only leads to an inaccuracy in determining ΔH_{cal} by no more than 2% (Privalov & Khechinashvili, 1974). The T_d values for C77A and C77/95A at pH 3.0 were 56.9 and 57.1 °C, respectively, which is about 14 °C less stable than the wild type (T_d = 71.4 °C). This destabilization was observed in all pH regions measured (Figure 2). The ΔH_{cal} values at 57 °C and pH 3.0 of C77A and C77/95A lysozymes were determined to be 95.1 and 91.9 kcal·mol^{-1}, respectively (Table I), which are again smaller than those of the wild type over the entire temperature region. In Figure 3, the temperature dependencies of ΔH_{cal} for the wild-type, C77A, and C77/95A lysozymes are shown. The least-squares fits of ΔH_{cal} values as a function of denaturation temperature yielded straight lines with slopes of 1.55 kcal·mol^{-1}·K^{-1} for the wild-type (line 1), 1.74 kcal·mol^{-1}·K^{-1} for C77A (line 2), and 1.40 kcal·mol^{-1}·K^{-1} for C77/95A (line 3). The average value of the ratio of $\Delta H_{cal}/\Delta H_{vH}$ for both C77A and C77/95A lysozymes was 0.95 ± 0.02, similar to that of the wild type, indicating a two-state denaturation (Kidokoro & Wada, 1987). The average values of ΔC_p obtained from each excess heat capacity curve of the C77A and C77/95A lysozymes were 1.55 ± 0.22 and 1.50 ± 0.19 kcal·mol^{-1}·K^{-1}, respectively, which were same as that of the wild type. These values were coincident with the ΔC_p values (1.74 kcal·mol^{-1}·K^{-1} for C77A and 1.40 kcal·mol^{-1}·K^{-1} for C77/95A) obtained from the slope after least-squares fitting of the temperature dependence of ΔH_{cal}.

X-ray Structure of Mutant Human Lysozyme (C77A). The three-dimensional structure of the mutant human lysozyme (C77A) was analyzed at 1.8-Å resolution by X-ray crystallography and compared to those of the wild type and mutant C77/95A, which were reported previously (Inaka et al.,

Table I: Thermodynamic Parameters for the Unfolding of the Mutant Human Lysozymes (C77A and C77/95A)

lysozyme	pH	T_d (°C)	ΔH_{cal} (kcal·mol^{-1})	ΔH_{vH} (kcal·mol^{-1})	ratio	ΔC_p (kcal·mol^{-1}·K^{-1})
C77A	2.30	42.3	68.5	72.5	0.94	1.90
	2.50	45.4	74.4	79.1	0.94	1.61
	2.60	46.4	77.0	82.3	0.94	1.48
	2.75	50.5	84.4	89.8	0.94	1.63
	2.93	55.0	90.7	91.6	0.99	1.13
	3.01	56.8	95.1	102.1	0.93	1.48
	3.06	57.1	94.1	100.0	0.94	1.62
					av 0.95 ± 0.02	1.55 ● 0.22
C77/95A	2.32	42.7	69.9	76.3	0.92	1.68
	2.51	45.6	74.0	75.6	0.98	1.84
	2.60	46.4	76.6	81.7	0.94	1.50
	2.80	52.8	86.5	91.2	0.95	1.48
	3.01	56.7	91.9	96.9	0.95	1.19
	3.06	56.9	91.2	98.6	0.92	1.35
	3.49	63.6	99.9	107.5	0.93	1.39
	4.50	65.6	101.7	102.7	0.99	1.58
					av 0.95 ± 0.02	1.50 ± 0.19

Table II: Crystallographic Data Processing and Refinement Statistics of the Mutant Human Lysozyme (C77A)

Crystal Data and X-ray Data Processing Statistics	
space group	$P2_12_12_1$
cell constants (Å)	
a	56.82
b	60.86
c	33.33
resolution (Å)	∞–1.76
no. of measured reflections	40579
no. of independent reflections	11258
av R_{symm}[a]	3.21
R_{merge}[b]	5.56
Final Refinement Parameters	
no. of atoms	1073
no. of solvent atoms	57
resolution range (Å)	5.0–1.80
no. of used reflections	10265
$\langle \|F_o\| - \|F_c\| \rangle$	27.74
R-factor[c] (final)	0.188

[a] R_{symm} gives the agreement between symmetry-related intensities on the same file. [b] $R_{merge} = (\Sigma|I - \langle I \rangle|)/(\Sigma|\langle I \rangle|)100$. [c] *R*-factor = $(\Sigma||F_o| - |F_c||)/\Sigma|F_o|$.

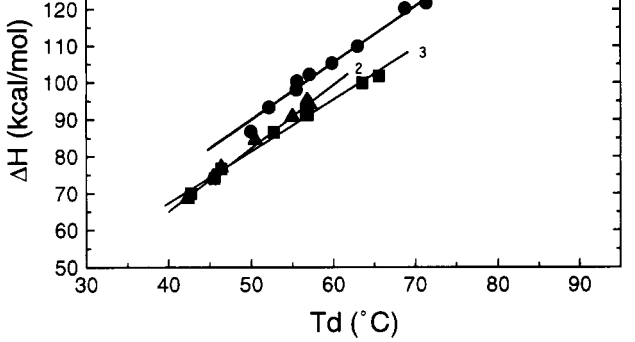

FIGURE 3: Temperature dependencies of the enthalpy change for the denaturation of the wild-type (●), C77A (▲), and C77/95A (■) lysozymes. Lines were obtained by a least-squares fit of ΔH_{cal} values as a function of temperature for the wild-type (line 1), C77A (line 2), and C77/95A (line 3) lysozymes.

1991a). Data collection and refinement statistics for C77A are summarized in Table II. The overall structure of C77A is quite similar to that of the wild type and C77/95A. The root-mean-square (rms) deviation between the wild type and C77A for the backbone atoms (N, Cα, C, and O) is 0.167 Å, which is quite similar to that observed between the wild type and C77/95A (0.141 Å). The structures in the vicinity of Cys-77 and Cys-95 are also shown in Figure 4. The differences observed were the disappearance of the sulfur atoms in the structures of C77A and C77/95A. The plots of the average thermal factors (*B*-factor) of the main chain atoms versus each residue for the wild-type, C77A, and C77/95A lysozymes are shown in Figure 5. All *B*-factors for C77A were predominantly larger than those of the wild type. Moreover, the *B*-factors for the region around Cys-77 are especially larger than those of the wild type. These findings are also similar to that of C77/95A as reported previously (Inaka et al., 1991a).

Enthalpy Change in the Binding of $(NAG)_3$ to the Wild-Type and Both Mutant Human Lysozymes (C77A, C77/95A). One way to quantitatively investigate the change occurring in the native state of the proteins is to compare the thermodynamic parameters for the binding of substrate analogue to them. A titration calorimeter (OMEGA) was used to obtain the thermodynamic parameters for the binding of substrate analogue [$(NAG)_3$] to the wild type and mutants at 40 °C and pH 4.5. The typical titration process of the wild type is shown in Figure 6. The binding constant (K_a) and the enthalpy change (ΔH_a) for the binding of $(NAG)_3$ (listed in Table III) are obtained by an analysis of this curve. The K_a values for binding of $(NAG)_3$ to C77A and C77/95A were 3.80×10^4 and 4.02×10^4 M^{-1}, respectively, which were smaller than that of the wild type ($K_a = 9.26 \times 10^4$ M^{-1}). The ΔH_a values ($\Delta H_a = -12.5$ kcal·mol^{-1} for C77A and −12.8 kcal·mol^{-1} for C77/95A) were about 2 kcal·mol^{-1} more favorable for $(NAG)_3$ binding to the mutants compared with that of the wild type ($\Delta H_a = -10.3$ kcal·mol^{-1}).

DISCUSSION

In order to fully understand the effect of cross-linking on the conformational stability of a protein, it is necessary to elucidate the thermodynamic change in denaturation. The thermodynamic parameters for the denaturation [$\Delta H(T)$, $T\Delta S$, and $\Delta G(T)$] of the C77A and C77/95A lysozymes at 57 °C were obtained according to eq 1–3 using the data listed in Table I. The ΔC_p values obtained from the slope of the temperature dependency of ΔH_{cal} were also used for calculation. At this temperature (57 °C), the accuracy of the ΔC_p values is not critical for extrapolation, since the ΔH values of all three lysozymes (the wild-type, C77A, and C77/95A) were observed experimentally. The data for denaturation of the wild-type human lysozyme were taken from previous results (Kuroki et al., 1992). The parameters obtained are listed in

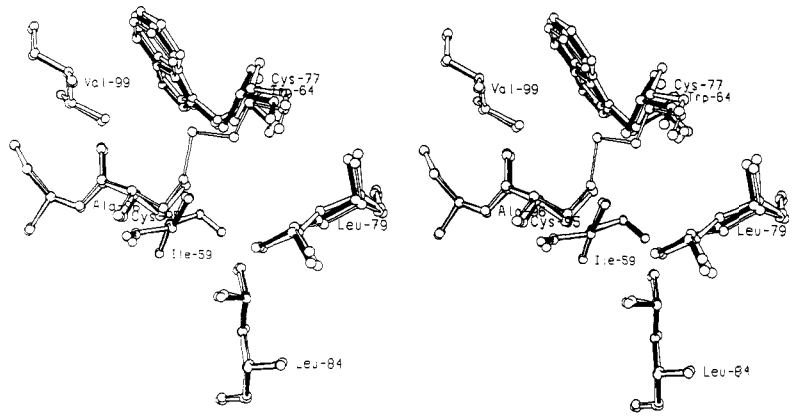

FIGURE 4: Stereo drawing of the refined structures around Cys-77 and Cys-95 in the wild-type (shown in thin open bonds), C77A (thick open bonds), and C77/95A (closed bonds) lysozymes after least-squares fit of the main chain atoms from each structure.

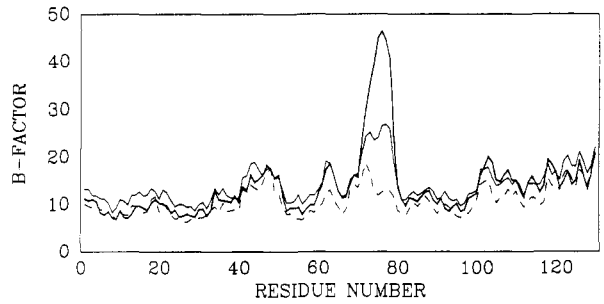

FIGURE 5: *B*-Factors averaged for the main chain atoms versus residue number for the wild-type (dashed curve), C77A (thick solid curve), and C77/95A (thin solid curve) lysozymes.

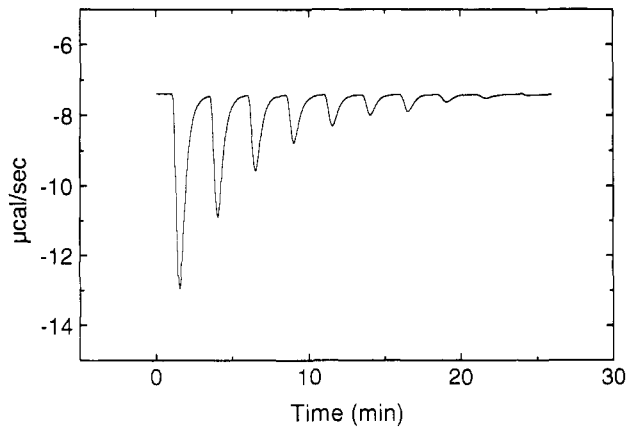

FIGURE 6: Typical calorimetric titration of wild-type human lysozyme (7.0×10^{-5} M) with 10 mM $(NAG)_3$ solution at pH 4.5 and 40 °C.

Table IV. The ΔG values for both C77A and C77/95A are 4.6 kcal·mol^{-1} less stable than the wild type at 57 °C. These values are not affected by the number of cysteines replaced with alanines. The thermodynamic contributions of ΔH and $T\Delta S$ to the stability (ΔG) of the wild-type, C77A, and C77/95A lysozymes are shown in Figure 7a,c,d. In this figure, the contributions of the ΔH and $T\Delta S$ terms to the stability at 57 °C are represented by the left and right rectangles, respectively. ΔG is indicated under the rectangles showing the ΔH and $T\Delta S$. The ΔH values for the denaturation of both C77A (94.8 kcal·mol^{-1}) and C77/95A (90.8 kcal·mol^{-1}) are smaller than that of the wild type (100.6 kcal·mol^{-1}). In addition, the entropy terms ($T\Delta S$) in the denaturation of C77A (94.8 kcal·mol^{-1}) and C77/95A (90.8 kcal·mol^{-1}) are also smaller than that of the wild type (96.0 kcal·mol^{-1}). These evaluations indicate that the major term destabilizing both mutants is the enthalpy term. The entropy term seems to compensate for the enthalpic destabilization of the mutant human lsyozymes.

The increase of the stability (ΔG) of a protein induced by introducing cross-links has been estimated from the entropy change in the denatured state (Flory, 1956; Poland & Scheraga, 1965; Lin et al., 1984; Pace et al., 1988). Indeed, the effect of cross-linking on the stability (ΔG) of lysozyme (Johnson et al., 1978; Ueda et al., 1985), RNase A (Lin et al., 1984), C_L (λ) and $C_L(\kappa)$ (Goto et al., 1987), and RNase T_1 (Pace et al., 1988) was explained by the estimation of the entropy change in the denatured state. According to Lin et al. (1984), the increase in entropy in the denatured state due to the removal of a disulfide bridge between Cys-77 and Cys-95 can be calculated to be about 4.9 kcal·mol^{-1} (Figure 7). Most of the destabilization of ΔG from the removal of the disulfide bridge (4.6 kcal·mol^{-1} at 57 °C) seems to correspond to the increase in entropy in the denatured state from the theoretical calculation. An increase in entropy from the removal of the disulfide bridge was expected to occur, but the thermodynamic parameters observed by calorimetry did not show such an increase. It should be considered, then, that there are other factors affecting the thermodynamic parameters in the denaturation of the mutants, which we will consider below.

One possibility concerns the difference of the hydration effect after the replacement of a cysteine with an alanine in C77A and C77/95A. However, this effect can be rejected as follows: According to Oobatake et al. (1988), the hydration effect of an amino acid can be calculated to be $\Delta G_h = -0.10$ kcal·mol^{-1} and $\Delta H_h = -1.78$ kcal·mol^{-1} for alanine and $\Delta G_h = -1.14$ kcal·mol^{-1} and $\Delta H_h = -3.43$ kcal·mol^{-1} for cysteine at 57 °C. It is also known that the hydration effect involves the accessible surface area of each residue (Eisenberg & McLachlan, 1986; Ooi et al., 1987). X-ray crystallographic result of the wild type and mutants showed that the side chains of Cys-77 and Cys-95 are not solvent-exposed in the X-ray structure. Therefore, it can be estimated that the substitution of a cysteine by an alanine should stabilize the mutant at most by 1.0 kcal·mol^{-1} in the ΔG of denaturation and increase the ΔH of denaturation by 1.7 kcal·mol^{-1} per one substitution of a cysteine to an alanine. The replacement may slightly affect the thermodynamic parameters of denaturation of the mutant lysozymes, but the direction of the contribution is different from the changes experimentally observed in the denaturation.

The second factor in explaining the discrepancy involves the thermodynamic character of the native state. The present results using calorimetry include the overall change occurring both in the native and in the denatured states. Therefore, to decrease both the change in enthalpy and the change in entropy for denaturation, the entropy of the native conformation must

Table III: Thermodynamic Parameters for the Binding of the Trimer of *N*-Acetylglucosamine, $(NAG)_3$, to the Wild-Type and Mutant Human Lysozymes (C77A and C77/95A)

lysozyme	pH	temp (°C)	n	K_a (M^{-1})	ΔG (kcal·mol^{-1})	ΔH (kcal·mol^{-1})	$T\Delta S$ (kcal·mol^{-1})
wild type	4.50	40.0	1.05 ± 0.02	9260 ± 179	−5.7	−10.5 ± 0.3	−4.8
C77A	4.50	40.5	1.02 ± 0.02	3800 ± 118	−5.1	−12.0 ± 0.8	−6.9
C77/95A	4.50	40.0	1.01 ± 0.02	4020 ± 85	−5.2	−12.4 ± 0.6	−7.2

Table IV: Thermodynamic Parameters for the Denaturation of the Wild-Type and Mutant Human Lysozymes (C77A and C77/95A) at 57 °C

lysozyme	T_d (°C)	ΔH^a (kcal·mol^{-1})	$T\Delta S^a$ (kcal·mol^{-1})	ΔG^a (kcal·mol^{-1})
wild type	71.4	100.6	96.0	4.6
C77A	57.1	94.8	94.8	0.0
C77/95A	56.9	90.8	90.8	0.0

[a] These values are extrapolated to 57.0 °C by eq 1–3.

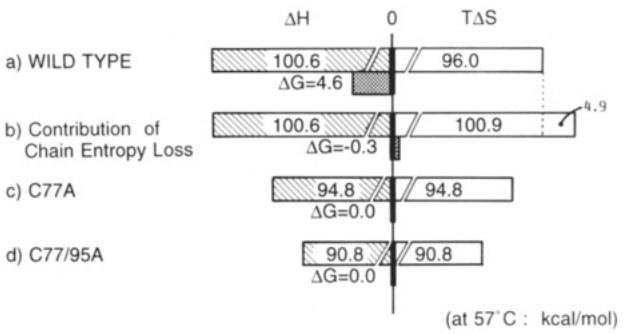

FIGURE 7: Histogram showing the thermodynamic change experimentally observed during the unfolding of the wild-type (a), C77A (c), and C77/95A (d) lysozymes at 57 °C. The hatched rectangles to the left indicate the contribution of the enthalpy for unfolding; the blank rectangles to the right show the entropic contribution for unfolding. The Gibbs free energy for unfolding is represented by a stippled retangle. The increase of entropy in the denatured state due to the removal of a disulfide bridge (4.9 kcal·mol^{-1}) represented in (b) was calculated according to Lin et al. (1984).

be increased much more than the increase in the entropy of denatured conformation. Therefore, it is necessary to investigate the change in thermodynamic character of the native state due to removal of the disulfide bridge.

X-ray crystallography is one of the most powerful methods to obtain the structural information for the native state. The results of X-ray crystallography showed that the tertiary structure of the C77A lysozyme was quite similar to those of the wild-type and C77/95A lysozymes reported previously (Inaka et al., 1991a). A disappearance of the sulfur atoms in both Cys-77 and Cys-95 was also observed. Such a packing defect at the positions of the sulfur atoms was also observed in the X-ray structure of the C77/95A lysozyme (Inaka et al., 1991a; see Figure 4). The sulfur atoms constructing the disulfide bridge between Cys-77 and Cys-95 make contacts with the side chains of Phe-57, Ile-59, Trp-64, Leu-79, Leu-84, Val-99, and Trp-109. The disappearance of the van der Waals contact between each sulfur atom and these residues should enthalpically destabilize the native conformation of human lysozyme.

As shown in Figure 5, an increase in *B*-factors throughout the molecule was observed in C77A and C77/95A; this was especially pronounced in the region from residues 70 to 80. This increase indicates that the native conformation of the mutant lysozymes became more flexible after the cleavage of the disulfide bridge. Such effects should contribute to decrease the $T\Delta S$ term of denaturation of the mutants, and may be also expected to accompany the decrease of the ΔH of denaturation due to the loss of contacts. These effects are in good agreement with the experimental results in that the ΔH and $T\Delta S$ values for denaturation of the mutants were decreased (Table IV).

The structural and enzymatic characteristics of the mutant human lysozymes are similar to those of the wild type (Taniyama et al., 1988, 1990; Inaka et al., 1991a). However, if there are significant changes occurring in the native state, these changes should affect thermodynamic parameters for the binding of substrate analogues such as $(NAG)_3$. The ΔH values for the binding of $(NAG)_3$ to both mutants (as obtained from isothermal calorimetry) were found to be about 2 kcal·mol^{-1} smaller than that of the wild type. This indicates that the enthalpy change for the binding of $(NAG)_3$ is favorable for binding to mutant lysozymes. Nonetheless, the binding constant (K) for the binding of $(NAG)_3$ to the mutant lysozymes decreased to about half of the wild-type value. Therefore, the entropy term worked unfavorably for the binding of $(NAG)_3$ to the mutants as if the binding of $(NAG)_3$ restricted the fluctuation of their respective native conformations. These findings suggest that the native conformations of the mutant lysozymes (C77A and C77/95A) are more flexible than that of the wild type due to the removal of the disulfide bridge. It is considered that the disulfide bridge between Cys-77 and Cys-95 not only suppresses the conformation of the denatured state but also tightly maintains the folded conformation.

Recently, Doig and Williams (1991) have reported that cross-links destabilize folded structures entropically but stabilize them enthalpically to a greater extent. In their calculation, cross-links decrease the hydrophobic effect by reducing the exposure of nonpolar side chains to water in the denatured state. The removal of the disulfide bridge, therefore, should result in a decrease of both the ΔH and $T\Delta S$ values for denaturation (due to increasing the exposure of nonpolar groups in the denatured state). Although the thermodynamic data presented here from DSC measurements for the mutant proteins do not contradict their conclusion, the changes in the thermodynamic characteristics of the mutant proteins in the native state are so significant that we cannot ignore the increase in the conformational flexibility in the native state of the mutants. Such effects should result in a decrease of the $T\Delta S$ term of denaturation of the mutants, and also be accompanied by a decrease of the ΔH of denaturation due to the loss of contacts. Using both calorimetry and X-ray crystallography has enabled us to identify significant changes of the thermodynamic character in the native state due to removal of the disulfide bond. Thus, it is concluded that removal of a disulfide bridge may significantly affect the thermodynamic character in the native state because some disulfide bridges have an important role in tightly maintaining tertiary structure. The effect of cross-linking on the stability of a protein cannot be explained simply as being due only to the entropy change in denaturation.

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