The Mechanism of Irreversible Inactivation of Lysozyme at pH 4 and 100 °C

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ABSTRACT: The mechanism of irreversible inactivation of lysozyme at pH 4, 100 °C, was investigated. It was elucidated that the inactivation was caused by production of molecules in an irreversibly denatured state. From analyses of the mechanism of production of the inactive enzyme, the inactivation was not evoked by a single chemical reaction. The free energy change between the folded and unfolded states decreased by the accumulation of chemical reactions (isomerization of Asp-Gly, deamidation of Asn, racemization of Asp and Asn, and cleavage of the Asp–X peptide bond) induced at high temperature. Thus, certain molecules were ultimately in the unfolded state even at low temperature and lost activity. Moreover, a good correlation between the stability (free energy change) and the averaged number of chemical reactions that leads to the inactivation was obtained on the basis of some assumptions.

Enhanced stabilization of proteins is one of the ultimate goals of protein engineering. For the long-term stabilization of proteins, it is important to take measures against irreversible chemical reactions. We can investigate these reactions in a short time by elevating temperature. On the other hand, knowledge of the thermal stability of proteins is required for the industrial use of proteins at high temperature. During the thermal stability of proteins, the notion of both reversible and irreversible heat denaturation should be considered. Complete renaturation by cooling after short-term heat denaturation has been well-known and investigated as reversible thermal denaturation (Plivalov, 1979; Becktel & Schellman, 1987). However, such activities are not fully regained after long-term heat denaturation. Since the irreversible thermoinactivation is due to many chemical reactions, the mechanism is very complex (Tanford, 1968). Information on the nature of irreversible thermoinactivation may enable one to better design a protein with increased thermostability. Despite the accumulation of information in this area, clarification of the irreversible inactivation mechanism has not yet been achieved.

Klibanov et al. reported the mechanism of irreversible thermoinactivations of some enzymes (Ahern & Klibanov, 1985; Zale & Klibanov, 1986; Volkin & Klibanov, 1987; Ahern et al., 1987; Tomazic & Klibanov, 1988a,b). It was reported that irreversible inactivation of lysozyme at pH 4, 100 °C, was caused by deamidation of Asn and/or hydrolysis of the Asp–X peptide bond (Ahern & Klibanov, 1985). The conclusion was derived from a consistency of the rate of the inactivation and those of the above reactions. However, we found that the main chain cleaved lysozyme at Gly104 was about 90% (Yamada et al., 1990) and mono-deamidated lysozyme derived from incubation at alkaline pH was about 80% active (unpublished data). Moreover, racemization at Asp or Asn was also conjectured to be a cause of the lysozyme inactivation (Zhao, 1989).

In this study, we have reexamined the mechanism of irreversible inactivation of the lysozyme at pH 4, 100 °C. We report and discuss a correlation between the stability at ambient temperature and thermoinactivation.

EXPERIMENTAL PROCEDURES

Materials. Hen egg white lysozyme was isolated from hen egg white and recrystallized 5 times. *Micrococcus luteus*, a substrate of lysozyme, was obtained from Sigma. Sephadex G-75 was the product of Pharmacia. CM-Toyopearl 650S and a column of Wakosil 5C18–200 (4.6 × 250 mm) were obtained from Tohso (Tokyo, Japan) and Wako Pure Chemicals Institute (Osaka, Japan), respectively. Guanidine hydrochloride (Gdn·HCl)[1] obtained from Nacalai Tesque (Kyoto, Japan) was recrystallized twice from methanol and exhaustively dried. All other reagents were of analytical grade for biochemical uses.

Analytical Methods. Enzymatic activity of lysozyme against *M. luteus* was turbidimetrically determined at 450 nm in 0.05 M potassium phosphate buffer at pH 7, 30 °C (Inoue et al., 1992).

The time course of the irreversible inactivation of the lysozyme was measured by incubating in 0.1 M acetate buffer (pH 4) in boiling water (100°C), periodically removing samples and assaying them for lytic activity at 30 °C.

Amino acid analyses were performed on a Hitachi L-8500 amino acid analyzer after hydrolyses of protein and peptide samples in 6 N HCl under vacuum at 110 °C for 22 h. Digestions of reduced and S-carboxymethylated lysozymes with TPCK-trypsin (Sigma) and separations of tryptic peptides on RP-HPLC (Wakosil 5C18–200) were performed as previously described (Inoue et al., 1992).

The isolation of denatured and nondenatured lysozyme that had been incubated for 1.5 h, at pH 4, 100 °C, was carried out using size-exclusion chromatography on two Sephadex G-75 columns (1.5 × 150 cm) connected in tandem, which were eluted with 0.05 M acetate buffer at pH 4, a flow rate of 12 s/drop, and at room temperature. The protein elution was monitored by the absorbance of effluents at 280 nm.

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; TPCK, L-(tosylamino)-2-phenylethyl chloromethyl ketone; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; OPA, *o*-phthaldialdehyde; NAC, *N*-acetyl-L-cysteine; 1–15 CL lysozyme, stabilized lysozyme derivative that is cross-linked intramolecularly between Lys1 and His15 with the bis(bromoacetamide) derivative of poly(methylenediamine) (n = 4); 6,127 CAM lysozyme, destabilized lysozyme derivative that is S-carboxamidomethylated at Cys6 and Cys127, and has three disulfide bonds; 101-succinimide lysozyme, lysozyme derivative in which the aspartylglycyl sequence at Asp101–Gly102 is converted to a cyclic imide.

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SDS–PAGE was carried out according to the method of Laemmli (1970). Samples were subjected to electrophoresis at a constant voltage of 200 V on 15% polyacrylamide gels and a mini-protean 2 DUAL SLAB CELL. Proteins were stained with Coomassie Brilliant Blue R-250.

The time course of appearance of denatured lysozyme was determined using discontinuous acid PAGE according to the method of Reisfield et al. (1962). Samples were dissolved in the solution containing 15% sucrose/0.13 N acetic acid and then electrophoresed at a constant current of 12 mA, at 4 °C for 3 h, on 15% polyacrylamide gels using the same apparatus as SDS–PAGE. Proteins were stained with Coomassie Brilliant Blue R-250. Proportions of the nondenatured lysozyme were determined using gel densitometry (LKB:2202 ULTROSCAN Laser Densitometer).

In order to compare the degree of deamidation in denatured and nondenatured lysozymes, continuous acid PAGE was carried out according to the method of Hollecker and Creighton (1980). Denatured and nondenatured lysozymes, that had been reduced and S-carboxymethylated, were dissolved in the solution containing 15% sucrose/0.13 N acetic acid and then electrophoresed at a constant current of 20 mA, at 4 °C for 2 h, in 12% polyacrylamide gels (pH 3.6) containing 8 M urea using the same apparatus as SDS–PAGE. As a marker of the degree of deamidation, native lysozyme that was reduced and S-alkylated with the equivalent mixture of iodoacetamide and iodoacetic acid was prepared. Reduced and S-carboxymethylated native lysozyme was employed as a control. Proteins were stained with Coomassie Brilliant Blue R-250.

Comparison of the degree of racemization at Asp and Asn in denatured and nondenatured lysozymes was carried out using the modified method of Aswad (1984). The following procedure was adopted.

(1) Preparation of OPA-NAC Solution. Four milligrams of OPA was dissolved in 300 mL of methanol. To this solution were then added 250 μL of 0.4 M sodium borate (pH 9.4), 390 mL of distilled water, and 60 μL of 1.0 M NAC adjusted to pH 5.5 with NaOH. The OPA–NAC solution could be stored at 4 °C for 2 weeks.

(2) Preparation of Asp Solution. One milligram of lysozyme was hydrolyzed in 300 μL of 6 N HCl under vacuum at 110 °C for 4 h. The hydrolysate was freeze-dried, and 500 μL of distilled water was added.

(3) Preparation of the Sample Solution. Twenty microliters of the Asp solution was mixed with 10 μL of the OPA–NAC solution and kept for 3 min, and then 470 μL of 0.05 M sodium acetate (pH 5.2) was added.

(4) RP-HPLC Separation of OPA-NAC Derivatives of D- and L-Asp. The sample solution was centrifuged at 15 000*g* for 1 min, and 20 μL of supernatant was used for direct injection into RP-HPLC on Wakosil 5C18–200. The column was isocratically eluted with 0.05 M sodium acetate (pH 5.8) containing 4% acetonitrile at a flow rate of 0.4 mL/min. The column effluents were monitored by the absorbance at 350 nm.

Determination of lysozyme stability was accomplished by Gdn·HCl-induced denaturation as previously described (Inoue et al., 1992). The unfolding transition of lysozyme induced by Gdn·HCl was monitored by observing changes in the tryptophyl fluorescence (emission at 360 nm and excited at 280 nm) as a function of the Gdn·HCl concentrations at pH 5.5 and 35 °C.

Preparation of Modified Lysozyme. Stabilized 1–15 CL lysozyme was prepared as previously described (Yamada et al., 1985).

Destabilized 6,127 CAM lysozyme was prepared by a modification of the method of Radford et al. (1991). The following procedure was adopted.

(1) Two hundred milligrams of lysozyme in 10 mL of 0.1 M Tris–acetate (pH 7.8) was reduced by incubation with 7.7 mg of dithiothreitol at 0 °C for 1.5 h. The reduction was arrested by the addition of 46.5 mg of iodoacetamide. The reaction with iodoacetamide was allowed to proceed for 45 min at room temperature in the dark. The sample was then dialyzed against distilled water at 4 °C for 1 day. The soluble fraction was collected by centrifugation (3000*g* for 10 min at 4 °C).

(2) In order to remove unreacted lysozyme, the collected sample was subjected to size-exclusion chromatography on a column (1.5 × 150 cm) of Sephadex G-75, which was eluted with 10% acetic acid containing 4 M urea at a flow rate of 12 s/drop, at room temperature. Under the eluting conditions, the three-disulfide derivative was denatured and eluted on size-exclusion chromatography in shorter elution times than unreacted lysozyme. The preceding fraction was dialyzed against distilled water at 4 °C for 2 days. The material was rechromatgraphed by cation-exchange HPLC on CM-Toyopearl 650S (8 × 500 mm) at pH 7.

RESULTS

Time Course of Irreversible Inactivation of Lysozyme at pH 4, 100 °C. Lysozyme in 0.1 M acetate buffer (pH 4) was incubated in boiling water (100 °C). In appropriate time intervals, lysozyme solutions were removed, immediately cooled and assayed for enzymatic activity against *M. luteus*. As shown in Figure 1A, activation was observed during the initial incubation times. This activation was caused by the formation of 101-succinimide lysozyme, which is a succinimide intermediate with isomerization at Asp101–Gly102, as previously reported (Tomizawa et al., 1994). After incubation for 30 min, the activity decreased at a first order rate (k = 0.7 h^{-1}). In the wide protein concentration range (1–10 mg/mL) examined, irreversible inactivations at pH 4, 100 °C, showed the same patterns, and no precipitates were formed, suggesting that the inactivation did not result from an intermolecular interaction of the protein.

Structural Changes in Lysozyme during the Process of Irreversible Inactivation. In order to investigate structural changes in the lysozyme during the process of irreversible inactivation, discontinuous acid PAGE in the absence of urea was employed according to the literature (Reisfeld et al., 1962) (Figure 1B). The native band (nondenatured; N) gradually decreased while the denatured fractions (denatured; D) increased with incubation time. Nondenatured and denatured bands became broader with incubation time. In the case of the nondenatured fractions, the formation of succinimide derivatives at Asp-Gly (48–49, 66–67, 101–102), which evoked the disappearance of the carboxylate anion (Tomizawa et al., 1994) and a faster migration rate during electrophoresis, caused the broadening of the bands. Moreover, the proportion of nondenatured lysozyme in each incubation time was measured using gel scanning densitometry, and was plotted vs incubation time (Figure 1A). The proportions of the nondenatured lysozyme were well correlated to residual activities. This correlation suggested that inactivation of lysozyme at pH 4, 100 °C, was mainly caused by the production of an irreversibly denatured lysozyme. To confirm this suggestion, nondenatured and denatured lysozymes prepared by incubation for 1.5 h at pH 4, 100 °C, were separated and purified by size-exclusion chromatography on Sephadex G-75

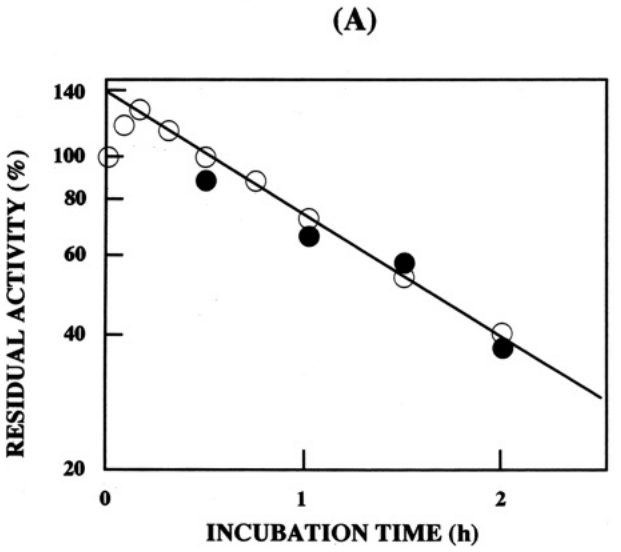

FIGURE 1: (A) Time course of irreversible inactivation of lysozyme at pH 4, 100 °C. Open circles, residual activity; closed circles, proportion of nondenatured lysozyme deduced from (B). (B) Discontinuous acid PAGE at pH 4.3, 4 °C, of lysozyme incubated at pH 4, 100 °C. D and N are denatured and nondenatured lysozymes, respectively.

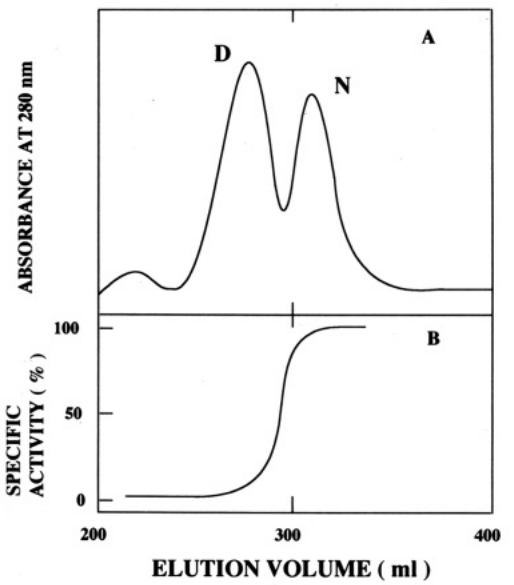

FIGURE 2: (A) Gel chromatography of lysozyme incubated at pH 4, 100 °C, for 90 min on Sephadex G-75 (1.5 × 300 cm), eluted with 0.05 M acetate buffer at pH 4. (B) Specific activity of each fraction.

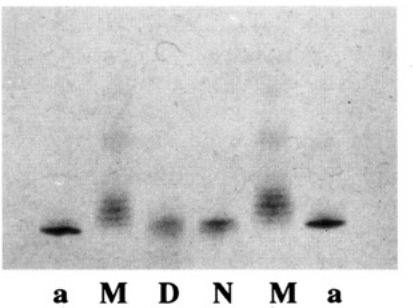

FIGURE 3: Continuous acid PAGE of lysozyme at pH 3.6, 4 °C, in the presence of 8 M urea. Lane a, native; M, the marker of deamidation; D, denatured; N, nondenatured lysozyme. a, D, and N, reduced lysozymes were reacted with iodoacetamide. M, reduced native lysozyme was reacted with an equivalent mixture of iodoacetamide and iodoacetic acid.

eluted with 0.05 M acetate buffer at pH 4. As shown in Figure 2, activities of nondenatured and denatured lysozyme were about 100% and <1%, respectively. This result indicated that irreversible inactivation of the lysozyme was caused by the production of the denatured lysozyme, that existed in the denatured state even in the absence of the denaturing reagent.

Comparison of the Primary Structure of Nondenatured and Denatured Lysozyme. It is possible that the production of irreversibly denatured lysozyme is caused by the chemical reactions of covalent bonds and/or conformational changes attributed to the disruption of noncovalent interactions. Denatured lysozyme at pH 4, 100 °C, was incubated in 6 M Gdn·HCl solution, exhaustively dialyzed against distilled water, and assayed for enzymatic activity. However, the activity of the denatured lysozyme was not recovered at all. Thus, some chemical reactions of covalent bonds clearly contribute to the production of the denatured lysozyme. To examine the covalent modifications, primary structural changes in the nondenatured and denatured lysozyme were compared by amino acid analyses and tryptic peptide mappings. Nondenatured lysozyme and denatured lysozyme were reduced and S-carboxymethylated, and then amino acid analyses and tryptic digestions were carried out. The amino acid compositions of nondenatured and denatured lysozyme were identical with that of the native lysozyme. No critical difference between these tryptic peptide mappings was detected, except for the appearance of noiselike minor peaks in tryptic peptides of the denatured lysozyme (data not shown). These results suggested that a particular chemical reaction on a specific residue (e.g., labile tryptophan and methionine residues) did not contribute to the production of the irreversibly denatured lysozyme. Since global information of particular chemical reactions (deamidation of Asn and Gln, disulfide exchange, or racemization) could not be obtained from comparisons of amino acid compositions and tryptic peptide mappings, precise investigations for each chemical reaction are shown below.

Deamidation of Asn and Gln. Ahern and Klibanov (1985) reported that the deamidation of Asn was the cause of the irreversible inactivation of lysozyme at pH 4, 100 °C. If this is the case, there must be a difference in the extent of deamidation between nondenatured and denatured lysozymes. The extents of deamidation of these lysozymes were compared using continuous acid PAGE in the presence of 8 M urea according to the literature (Hollecker & Creighton, 1980). As shown in Figure 3, a gap indicating a difference in the extent of deamidation between nondenatured and denatured lysozymes was not evident. Both fractions showed only some monodeamidated bands. This result indicated that the deamidation of Asn could not be the dominant cause of irreversible inactivation of the lysozyme at pH 4, 100 °C.

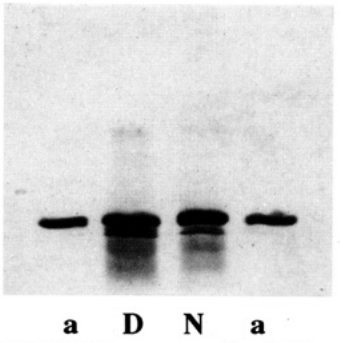

FIGURE 4: SDS–PAGE of lysozymes reduced with mercaptoethanol. D and N are denatured and nondenatured lysozymes, respectively. a, native lysozyme.

Hydrolyses of Asp–X Peptide Bonds. Ahern and Klibanov (1985) reported that hydrolyses of Asp–X peptide bonds were the other cause of the irreversible inactivation of lysozyme at pH 4, 100 °C. A comparison of cleavages of peptide bonds was carried out using SDS–PAGE after reduction of the nondenatured and denatured lysozymes. As shown in Figure 4, molecular weight bands lower than those of the native were observed in both lysozymes. While lower molecular weight bands increased in denatured lysozyme as compared to the nondenatured one, a considerably large band at the intact protein was observed in the denatured lysozyme. Thus, hydrolyses of Asp-X peptide bonds could not be the single cause of irreversible inactivation of the lysozyme at pH 4, 100 °C.

Racemization of Asp and Asn. It was theorized that racemizations of Asp or Asn contributed to irreversible inactivation of a lysozyme at 100 °C (Zhao et al., 1989). A comparison of Asp or Asn racemization between nondenatured and denatured lysozymes was performed using the modified method of Aswad (1984). A small amount of D-Asp was observed in both the nondenatured and denatured lysozymes, while the denatured lysozyme showed a slight increase in the D form compared to the nondenatured lysozyme.

Disulfide Exchange. Disulfide exchange partly contributed to the inactivation of ribonuclease A at neutral pH, 90 °C, and the inactivation of ribonuclease A was suppressed by the addition of $CuCl_2$ or *N*-ethylmaleimide, which inhibits heat-induced disulfide exchange (Zale & Klibanov, 1986). The effects of these disulfide exchange inhibitors were tested on the time course of lysozyme inactivation at pH 4, 100 °C, and the suppression of inactivation was not observed.

Isomerization of Asp-Gly. The aspartylglycyl sequence is easily converted to the β-aspartylglycyl sequence *via* the cyclic imide under mildly acidic conditions, and the formation of the imide increased with increases in the concentration of acetate buffer (Tomizawa et al., 1994). The effect of acetate buffer concentration was tested on the time course of lysozyme inactivation at pH 4, 100 °C (Figure 5). The formation of the cyclic imide derivative and lysozyme inactivation were accelerated with increasing concentration of acetate buffer. This result suggested that isomerization of Asp-Gly might affect the irreversible inactivation of lysozyme at pH 4, 100 °C.

Unfolding of Nondenatured Lysozyme. To investigate the stability against unfolding of the nondenatured lysozyme heated at pH 4, 100 °C, for a specific time, discontinuous acid-PAGE in the presence of urea was carried out. As shown in Figure 6, the nondenatured lysozyme after heating for a long time was considerably destabilized against unfolding, and consisted of the mixture with various stabilities against unfolding.

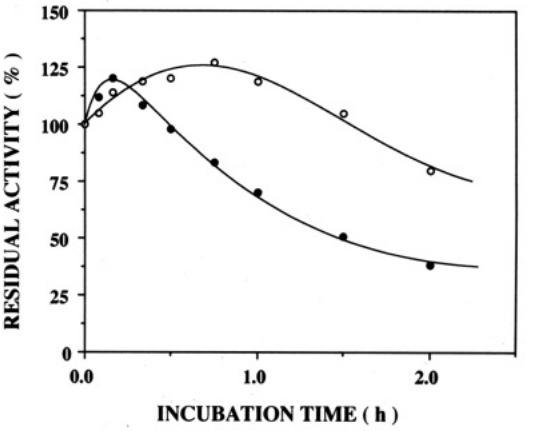

FIGURE 5: Dependence of concentration of acetate buffer on the time course of irreversible inactivation of lysozyme at pH 4, 100 °C. Open circles, in 0.01 M acetate buffer; closed circles, in 0.75 M acetate buffer.

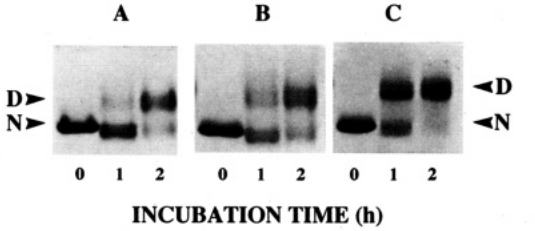

FIGURE 6: Discontinuous acid-PAGE in the presence of urea at pH 4.3, 4 °C, of lysozyme incubated at pH 4, 100 °C. (A) In the presence of 1 M urea; (B) 2 M urea; and (C) 4 M urea. D and N are denatured and nondenatured lysozymes, respectively.

Time Courses of Irreversible Inactivation of Stabilized and Destabilized Lysozyme at pH 4, 100 °C. On the basis of the previously mentioned results, it was postulated that the decrease in stability against unfolding by numerous chemical reactions would lead to the production of an irreversibly denatured lysozyme. If this is the case, the inherent stability of a lysozyme against unfolding should correlate to the rate of lysozyme inactivation. To prove this fact, we chose chemically modified lysozymes which have different stabilities against unfolding. They included the 1–15 CL lysozyme, a stabilized lysozyme derivative that is cross-linked intramolecularly between Lys1 and His15 with the bis(bromoacetamide) derivative of poly(methylenediamine) ($n = 4$); and the 6,127 CAM lysozyme, a destabilized lysozyme derivative that is S-carboxamidomethylated at Cys6 and Cys127, and has three disulfide bonds. Because the modification sites and chemical reaction sites induced at high temperature were different, a good relation between the stability and the irreversible thermoinactivation would be achieved. The time courses of the inactivations of stabilized 1–15 CL, destabilized 6,127 CAM, and native lysozyme were investigated, and the rate constants of the inactivations of these lysozymes were 0.59, 1.56, and 0.70 h^{-1}, respectively (Figure 7). The free energy changes (ΔG) of 1–15 CL, 6,127 CAM, and the native lysozyme measured according to the literature (Inoue et al., 1992) were 11.70, 4.45, and 9.70 kcal/mol, respectively, at pH 5.5, 35 °C. The results indicated that the lysozyme stabilized against unfolding was stable against irreversible thermoinactivation.

DISCUSSION

It was indicated that irreversible thermoinactivation of a lysozyme at pH 4 was caused by the production of an irreversibly denatured lysozyme. From the investigation of

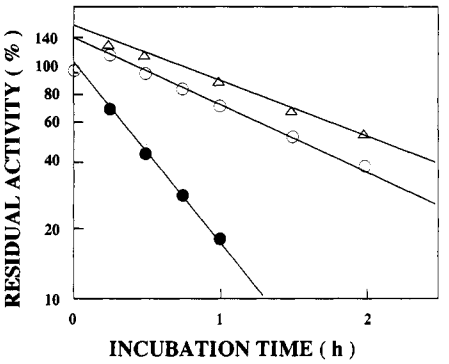

FIGURE 7: Time course of irreversible inactivation of modified lysozymes at pH 4, 100 °C. Open circles, native; closed circles, 6,127 CAM; triangles, 1–15 CL lysozyme.

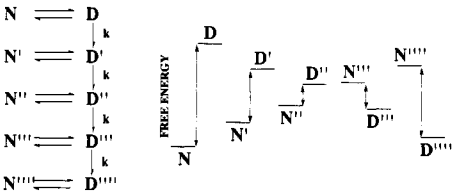

FIGURE 8: Schematic representation of the mechanism of irreversible inactivation of lysozyme at pH 4, 100 °C. k is the rate constant of the averaged chemical reaction. As the averaged reaction proceeds, molecules in the denatured state (D, D′, D″, D‴, D⁗) are stabilized compared with those in the folded state (N, N′, N″, N‴, N⁗).

the production mechanism of an irreversibly denatured lysozyme, the inactivation was not evoked by a single chemical reaction (deamidation of Asn and Gln, racemization of Asp and Asn, cleavage of Asp–X peptide bond, or isomerization of Asp-Gly). We propose the inactivation mechanism as shown in Figure 8. Thus, if multiple chemical reactions are averaged and the averaged reaction has a rate constant with units of k, the equilibrium between N and D will change to that between N′ and D′, - - -, N⁗ and D⁗, as the reaction proceeds. The reacted molecule will be destabilized against unfolding. Until two averaged reactions occur (the equilibrium between N″ and D″), the activity will remain constant. However, after three averaged reactions, a denatured state will become stabilized rather than a folded state, the activity will be completely lost. On the basis of the mechanism, the initial stability against unfolding at moderate temperature correlates with the averaged reaction number which causes irreversible denaturation. As shown in Figure 2, in spite of destabilization by multiple chemical reactions, the activity of lysozyme was maintained, if a folded state as native lysozyme can be retained at that condition. The above discussions are also supported by the results that the main chain cleaved lysozyme at Gly104 is about 90 % active (Yamada et al., 1990) and monodeamidated lysozyme derived from incubation at alkaline pH is about 80% active (unpublished data). On the other hand, the formation of the 101-succinimide lysozyme enhanced the total activity about 20%, and this might compensate for the slight inactivations already mentioned. Thus, the systematic errors in activity evoked by these modifications might be below 20%.

It was indicated that the inherent stability against unfolding was correlated to the rate of thermoinactivation (Figure 7). To analyze the correlation between the accumulation of chemical damages and inactivation, some assumptions were made. Since the chemical reactions mainly occurred in the aspartate residue at pH 4, 100 °C, it is theorized that only seven aspartate residues of the lysozyme are suffering from chemical reactions with the averaged rate constant of k. It is then, assumed that when the number of chemical reactions

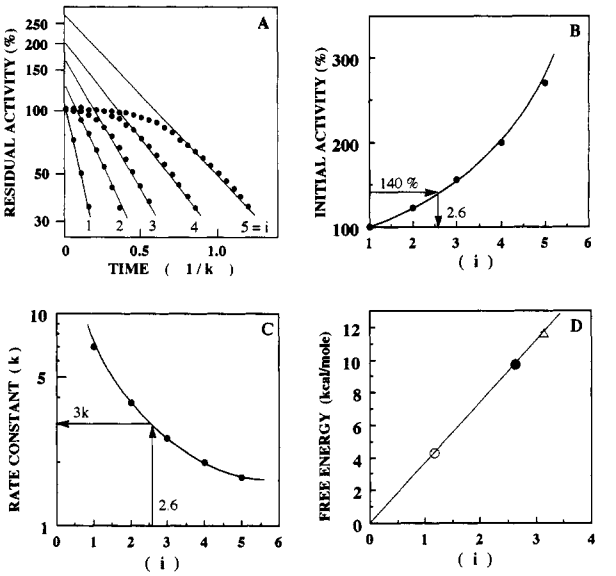

FIGURE 9: (A) Computer-simulated time course of irreversible inactivation of lysozyme using eq 1. i is the number of chemical reactions which could inactivate lysozyme. (B) Correlation between i and the initial activity. The initial activity of each i was determined with the extrapolation to zero time of the first-order line in (A). i (2.6) of native lysozyme was determined from the initial activity (140%) in Figure 1A. (C) Correlation between i and the first-order rate constant. Each rate constant, with units of k corresponding to each i, was determined from (A). To determine k for the averaged chemical reaction, i (2.6) of native lysozyme was used. (D) Correlation between i and the free energy change. The rate constants of 6,127 CAM and 1–15 CL lysozyme, which have units of k, were determined using each inactivation rate constant in Figure 8 and a value of k = 0.233 derived from $3k$ (Figure 9C) = 0.7 (Figure 1A), resulting in $2.53k$ and $6.70k$, respectively. i values of for 6,127 CAM and 1–15 CL lysozyme were determined from the correlation in (C) using the respective rate constant with units of k. Open circle, 6,127 CAM; closed circle, native; triangle, 1–15 CL lysozyme.

is more than i or less than $i - 1$, the activity is completely lost or held, respectively. Thus, i is the number of chemical reactions which cause irreversible denaturation. Therefore, the residual activity (A) of lysozyme is a proportion of the lysozyme suffering from the number of the reactions, that is, less than $i - 1$, as expressed by the equation:

$$A = \sum_{n=0}^{j-1} P_n \qquad (1)$$

where n is the number of chemical reactions and P_n is the proportion of lysozyme suffering from n chemical reactions. According to eq 1, when i was from 1 to 5, the residual activities after appropriate times, which have units of $1/k$, were computed (Figure 9A). Each i was then plotted vs an initial activity determined by extrapolation of the first-order line to zero time (Figure 9B). As shown in Figure 1A, since the initial activity of a native lysozyme was 140%, i of the native lysozyme was estimated to be 2.6 from Figure 9B. Moreover, in Figure 9C, each i was plotted vs the rate constant of inactivation, which has k units, determined from part of the first-order line in Figure 9A. The rate constant of inactivation of native lysozyme was found to be $3k$ units. Since the experimental rate constant of inactivation of native lysozyme in Figure 1A was 0.7 h^{-1}, k, which is the rate constant of the averaged chemical reaction, was found to be 0.233 h^{-1}. On the other hand, in the cases of stabilized 1–15 CL and destabilized 6,127 CAM lysozyme, since the rate constants of inactivation were 0.59 (2.53 k) and 1.56 h^{-1} (6.70 k), respectively, i *values* of these lysozymes were 3.1 and 1.1,

respectively, from Figure 9C. Thus, we could obtain the correlation between the inherent stability against unfolding and thermoinactivation of the lysozyme. Each free energy change (ΔG) of the modified and native lysozymes at pH 5.5, 35 °C, was plotted vs each i. As shown in Figure 9D, the plot of ΔG as a function of i gave a straight line with a slope of 3.8 kcal/mol and an intercept of around 0. The result could be interpreted that 3.8 kcal/mol of the free energy decreases for every averaged chemical reaction with a 0.233 h^{-1} rate constant, and when the free energy change is around 0, the activity cannot be detected.

On the basis of some assumptions, the linear correlation between the free energy changes (ΔG) and the numerical values (i) of the averaged chemical reaction, which leads to irreversible denaturation, was obtained (Figure 9D). In addition, the correlation suggested that the rate constant ($k = 0.233\ h^{-1}$) of the averaged chemical reaction, the destabilized free energy (3.8 kcal/mol) by one averaged chemical reaction, was equivalently adapted to the modified and native lysozymes. Since the most stable 1–15 CL lysozyme completely lost its enzymatic activity at 100 °C (Ueda et al., 1985), it is reasonable to think that the modified and native lysozymes employed here are completely in the denatured state at that temperature (100 °C). It is supposed that, in spite of the inherent stability against unfolding, all lysozymes in the completely denatured state are equivalently affected by the chemical reactions.

We conclude that the irreversible thermoinactivation at pH 4 is the result of the accumulation of chemical reactions, which mainly occur at aspartic acid and cause denaturation of the protein. Since these chemical reactions usually take place in the unfolded state, the stable proteins can maintain a folded conformation to prevent the accumulation of chemical reactions. Thus, stabilization can suppress these irreversible inactivations in double meanings.

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