An Intramolecular Cross-Linkage of Lysozyme. Formation of Cross-Links between Lysine-1 and Histidine-15 with Bis(bromoacetamide) Derivatives by a Two-Stage Reaction Procedure and Properties of the Resulting Derivatives

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ABSTRACT: Hen egg white lysozyme was treated at pH 5.5 with four bifunctional reagents, bis(bromoacetamide) derivatives [$BrCH_2CONH(CH_2)_nNHCOCH_2Br$, **1**-*n*, *n* = 0, 2, 4, and 6], to alkylate His-15 monofunctionally. The excess bifunctional reagent was then removed, and the pH was raised to 9.0 to allow the other end of the reagent molecule to react. The shortest reagent (**1**-0) gave no intramolecularly cross-linked lysozyme derivative but only histidine-15-modified lysozyme monomer and intermolecularly cross-linked lysozyme dimer. However, the reagents with longer arms (**1**-2, **1**-4, and **1**-6) gave lysozyme derivatives cross-linked intramolecularly between the nitrogen at ϵ_2 of His-15 and the ϵ-amino group of Lys-1 without formation of any other intramolecularly cross-linked lysozyme derivative. These results are consistent with our previous proposal that lysozyme has a small hydrophobic pocket that binds small molecules in the direction from His-15 to Lys-1 [Yamada, H., Uozumi, F., Ishikawa, A., & Imoto, T. (1984) *J. Biochem. (Tokyo) 95*, 503–510]. The thermal stabilities of three cross-linked lysozymes thus obtained were investigated in 0.1 M acetate buffer containing 3 M guanidine hydrochloride at pH 5.5. All derivatives were stabilized but to different degrees. The derivative cross-linked with **1**-4 was most stabilized (2.3 kcal/mol), but the derivatives cross-linked with the reagents both shorter (**1**-2) and longer (**1**-6) than **1**-4 were less stabilized (both 1.6 kcal/mol). The decreased entropies of the denatured polypeptide chains due to the present cross-links were theoretically predicted to be −9.1 to −9.2 eu, which corresponded to stabilization energies of about 3.0 kcal/mol in all cases around the melting temperatures of the cross-linked derivatives. The lower than predicted stabilizations observed and the magnitude of the stabilizations suggest that the introduction of the cross-link causes a strain in the native state of lysozyme depending on the length of the cross-link and this strain effect of the destabilization of the denatured state mentioned above. Therefore, it is concluded that maximum stabilization can be achieved only when the proper length of a cross-link is introduced into a protein. The activities of the cross-linked lysozymes against glycol chitin at pH 5.5 increased first on increasing temperature, reached the maxima, then decreased, and vanished at 100 °C. Below 60 °C, all activities increased similarly to that of native lysozyme, but above 60 °C, the activities of the derivatives were signficantly higher than that of native lysozyme. The orders of the optimum temperatures and the maximum activities were exactly the same as the order of the thermal stabilities. The mechanism of the decrease of the activity at high temperatures was discussed.

Many proteins contain intramolecular covalent cross-links of disulfide groups. The function of cross-links is believed to be to increase the thermal stability of a protein due to the destabilization of the denatured state, but the effect on the native state is not known. In recent years, much effort has been made to introduce artificial intramolecular cross-links into proteins directly or indirectly by using a bifunctional reagent. In the direct methods, hen egg white lysozyme has been cross-linked between Glu-35 and Trp-108 as an ester bond by iodine oxidation (Imoto et al., 1973; Beddel et al., 1975). We have also reported the cross-linking of lysozyme between Lys-13 (ϵ-amino) and Leu-129 (α-carboxyl) as an amide bond by the carbodiimide reaction (Yamada et al., 1983). Bovine pancreatic trypsin inhibitor has also been cross-linked by the carbodiimide reaction (Goldenberg & Creighton, 1983). By use of 1,5-difluoro-2,6-dinitrobenzene as a bifunctional reagent, staphylococcal nuclease (Cuatrecasas et al., 1969), α subunit of *Escherichia coli* tryptophan synthetase (Hardman & Hardman, 1971), and ribonuclease A (Marfey et al., 1965a,b; Lin et al., 1984) have been intramolecularly cross-linked. We are interested in the effects of artificial cross-links on protein stability and function, but at the present stage, the accumulation of protein derivatives with artifical intramolecular cross-links is too few to evaluate these effects. Therefore, we must prepare many more protein derivatives in which the artificial cross-links are introduced at various sites.

Hen egg white lysozyme contains a single histidine residue (His-15), which is alkylated with haloacetic acids and their derivatives at pH 5.0–5.5 (Piszkiewicz & Bruice, 1968; Parsons et al., 1969; Kravchenko et al., 1964; Goux & Allerhand, 1979; Yamada et al., 1984). At pH 7–9, the ϵ-amino groups are also alkylated with iodoacetic acid (Kravchenko et al., 1964). Therefore, it might be reasonable to introduce an intramolecular cross-link to lysozyme with bifunctional bis(haloacetic acid) derivatives by using a two-stage cross-linking procedure (reaction at pH 5.0–5.5, removal ofthe excess reagent, and incubation at alkaline pH). In this paper, we describe the preparation of lysozyme derivatives cross-linked intramolecularly between His-15 and Lys-1 with the bis(bromoacetamide) derivatives of polymethylenediamines [$BrCH_2CONH(CH_2)_nNHCOCH_2Br$, *n* = 2, 4, and 6] according to this procedure. We also describe the thermal stabilities and the temperature dependences of the activities of the cross-linked lysozymes thus obtained.

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Table I: Preparation of Bis(bromoacetamide) Derivatives

compd	yield (%)	mp (°C)	found (%)			calcd (%)		
			C	H	N	C	H	N
1-0	38	201–203	17.50	2.21	10.14	17.54	2.21	10.23
1-2	34	150–151	24.18	3.39	9.27	23.87	3.34	9.28
1-4	60	139–140	29.29	4.32	8.33	29.12	4.28	8.33
1-6	57	144.5–145.5	33.72	5.07	7.83	33.54	5.07	7.82

Experimental Procedures

Materials. Five times recrystallized hen egg white lysozyme was donated by Eisai Co. (Tokyo, Japan). Bio-Rex 70 (100–200 mesh) was obtained from Bio-Rad Laboratories. Sephadex G-25 (medium) and Sephadex G-100 were from Pharmacia. Glycol chitin, a substrate of lysozyme, was prepared as described elsewhere (Yamada & Imoto, 1981).

Syntheses of 1,2-bis(bromoacetyl)hydrazine (**1**-0) and *N*,-*N*′-bis(bromoacetyl)polymethylenediamines [$BrCH_2CONH(CH_2)_nNHCOCH_2Br$, $n = 2$ (**1**-2), $n = 4$ (**1**-4), and $n = 6$ (**1**-6)] were carried out according to the method for iodoacetamide derivatives of Ozawa (1967) except that bromoacetyl bromide was used here instead of iodoacetyl chloride. The yields, melting points, and elemental analysis data of the products are listed in Table I.

Analytical Methods. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer after hydrolyses of protein or peptide samples in 6 N HCl under vacuum at 110 °C for 20 h. Retention times of 1- and 3-(carboxymethyl)-histidines and N^{α}-and N^{ϵ}-(carboxymethyl)lysines were determined by using the authentic samples, respectively. Chromatography of lysozyme derivatives was performed on a column (1.4 × 65 cm) of the carboxylic acid cation exchanger Bio-Rex 70 with gradient elution from 1 L of 0.02 M borate buffer to 1 L of the same buffer containing 0.15 M NaCl at pH 10.0. Protein elution was monitored by absorbance of effluents at 298 nm instead of 280 nm to obtain the reduced intensity with a Hitachi 200-10 double-beam spectrophotometer. Digestions of reduced and S-carboxymethylated lysozyme derivatives with L-1-(*p*-tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin (Worthington, 1% lysozyme by weight) and separations of tryptic peptides on reversed-phase high-performance liquid chromatography (TSK gel ODS-120A, 4 × 300 mm, Toyo Soda, Japa) were accomplished as described previously (Yamada et al., 1981; Okazaki et al., 1982). Activities of lysozyme and its derivatives against glycol chitin at various temperatures were determined in 0.1 M acetate buffer at pH 5.5 by measuring the reducing power produced after 30-min reaction as described elsewhere (Imoto & Yagishita, 1971). Thermal stabilities of lysozyme and its derivatives in 0.1 M acetate buffer containing 3 M guanidine hydrochloride at pH 5.5 were determined by using the difference spectral method at 301 nm (Aune & Tanford, 1969) with a Hitachi 557 double-wavelength double-beam spectrophotometer equipped with temperature controller SPR-6.

Reactions of Lysozyme with Bis(bromoacetamide) Derivatives (**1**-*n*). Lysozyme (300 mg, 0.021 mmol) was dissolved in 10 mL of water, the pH of the solution was adjusted to 5.5 with dilute acetic acid, and then excess bis(bromoacetamide) derivative (**1**-*n*, 10 or 20 times excess) was added with stirring. Because of the different solubilities of the bifunctional reagents employed here, they were added in different ways. As 1,2-bis(bromoacetyl)hydrazine (**1**-0) was not soluble enough in either water, ethanol, acetone, or acetonitrile, it was added as a powder directly (114 mg, 0.42 mmol) to make a suspension. As **1**-2 was very soluble in water, it was added as a solid (126 mg, 0.42 mmol). As **1**-4 and **1**-6 were hardly soluble in water but soluble enough in a water–ethanol mixture, solutions of 69.2 mg (0.21 mmol) of **1**-4 in 3 mL of ethanol and 75 mg (0.21 mmol) of **1**-6 in 4 mL of ethanol were added respectively. The mixture was stirred at 40 °C for 24 (**1**-0), 6 (**1**-2), 30 (**1**-4), or 40 h (**1**-6), and the pH of the solution was maintained at 5.5 by addition of 0.2 N NaOH with titration system RTS 662 (Radiometer) during the reaction. After the reaction, excess reagent was removed by gel filtration through a Sephadex G-25 column (3 × 70 cm) with 0.05 M KH_2PO_4. In the case of **1**-0, insoluble reagent was removed by centrifugation prior to gel filtration. The protein fraction was diluted to 300 mL with 2 mM sodium tetraborate, and the pH was raised to 9.0 with NaOH. After a 1-day incubation at room temperature, the protein was precipitated by adding 180 g of $(NH_4)_2SO_4$, collected by centrifugation, redissolved in about 5 mL of water, and then passed through a Sephadex G-100 column (1.5 × 150 cm) with 0.05 M phosphate buffer at pH 7.0 to remove polymerized lysozyme if any. The monomer fraction was dialyzed against distilled water and then analyzed by ion-exchange chromatography on Bio-Rex 70.

Results

Three hundred milligrams of hen egg white lysozyme was allowed to react with four bifunctional reagents, $BrCH_2CONH(CH_2)_nNHCOCH_2Br$ (**1**-n, n = 0, 2, 4, and 6), at pH 5.5 and 40 °C to modify His-15 monofunctionally. The excess reagents were then removed by gel filtration. The protein fraction was diluted to 300 mL to minimize the formation of the intermolecularly cross-linked lysozyme, and the pH of the solution was raised to 9.0 to allow the other end of the reagent molecule to react. After a 1-day incubation at room temperature, the solution was concentrated to about 5 mL. Gel filtration of the resulting solution on Sephadex G-100 indicated that lysozyme was dimerized in yields of 9 and 6% in the cases of **1**-0 and **1**-2, respectively, but no dimer was formed in the cases of **1**-4 and **1**-6 under the conditions employed. The monomer fractions were chromatographed on Bio-Rex 70 at pH 10, and the chromatographic patterns are shown in Figure 1A–D, respectively. In every case, only one major peak (I, II, III, or IV) appeared before the native peak, which is indicated by the arrow in Figure 1. Amino acid compositions of the derivatives in peaks I–IV are listed in Table II. Every derivative did not contain histidine but did contain one 3-(carboxymethyl)histidine per molecule. Since 3-(carboxymethyl)histidine appeared just after the alanine peak under routine amino acid analysis conditions and since their separation was not enough to calculate their peak areas individually, both are included in the listing for alanine in Table II. Furthermore, three derivatives (II, III, and IV, but not I) contained five lysines (six lysines in native lysozyme) and one N^{ϵ}-(carboxymethyl)lysine per molecule. Since the separation of N^{ϵ}-(carboxymethyl)lysine from valine was not enough for the calculation of their peak areas individually, both are included in the listing for valine. 1-(Carboxymethyl)histidine and N^{α}-(carboxymethyl)lysine, which appeared near the proline and cystine peaks, respectively, were not detected at all. These results indicate that the derivatives in peaks II, III, and IV are the lysozymes cross-linked intramolecularly with

Table II: Amino Acid Compositions of Lysozyme Derivatives[a]

amino acid	native lysozyme theory	native lysozyme control	I[b]	II	III	IV
Asp	21	20.5	20.8	20.7	20.9	20.5
Thr	7	6.8	6.9	6.9	6.7	6.6
Ser	10	9.0	9.1	9.2	9.2	8.9
Glu	5	5.3	5.3	5.4	5.3	5.3
Pro	2	1.9	2.0	2.0	2.0	2.0
Gly	12	11.9	11.9	12.0	11.9	11.9
Ala + 3-CM-His[c]	12	11.8	12.7 (+1)	12.6 (+1)	12.7 (+1)	12.7 (+1)
Val + ϵ-CM-Lys[d]	6	5.6	5.5	6.6 (+1)	6.5 (+1)	6.6 (+1)
Met	2	2.0	1.9	1.9	1.9	1.9
Ile	6	5.6	5.5	5.5	5.5	5.5
Leu	8	8	8	8	8	8
Tyr	3	2.9	3.0	3.0	3.0	3.0
Phe	3	3.0	3.0	2.9	3.0	3.0
Lys	6	5.9	5.9	5.0 (−1)	5.0 (−1)	5.0 (−1)
His	1	1.0	0.1 (−1)	0.0 (−1)	0.0 (−1)	0.0 (−1)
Arg	11	11.0	11.1	11.0	10.9	10.9

[a] All values are expressed as molar ratios normalized to a value of 8.0 for leucine. [b] See Figure 1. [c] 3-(Carboxymethyl)histidine. [d] N^{ϵ}-(Carboxymethyl)lysine.

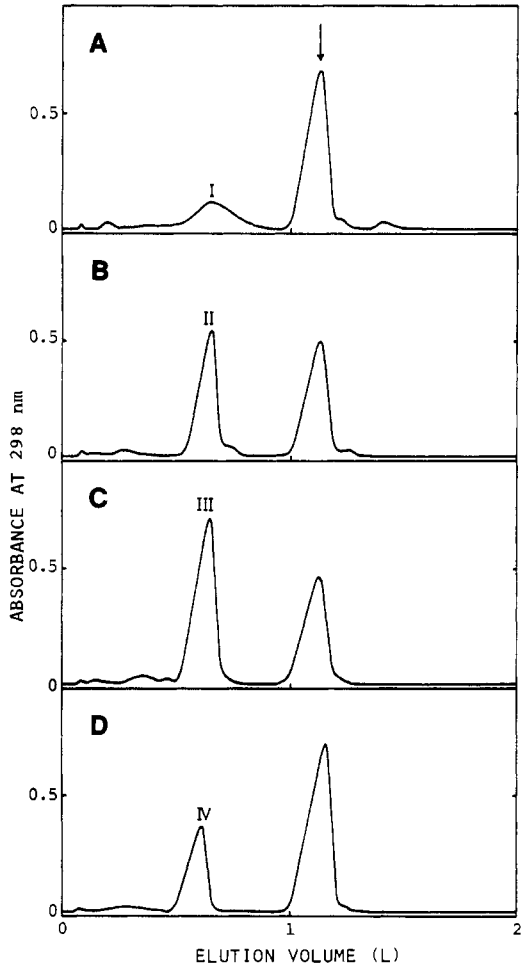

FIGURE 1: Ion-exchange chromatography of reaction mixtures of lysozyme with bis(bromoacetamide) derivatives (**1-*n***) on Bio-Rex 70 at pH 10.0. The column (1.4 × 65 cm) was eluted with a gradient of 1 L of 0.02 M borate buffer and 1 L of the same buffer containing 0.15 M NaCl. (A) Reaction with $BrCH_2CONHNHCOCH_2Br$ (**1**–0); (B) reaction with $BrCH_2CONH(CH_2)_2NHCOCH_2Br$ (**1**–2); (C) reaction with $BrCH_2CONH(CH_2)_4NHCOCH_2Br$ (**1**-4); (D) reaction with $BrCH_2CONH(CH_2)_6NHCOCH_2Br$ (**1**-6). The *arrow* indicates the elution position of native lysozyme.

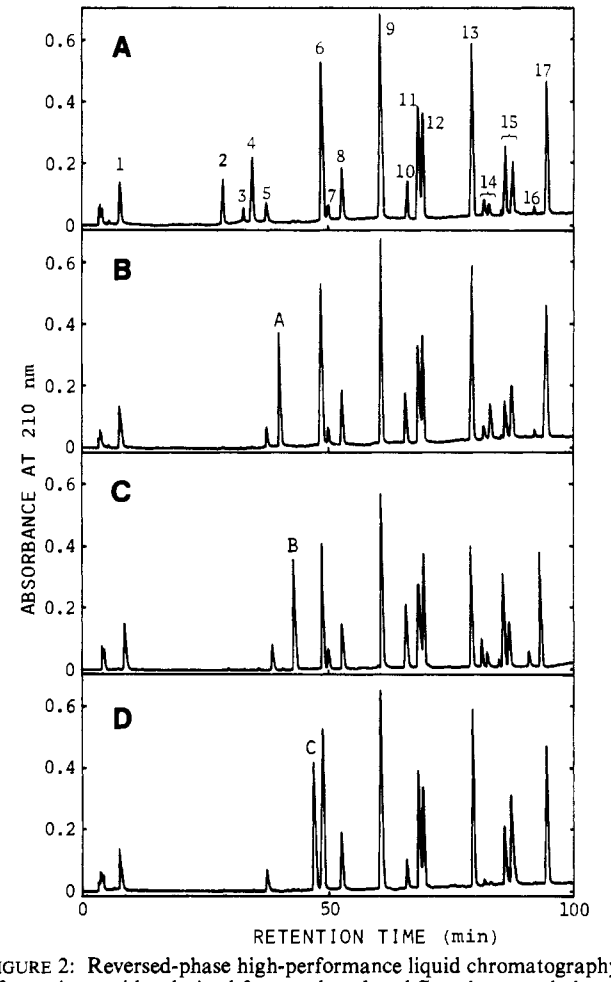

FIGURE 2: Reversed-phase high-performance liquid chromatography of tryptic peptides derived from reduced and S-carboxymethylated lysozyme derivatives on a 4 × 300 mm column of TSK gel ODS-120A (5 μm, Toyo Soda). The column was eluted with a gradient of 40 mL of 1% acetonitrile and 40 mL of 40% acetonitrile, both containing 0.1% concentrated HCl at a flow rate of 0.8 mL/min. (A) Native lysozyme; (B) derivative II in Figure 1B; (C) derivative III in Figure 1C; (D) derivative IV in Figure 1D. Assignments of peaks are as follows: T_{10} (1), T_{1+2} (2), T_2 (3), T_5 (4), T_{17+18} (5), T_7 (6), T_{3+4} (7), T_3 (8), T_9 (9), T_{15+16} (10), T_{16} (11), T_8 (12), T_6 (13), T_{12+13} (14), T_{13} (15), T_{11+12} (16), and T_{11} (17).

reagents **1**-2, **1**-4, and **1**-6, respectively, between the nitrogen at ϵ_2 of His-15 and one of the ϵ-amino groups of the six lysine residues. The derivative in peak I contained six lysines, indicating it to be the lysozyme modified at N^{ϵ_2} of His-15 with **1**-0 monofunctionally.

Table III: Amino Acid Compositions of Peptides[a]

amino acid	$T_{1+2} + T_5$, theory	amino acid ratio in peptide A[b]	B	C
Asp	2	2.2	2.0	1.7
Gly	2	2.3	2.0	2.2
3-CM-His[c]		1.0 (+1)	1.0 (+1)	0.8 (+1)
Val, ϵ-CM-Lys[d]	1	2.0 (+1)	1.8 (+1)	1.9 (+1)
Leu	1	1	1	1
Tyr	1	0.6	0.6	0.9
Phe	1	1.1	1.0	0.8
Lys	1	0.1 (−1)	0.0 (−1)	0.1 (−1)
His	1	0.0 (−1)	0.0 (−1)	0.0 (−1)
Arg	2	2.1	2.0	2.0

[a] Normalized to a value of 1 for leucine. [b] See Figure 2. [c] 3-(Carboxymethyl)histidine. [d] N^{ϵ}-(Carboxymethyl)lysine.

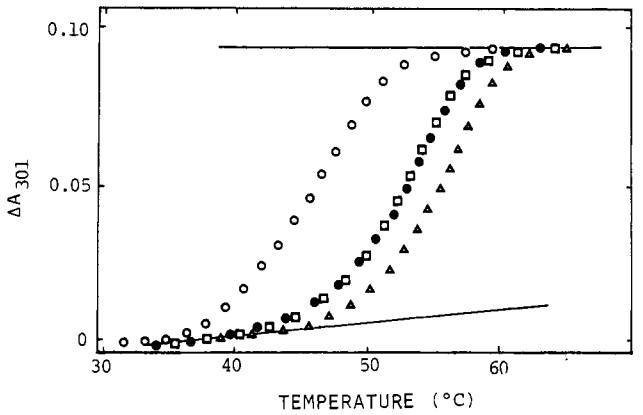

FIGURE 3: Thermal denaturations of lysozyme and its cross-linked derivatives in 0.1 M acetate buffer containing 3 M guanidine hydrochloride at pH 5.5. Difference absorbances at 301 nm were plotted against temperature for native lysozyme (○), derivative II (●, n = 2), derivative III (△, n = 4), and derivative IV (□, n = 6). The concentration of each protein was 1.3 mg/mL.

For the identification of the cross-linked sites, the derivatives in peaks II, III, and IV were reduced, S-carboxymethylated, and then hydrolyzed to peptides with trypsin. The elution patterns of the tryptic peptides on reversed-phase high-performance liquid chromatography are shown in Figure 2B–D, respectively. For comparison, the pattern from native lysozyme is shown in Figure 2A, where the assignment of each peak is also shown. In every case, peaks of the native peptides 2 (T_{1+2}, Lys-1–Arg-5), 3 (T_2, Val-2–Arg-5), and 4 (T_5, His-15–Arg-21) disappeared, and a new peak (peak A, B, or C in Figure 2) appeared before peak 6 (peptide T_7). T refers to the nomenclature of Canfield (1963) for the tryptic peptides of lysozyme. All other peptides appeared at the same positions as those of native peptides. Peptides A, B, and C were subjected to amino acid analyses. As shown in Table III, all new peptides had the same amino acid composition and are assigned to the peptides T_{1+2} plus T_5, where histidine and lysine are substituted with 3-(carboxymethyl)histidine and N^{ϵ}-(carboxymethyl)lysine, respectively. All of these results clearly indicate that the derivatives in peaks II, III, and IV are all the intramolecularly cross-linked lysozymes between the ϵ-amino group of Lys-1 and the nitrogen at ϵ_2 of His-15 with bifunctional reagents **1**-2, **1**-4, and **1**-6, respectively.

Stabilities of cross-linked lysozyme derivatives II, III, and IV were investigated with the thermal denaturation experiments in 0.1 M acetate buffer containing 3 M guanidine hydrochloride at pH 5.5. In Figure 3, the denaturation curves of derivatives II, III, and IV obtained from the absorbance at 301 nm vs. temperature are shown as well as that of native lysozyme. The high- and low-temperature base lines were used to calculate the equilibrium constants (K's, K = D/N), assuming two-state transitions between the native (N) and denatured (D) states of the proteins. The respective melting temperatures were 45.4 (native lysozyme), 52.7 (II), 55.4 (III), and 52.6 °C (IV).

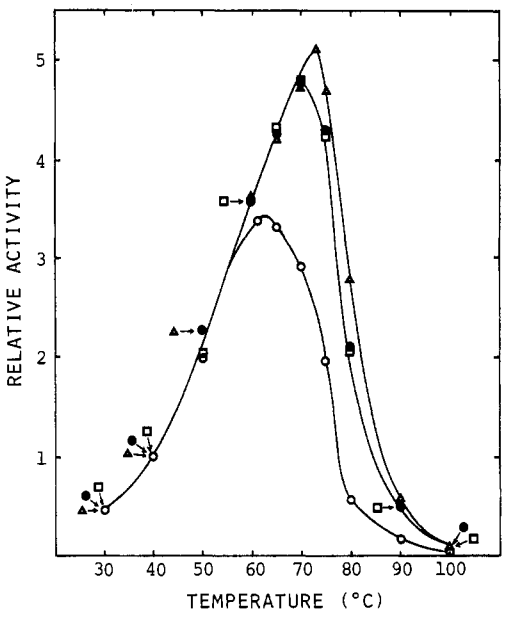

FIGURE 4: Temperature dependences of the activities of lysozyme and its cross-linked derivatives against glycol chitin in 0.1 M acetate buffer at pH 5.5. The activities relative to that of native lysozyme at 40 °C were plotted against temperature for native lysozyme (○), derivative II (●, n = 2), derivative III (△, n = 4), and derivative IV (□, n = 6).

The temperature dependences of the activities of lysozyme and its cross-linked derivatives were determined at pH 5.5 by using glycol chitin as a substrate in 0.1 M acetate buffer. The activities were expressed as values relative to the activity of native lysozyme at 40 °C and plotted against temperature in Figure 4. Below 60 °C, the activities of the cross-linked derivatives were similar to that of native lysozyme. Above 60 °C, however, the activities of the derivatives were significantly higher than that of native lysozyme.

Discussion

Lysozyme derivatives intramolecularly cross-linked between Lys-1 and His-15 were prepared by the two-stage reaction procedure described above. With this cross-linking procedure, three of the reagents (**1**-2, **1**-4, and **1**-6) gave lysozymes cross-linked between the ϵ-amino group of Lys-1 and the nitrogen at ϵ_2 of His-15 selectively without any formation of monofunctionally modified lysozyme derivatives, although some lysozyme dimer was also obtained in the case of **1**-2. These facts indicate that the second cross-linking reaction with Lys-1 occurred very efficiently without significant hydrolysis of the second bromoacetyl group in these reagents. However, the shortest reagent, **1**-0, did not give any intramolecularly cross-linked lysozyme but did give lysozyme modified at $N^{\epsilon 2}$ of His-15 monofunctionally and lysozyme dimer, indicating that the second bromoacetyl group was hydrolyzed or reacted with another lysozyme molecule in this case. In the crystalline state of lysozyme, the distance between the two nitrogens at ϵ of Lys-1 and ϵ_2 of His-15 is 11.8 Å and is longer than the distance between N^{ϵ} of Lys-96 and $N^{\epsilon 2}$ of His-15 (7.8 Å; Imoto et al., 1972). Molecular model building suggests that the reagents employed here could cross-link two residues within distances of 8.7 (**1**-0), 11.0 (**1**-2), 13.4 (**1**-4), and 16.0 Å (**1**-6).

Therefore, all of the four reagents are long enough for cross-linking occurred between His-15 and Lys-96. Nevertheless, the cross-linking occurred between His-15 and Lys-1 exclusively. Thus, we can conclude that the reagent molecule attached to His-15 does not move around freely but is fixed to the direction of Lys-1, which is opposite to the direction of Lys-96. This conclusion is consistent with our previous proposal that lysozyme has a small hydrophobic pocket that binds small molecules in the vicinity of His-15 and that this binding site is a pocket surrounded by Arg-14 and Asp-87 in the direction of Lys-1 from His-15 (Yamada et al., 1984). Thus, the shortest reagent, **1**-0, is too short to cross-link Lys-1 and His-15 intramolecularly, and therefore, at alkaline pH, the remaining bromoacetyl end of the reagent molecule is just hydrolyzed to give a monofunctionally modified lysozyme or otherwise reacts with another lysozyme molecule to give lysozyme dimer. In the case of **1**-2, the length of the reagent is a little shorter (about 0.8 Å) than the distance between N^{ϵ} of Lys-1 and $N^{\epsilon 2}$ of His-15. This may reduce somewhat the efficiency for intramolecular cross-linking, leading to the formation of a small amount of lysozyme dimer besides the intramolecularly cross-linked lysozyme. The reagents **1**-4 and **1**-6 are long enough for cross-linking between His-15 and Lys-1, and this may be a reason for no significant formation of lysozyme dimer. Since the two-stage reaction procedure was employed here, the yields of the intramolecularly cross-linked lysozymes should depend mainly on the initial yields of the monofunctionally modified lysozymes. Furthermore, the initial reaction conditions were not the same because of the different solublities of the bifunctional reagents employed. Therefore, the chromatographic patterns in Figure 1 do not offer any information about the rates of the second cross-linking reactions.

All of the cross-linked lysozymes obtained here were found to be fully active below 60 °C (vide infra), suggesting that no serious conformational change in the lysozyme molecule is caused by the cross-linking. Thus, the stabilities of these lysozyme derivatives cross-linked intramolecularly with various bridge spans are interesting. In 3 M guanidine hydrochloride at pH 5.5, all cross-linked lysozymes obtained here showed higher melting temperatures than native lysozyme in the thermal transition between the native (N) and denatured (D) states, indicating that the present intramolecular cross-links stabilized lysozyme. However, the degree of stabilization depended on the cross-linking bridge length, and the order was III (n = 4) > II (n = 2) $\simeq$ IV (n = 6). The stabilization energy due to cross-link formation can be obtained by changing the sign of the free energy change (ΔG) of the denaturation of native lysozyme at the melting temperature of each cross-linked lysozyme. As the temperature range of the transition zone of the thermal denaturation of native lysozyme was limited, the latter value was determined by extrapolation according to the equation (Ahmad & McPhie, 1978)

$$\ln K = -a + (\Delta C_p/R) \ln T + b/T$$

Here a and b are constants independent of temperature (T), ΔC_p is the heat capacity difference between the denatured and native states of lysozyme, and R is the gas constant. The curve fitting of the data of native lysozyme allowed us to determine a (3045), b (114330), and ΔC_p [926 cal/(mol·°C)]. The value of ΔC_p obtained here is in good agreement with that [950 cal/(mol·°C)] obtained by Tanford & Aune (1970) for the thermal transition of native lysozyme between the N and "X" states, where the X state is an unfolded lysozyme at high temperatures and moderate concentration of guanidine hydrochloride. Therefore, the denatured state (D) designated here might be similar to the X state referred to by Tanford & Aune (1970). In Table IV, the free energy changes (ΔG's) thus calculated are summarized. The net effect of a cross-link on protein stability is the difference between the effects on the native and denatured states. The effect on the denatured state is considered to be the entropy loss due to the cross-link. Several theoretical methods to estimate the entropy loss in the denatured state due to a cross-link have been developed by Flory (1956) and Poland & Scheraga (1965). Johnson et al. (1978) and Lin et al. (1984) have successfully applied these methods to explain the stabilizations of the lysozyme derivative cross-linked between Glu-35 and Trp-108 and of the ribonuclease A derivative cross-linked between Lys-7 and Lys-41, respectively. Therefore, we also calculated the entropy loss in the denatured state due to the present cross-linking. By use of the method of Poland & Scheraga (1965), the present entropy loss in the denatured state is calculated to be −7.2 eu, which corresponds to ΔG = 2.3–2.4 kcal/mol at around the melting temperatures (52.6–55.4 °C) of derivatives II, III, and IV. The stabilization energy of derivative III (2.3 kcal/mol) obtained from the thermal denaturation experiments is in good agreement with this value. In the cases of derivatives II and IV, however, the stabilization energies observed (both 1.6 kcal/mol, Table IV) are considerably smaller than expected. Since the length of the cross-link is not considered in the method of Poland & Scheraga (1965), we calculated this entropy loss by using eq 60 and 63′ of Flory (1956), where we can introduce the length of the cross-link into the volume element $\Delta\tau$. As a first approximation, $\Delta\tau$ is assumed to be independent of the length of the cross-link and equal to the volume of a sphere of 17.4-Å diameter, the distance between the α-carbon atoms of Lys-1 and His-15 in the crystalline state of lysozyme (Imoto et al., 1972). Setting the length of the statistical element (l') of the chain as 3.8 Å, the C^{α}–C^{α} distance of a polypeptide chain, the present entropy loss is calculated to be −7.4 eu (ΔG = 2.4 kcal/mol at 52.6–55.4 °C), which is consistent with the value calculated above. A more realistic $\Delta\tau$ that takes account of the length of the cross-link may be obtained by setting $\Delta\tau$ to be the mean volume of the sphere within the diameter of the most expanded distance between two α-carbons of Lys-1 and His-15. This $\Delta\tau$ can be calculated if the distribution of the end-to-end distance (d) between these cross-linked α-carbons is known. Therefore, the distribution of d was examined by 1000 times computations

Table IV: Free Energy Changes on Thermal Denaturation of Native and Intramolecularly Cross-Linked Lysozymes in 0.1 M Acetate Buffer Containing 3 M Guanidine Hydrochloride at pH 5.5

lysozyme		T (°C)	K (D/N)	ΔG^a (kcal/mol)
native	exptl	45.4	1	0
II (n = 2)	exptl	52.7	1	0
native	calcd[a]	52.7	12.4	−1.6
III (n = 4)	exptl	55.4	1	0
native	calcd[a]	55.4	32.4	−2.3
IV (n = 6)	exptl	52.6	1	0
native	calcd[a]	52.6	11.9	−1.6

[a] See the text. A comparison at a common temperature (the melting temperature of native lysozyme) may be more realistic than the present comparison. To do this, we must calculate K of each cross-linked lysozyme at this temperature by extrapolation of the temperature dependence of each K. However, the precise determination of K at high and low temperatures was difficult in the present method, and we do not know how reliable our data are. On the other hand, the melting temperature of each cross-linked derivative could be easily determined with considerable precision. As for native lysozyme, we judged the temperature dependence of K obtained here to be more reliable because the value of ΔC_p was in good agreement with that reported by Tanford & Aune (1970) as mentioned in the text. Thus, the present comparison was employed here.

with the Monte Carlo method where free bond rotations around single bonds in a cross-linking bridge were assumed, except that two amide bonds were fixed to trans conformations. In every case, the distribution obtained was fairly consistent with a Gaussian distribution, and the mean distances ($\bar{d}$'s) ± the standard deviations (σ's) were 12.86 ± 3.48 Å for derivative II, 12.41 ± 4.14 Å for derivative III, and 12.66 ± 4.48 Å for derivative IV. Since the mean cube ($\overline{d^3}$) of d is equal to $\bar{d}^3 + \bar{d}\sigma^2$ when the distribution of d is Gaussian, $\Delta\tau$ is expressed by $(4/3)\pi(\overline{d^3}/2^3)$ or $(1/6)\pi(\bar{d}^3 + \bar{d}\sigma^2)$. With $\Delta\tau$ obtained, the entropy losses were calculated to be −9.1 eu for derivatives II and IV and −9.2 eu for derivative III, which corresponded to ΔG = 3.0 kcal/mol at around the melting temperatures of derivatives II, III, and IV, respectively. These results indicate that the entropy losses or the free energy changes (ΔG's) in the denatured state of lysozyme due to the present cross-links are almost constant even though the lengths of the cross-links are different. Furthermore, the stabilization energies observed were all lower than predicted. Therefore, we must consider the destabilization effect in the native state, which partly cancels the destabilization of the denatured state. We propose that this effect is a strain caused by the cross-link in the native state and that the magnitude of this effect depends on the length of the cross-link. As mentioned above, the cross-linking bridge length in derivative II is little shorter than the distance between N^{ϵ} of Lys-1 and $N^{\epsilon 2}$ of His-15 in native lysozyme. This may cause a considerable strain in the native state of derivative II. In the case of derivative IV, the bridge is fairly flexible and able to expand up to 16 Å, which is much longer than the distance between two cross-linked nitrogens (11.8 Å). Nevertheless, the stabilization energy of this derivative is about half of the predicted value. Therefore, we suppose that a longer cross-link than the proper length (the distance between two cross-linked nitrogens) may also cause a considerable strain in the native state of lysozyme. As for derivative III, the cross-linking bridge length (13.4 Å in the expanded conformation) is just a little longer than the distance between the cross-linked nitrogens, and therefore the strain may be minor, and the highest stabilization was observed in this derivative. From these considerations, we might draw the following conclusions. An intramolecular cross-link destabilizes both the native and denatured states of a protein. The destabilization of the native state is due to strain, and that of the denatured state is due to entropy loss. The length of the cross-link affects greatly the degree of strain but does not so much affect the extent of entropy loss. Both shorter and longer cross-links than the proper length cause considerable strains in the native state of a protein. Therefore, the maximum stabilization of a protein by an intramolecular cross-link can be achieved only when the proper length of the cross-link is introduced into the protein.

Since the cross-linked derivatives obtained here were found to be more thermally stable than native lysozyme, and since the modified sites (Lys-1 and His-15) are located at the surface opposite to the sachharide binding site of lysozyme (Imoto et al., 1972), it is expected that these derivatives would be more active than native lysozyme at high temperatures as long as the enzyme in the native state is always active. Therefore, the temperature dependences of the enzymatic activities of these derivatives as well as that of native lysozyme were investigated by using glycol chitin as a substrate at pH 5.5 in the temperature range of 30–100 °C. As shown in Figure 4, as temperature increased, the activity of every lysozyme increased first, reached a maximum, then decreased, and vanished at 100 °C. Below 60 °C, the activities of the cross-linked derivatives were almost identical with that of native lysozyme and increased with temperature. While the activity of native lysozyme reached its maximum at 62 °C, the activities of the cross-linked lysozymes increased further and reached their maxima at 70 °C for derivatives II (n = 2) and IV (n = 6) and at 73 °C for derivative III (n = 4). As for the maximum activity, the order of the magnitude was III > II ≃ IV > native lysozyme. This order was exactly the same as that of the degree of stabilization as expected. The temperature dependence of the activity may reflect the following three factors: enzyme–substrate complex formation, catalytic ability, and denaturation. Under the substrate-saturated condition, catalytic ability would increase with temperature, explaining the initial increase of the activities below 60 °C. On the other hand, denaturation would decrease the activity. However, the activity of native lysozyme decreased above 62 °C. This temperature seems somewhat lower than expected from the denaturation, because native lysozyme does not undergo the conformational change up to 70 °C and is half-denatured at about 77 °C in the absence of a denaturant at around neutral pH (pH > 5; Imoto et al., 1972). Moreover, the substrate in the system of the activity measurements should stabilize the native conformation of lysozyme (Pace & McGrath, 1980). Therefore, another explanation must be required for the decrease of the activity of lysozyme prior to denaturation. One possibility is the loss of the capability to form the enzyme–substrate complex at high temperatures. Although the ability for complex formation between lysozyme and the trimer of *N*-acetyl-D-glucosamine has been reported to be reduced with temperature (Banerjee & Rupley, 1973), Hayashi et al. (1968) found that the enzyme–substrate complex was fully formed up to 70 °C under the conditions employed here. Therefore, this possibility may be ruled out. The second possibility is that there is an inactive form in the native state of lysozyme and the inactive form becomes predominant at high temperatures prior to denaturation. However, as discussed above, the stabilization of lysozyme by cross-links is mainly attributed to destabilization of the denatured state, and we cannot find a reasonable correlation between the amount of the inactive form mentioned above and protein stabilization by cross-linking. Therefore, this possibility may be also ruled out. Lysozyme is irreversibly inactivated at high temperatures (Imoto et al., 1972). It may be possible that the present decrease of the activity is due to this irreversible inactivation, which follows the reversible thermal denaturation. To confirm this, native lysozyme was preincubated at 70 °C under the conditions of activity measurements (pH 5.5, 30 min), except that substrate was omitted, and cooled in an ice bath, and then the activity was measured at 40 °C. The activity of the preincubated sample was reduced to 90% of its original activity and was not recovered after a 1-day storage at room temperature. Therefore, this might partly explain the decrease of activity prior to the thermal denaturation equilibrium. The presence of another inactive intermediate that is kinetically controlled via the denatured state at high temperatures might afford an additional explanation. More vigorous examinations are under way.

Anyway, we can conclude that the stabilization of lysozyme by intramolecular cross-links increases its activity at high temperatures. This finding may be applied to fields of biotechnology in order to utilize the enzyme reaction for preparations of useful materials.

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