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A Structural Phase of Heat-Denatured Lysozyme with Novel Antimicrobial Action

Hisham R. Ibrahim,*,† Shenji Higashiguchi,‡ Lekh R. Juneja,‡ Mujo Kim,‡ and Takehiko Yamamoto§

Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, Kagoshima 890, Japan, Central Research Laboratories, Taiyo Kagaku Company, Ltd., Yokkaichi, Mie 510, Japan, and Department of Biotechnology, Fukuyama University, Fukuyama, Hiroshima 729-02, Japan

The structure and antimicrobial function of hen egg white lysozyme was investigated by means of thermal denaturation at 80 °C (pH 7.2), which leads to irreversible denaturation. With an increase in the heating time (up to 30 min) of lysozyme, the soluble fraction showed progressive decrease in its enzyme activity that coincided with the formation of a slower migrating band on the acid PAGE. Fluorescence spectra revealed that, as the extent of denaturation increases, the surface hydrophobicity and the exposure of tryptophan residues were greatly promoted. In parallel to these conformational changes of lysozyme there has been consistent increase in its antimicrobial activities against Gram-negative bacteria, with no detrimental effect on its inherent action to Gram-positive bacteria. Interestingly, lysozyme heated for 20 min, devoid of enzyme activity (HDLz), killed *Escherichia coli* K12 in a dose-dependent manner, while its bactericidal activity to *Staphylococcus aureus* was almost similar to that of the native lysozyme. The binding capacity of HDLz to membrane fractions of *E. coli* K12 was greatly promoted, particularly to the inner membrane, as determined by ELISA. The HDLz permeabilized liposomal membranes made from *E. coli* phospholipids, as demonstrated by calcein efflux, in a protein concentration-dependent manner. Good correlations between the degree of heat inactivation of lysozyme (or dimerization), increased hydrophobicity, and enhanced bactericidal activity against Gram-negative *E. coli* K12 were observed. The results of this study, first of all, suggest that susceptibility of Gram-negative or even Gram-positive bacteria to lysozyme is independent of enzymatic activity. It is likely that denatured lysozyme, e.g., the dimeric form, has an intrinsic structural motif which is generally lethal to the bacteria through membrane perturbation.

Keywords: *Lysozyme; denaturation; conformational changes; antimicrobial action; membrane interaction; liposome*

INTRODUCTION

Lysozyme is a ubiquitous enzyme that belongs to a class of enzymes that lyse the cell walls of certain Gram-positive bacteria, as they split the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan in the bacterial cell wall. Lysozyme is widely distributed in various biological fluids and tissues, including avian egg, plant and animal secretions, tears, saliva, and respiratory and cervical secretions, and is secreted by polymorphonuclear leukocytes (Jolles and Jolles, 1984). Although lysozyme is one of the simplest enzymes—its structural and physiological (Blake et al., 1965; Imoto et al., 1972) as well as enzymatic (Jolles and Jolles, 1984; Muraki et al., 1988) characteristics have been considerably elucidated—its structure—bactericidal relationship has yet to be unraveled. This is because it has long been believed that the antimicrobial action of lysozyme would merely be attributed to its catalytic function on bacterial cell wall. In avian egg, lysozymes are thought to function as bactericides, although conclusive evidence on this point is lacking. A rise in the pH of the albumin begins once the egg is laid. The pH increases with the time from 7.6 to 9.5 (Burley and Vadehra, 1989). At this range of pH lysozyme is enzymatically inactive (Ibrahim et al., 1994b; Muraki et al., 1988) but substantially bactericidal, particularly at pH 9.5 (Wang and Shelef, 1991). Lysozyme has been reported to exist as a reversible dimer between pH 5.0 and 9.0, and the active site is involved in this type of dimerization (Sophianopoulos and Van Holde, 1964; Sophianopoulos, 1969). Back (1984) found that when hen eggs are stored, an irreversible dimeric form of lysozyme is generated. In line with these physicochemical properties of lysozyme, the pH- and temperature-dependent phase transition in lysozyme crystals has been reported (Jolles and Jolles, 1984). Lysozyme has also been found to possess an ability to inactivate certain viruses, regardless of its enzyme activity, by forming an insoluble complex (Hasselberger, 1978). The basicity of the lysozyme molecule has been shown to be important in resisting microorganisms (Ng and Garibaldi, 1975). In spite of a wealth of circumstantial evidence, it is still not certain that the enzymatic activity of lysozyme makes a large contribution to the defense against infections, because the lytic action of lysozyme does not kill the susceptible bacteria under physiological conditions, osmotically balanced

* Address correspondence to this author at the Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, 21-24 Korimoto 1-Chome, Kagoshima 890, Japan (telephone +81-99-285-8658; fax +81-99-285-8525; e-mail hishamri@chem.agri.kagoshima-u.ac.jp).

† Kagoshima University.

‡ Taiyo Kagaku Co. Ltd.

§ Fukuyama University.

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(Brooks et al., 1991). Indeed, the interrelationships between structural elements and their influence on the antimicrobial behavior or the biological function of the multifunctional lysozyme are not understood.

Previously we have reported that the antimicrobial activity of lysozyme can be switched to include Gram-negative bacteria through genetic fusion of a hydrophobic peptide to the C terminus (Ibrahim et al., 1992, 1994b) or by chemical modification of lysyl residues with saturated fatty acids (Ibrahim et al., 1991, 1993). The data were interpreted that increased surface hydrophobicity associated with conformational changes contributes to a large extent to the enhanced antimicrobial action of the modified lysozymes. More recently, we have found that when lysyl residues of lysozyme were reacted with a phenolic aldehyde, perillaldehyde, considerable conformational changes occurred in the lysozyme molecule, and the antimicrobial activity of the enzyme was greatly promoted against both Gram-negative and Gram-positive bacteria (Ibrahim et al., 1994a). While conducting experiments on the perillaldehyde-modified lysozyme, we found a surprising phenomenon that heating of the unmodified lysozyme produces a potent bactericidal molecule against various kinds of bacteria. In the present study, we report that lysozyme thermally denatured at 80 °C (pH 7.2) has a high tendency to dimerize and this dimeric form is bactericidal against either Gram-positive or Gram-negative bacteria, regardless of the residual enzyme activity. Conversion of lysozyme from monomeric to dimeric form was proportional to the degree of inactivation. A good correlation between the degree of inactivation and the promoted antimicrobial effects against the rough strain *Escherichia coli* K12 was obtained. The bactericidal effects of heat-inactivated lysozyme were interpreted in the light of its capability to bind bacterial membrane fractions and its ability to disrupt the integrity of liposomal membranes made from *E. coli* phospholipids.

MATERIALS AND METHODS

Materials. Hen egg white lysozyme was purified from fresh egg white and recrystallized five times as previously reported (Alderton and Fevold, 1946). *Micrococcus lysodeikticus*, a substrate of lysozyme, and ovalbumin were purchased from Sigma (St. Louis, MO). Calcein and *cis*-parinaric acid (CPA) were purchased from Wako Pure Chemicals (Osaka, Japan). Anti-lysozyme IgG monoclonal antibody was provided by Dr. Y. Yamaguchi (Fukuyama University, Fukuyama, Japan). Alkaline phosphatase coupled to goat anti-mouse IgG was from Zymed (San Francisco, CA). Phospholipids were isolated from *E. coli* K12 according to the method described previously (Viitanen et al., 1986). The inner (cytoplasmic) and outer membranes were isolated from *E. coli* K12 according to the method of Yamato et al. (1975). Unless otherwise stated, all other chemicals were of the highest grade commercially available.

Bacteria and Growth Medium. Brain heart infusion (BHI), nutrient, desoxycholate hydrogen sulfide lactose (DHL), and MacConkey medium were from Difco (Detroit, MI). As test microorganisms for antimicrobial assay *E. coli* IFO 3301, *Proteus mirabilis* IFO12668, *Salmonella enteritidis* IFO 3313, *Bacillus cereus* IFO 13690, and *Staphylococcus aureus* IFO 14462 were obtained from the Institute of Fermentation Osaka (Japan). *Streptococcus mutans* ATCC 25175 was from the American Type Culture Collection (Rockville, MD).

Thermal Denaturation of Lysozyme. Heat-induced inactivation of the lysozyme was performed by incubating 2 mL of 1 mg of lysozyme per milliliter in 10 mM sodium phosphate buffer (pH 7.2) in a screw-capped tube at 80 °C for various lengths of time. Periodically, samples were removed and placed in an ice−water bath for 10 min. Lysozyme aggregates formed during heating were removed by centrifugation (3000*g* for 15 min). The clarified solution was then used in the following experiments after the determination of the protein content according to the Lowry method as modified by Miller (Miller, 1959). In some cases, supernatant thus obtained was dialyzed in a Spectra/por dialysis tube (MWCO = 6000−8000; Spectrum Medical Inc., Los Angeles) against distilled water and lyophilized.

Acid Polyacrylamide Gel Electrophoresis (PAGE). The time course of denaturation of lysozyme was chased by using discontinuous acid PAGE according to the method of Lewis et al. (1968). Protein samples were electrophoresed at a constant current of 6 mA per plate for 4 h on nondenaturing 15% polyacrylamide gels at pH 4.5. Protein bands were visualized by Coomassie brilliant blue R-250.

SDS−PAGE. Protein samples were incubated for 2 h in 0.125 M Tris-HCl buffer (pH 6.8) containing 2% SDS and 15% glycerol. Electrophoresis was performed according to the method of Laemmli (1970), and then the gels were stained with Coomassie brilliant blue R-250.

Assay of Lysozyme Activity. The lysis of *M. lysodeikticus* cells was determined by monitoring the decrease in turbidity of a 1.9 mL cell suspension (170 μg of dry cells/mL) in 50 mM sodium phosphate buffer (pH 6.2) following the addition of 100 μL of lysozyme solution (20 μg/mL) after equilibration to achieve constant absorbance (0.75−0.8). The decrease in absorbance at 600 nm (25 °C) was monitored using a Shimadzu MPS-2000 recording spectrophotometer. The activity is presented as the rate of decrease in absorbance per minute of the initial velocity of reaction.

Assay of Antibacterial Activity. The bacteria were cultivated in BHI broth at 37 °C for 16 h with shaking and then diluted 1:100 in BHI broth. The diluted culture was further incubated for 3−4 h until mid-logarithmic phase was reached, based on absorbance measurements at 675 nm. Bacterial pellets (3000*g* for 7 min at 4 °C) were washed two times and resuspended in 10 mM sodium phosphate buffer (pH 7.2) or 0.65% peptone broth (pH 7.4) (Difco), to give absorbance at 675 nm of 0.002 cm^{-1}. One milliliter of the bacterial suspension was mixed with an equal volume of various concentrations of lysozyme in the same medium. The mixture was incubated at 37 °C for 1 h. A 100-μL portion or dilutions (in 0.85% NaCl) were plated onto nutrient agar plates for all strains tested. *E. coli* K12 and *S. enteritidis* were further plated onto MacConkey and DHL agar, respectively. Colony forming unit (CFU) values were obtained after the plates were incubated at 37 °C for 24 h. All assays were performed in triplicate, and the results, unless otherwise notified, are presented as percent survival ± SE to the controls, which consisted of bacteria incubated alone.

Preparation of Membrane Fractions of *E. coli* K12. The inner (IM) and outer (OM) membranes from the crude membranes obtained by sonication of osmotically lysed bacteria as described (Yamato et al., 1975) were separated using sucrose gradients. Membrane fractions were then dialyzed thoroughly against distilled water and kept under nitrogen at −30 °C. Protein and phospholipid contents in each fraction were determined as previously reported (Miller, 1959; Raheja et al., 1973).

Binding of HDLz to Membrane Fractions of *E. coli* K12. Microtiter plates (Nunc, EIA flat plate I) were coated with 100 μL/mL of the respective membrane fraction dilution (10 μg of protein/mL) in 0.1 M Na_2CO_3 (pH 9.6). Plates were allowed to stand at 37 °C for 3 h. Controls consisted of only buffer. The plates were then rinsed thoroughly with wash buffer PBS-Tw (0.05 % Tween-20 in 150 mM phosphate saline buffer, pH 7.2) and air-dried. Excess binding sites were then blocked by adding 200 μL of PBS-Tw containing 0.1% ovalbumin to each well for 1 h at 37 °C. After the wells were rinsed with PBS-Tw, 100 μL lysozyme dilutions in PBS-Tw were added to each well. The binding of lysozyme was allowed to proceed for 1 h at 37 °C. The plate was then washed thoroughly with PBS-Tw. A 100-μL aliquot of mouse anti-lysozyme IgG monoclonal antibody dilution (10 μg/mL) in PBS-Tw was added to each well and incubated for further 1 h. The

anti-lysozyme IgG was rinsed out with PBS-Tw and replaced for 1 h with 100 μL/well of alkaline phosphatase-conjugated goat anti-mouse IgG diluted 500 times in PBS-Tw as recommended by the manufacturer (Zymed). The plate was then incubated for 1 h at 37 °C. The solution was flicked out, and the plate was washed three times with PBS-Tw. Finally, 100 μL/well of *p*-nitrophenyl phosphate disodium in diethanolamine buffer (pH 9.8) (1 mg of PNPP/mL) was added and incubated at 25 °C. When sufficient color had developed (approximately 20 min), the reaction was stopped by the addition of 50 μL/well of 2 N NaOH. Absorbance at 405 nm was determined in a microtiter plate reader. Mean absorbance and standard errors of the means were calculated, after the value of controls was subtracted, from four replicates of each well.

Preparation of Calcein Liposomes. Calcein-loaded liposomes made from *E. coli* phospholipids were prepared as follows. A 100-μL aliquot (50 mg dry weight of lipids/mL of argon-saturated 2 mM β-mercaptoethanol) was added to 900 μL of 60 mM calcein solution (50 mM potassium phosphate buffer, pH 7.2) in a microfuge tube. The mixture was sonicated for 2 s at maximum output followed by a 3-s pause. The cycle was repeated until the turbid suspension had been clarified. The suspension was centrifuged at 356000*g* for 1 h at 4 °C. The liposome pellet was gently resuspended in 50 mM potassium phosphate buffer (pH 7.2), and centrifugation was repeated. Finally, the liposome pellet was resuspended in the same buffer and centrifuged for 3 min at 10000*g* to remove any multilamellar vesicles. The calcein-loaded liposomes were kept on ice and used within 3 h in the following experiments.

Calcein Release from Liposomes by HDLz. To 1 mL of 50 mM sodium phosphate buffer (pH 7.2) was added 2 μL of calcein-loaded liposome preparation (final 100 μg of phospholipids/mL) in a fluorescence spectrophotometer cuvette. An aliquot (30 μL) of the lysozyme solution (different protein concentrations in 10 mM sodium phosphate buffer, pH 7.2) was added, and fluorescence change was monitored at 25 °C in a Hitachi F-3000 fluorescence spectrophotometer of excitation at 495 nm and emission recorded at 520 nm. Complete release of calcein from liposome was achieved by the addition of Triton X-100 (final 0.1%). The percent leakage of liposome was presented as

$$\% \text{ leakage} = [(F_t - F_0)/(F_f - F_0)] \times 100$$

where F_0 and F_t are the fluorescence intensities before and 1 min after of liposome addition, respectively. F_f is the fluorescence intensity after addition of Triton X-100.

Determination of Surface Hydrophobicity. Surface hydrophobicity of lysozyme was determined according to the method described by Kato and Nakai (1980) using the fluorescence probe CPA. Ten microliters of an ethanolic solution of CPA was added to 2 mL of various lysozyme concentrations in 10 mM phosphate buffer (pH 7.2). The mixture was excited at 325 nm and the relative fluorescence intensity recorded at 420 nm in a Hitachi F-3000 fluorescence spectrophotometer. Hydrophobicity is presented as initial slope (S_0), which was calculated from the plot of fluorescence intensity versus protein concentration.

Estimation of Free Sulfhydryl Groups. To determine the extent of cleavage of disulfide bonds of lysozyme upon heating, the free thiol groups were determined by using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to the procedure of Ellman (1959).

Fluorescence Spectra. Fluorescence spectra were recorded in a Hitachi F-3000 fluorescence spectrophotometer, using 1-cm square cuvettes thermostated at 25 °C. Protein solution (0.1 mg/mL of 10 mM sodium phosphate buffer, pH 7.2) was excited at 280 nm, and the emission was recorded at right angles to the excitation with a 5-nm bandwidth in the range 300−400 nm.

RESULTS

Time Course of Thermal Inactivation of Lysozyme. Increasing durations of exposure of lysozyme to 80 °C at pH 7.2 showed gradual decrease in the amount of native protein band (N) with concomitant increase in the intensity of the denatured band (D) as shown in the acid PAGE patterns (Figure 1A). Addition of 4 M urea into the polyacrylamide gel did not resolve this denatured form (data not shown). However, on nonreducing SDS−PAGE this denatured form was almost dissociated; it showed the same mobility as the native lysozyme (0 time) with the presence of a marginal amount of undissociated oligomeric forms (Figure 1B), indicating that the nature of the chemical forces involved in the formation of such oligomeric form are mainly noncovalent interactions with a very small contribution of intermolecular disulfide interchange reactions. Strikingly, when the native enzyme or HDLz (a completely inactive form) was reduced and their sulfhydryl groups were blocked by S-aminoethylation (RAE-NLz and RAE-HDLz, respectively), the two lysozyme types exhibited the same electrophoretic mobilities but migrated more slowly than the nontreated lysozyme (NLz) on nondenaturing acid PAGE (Figure 1C). The results suggest that even the chemically denatured lysozyme tends to dimerize similarly as the heat-denatured ones. Since sulfhydryl groups of the S-aminoethylated lysozymes are not available for disulfide interchange, the important contribution of the noncovalent interactions between denatured molecules can be demonstrated. The progressive denaturation (disappearance of N bands) of lysozyme was accompanied by proportional loss in the enzyme activity with the heating time, and enzyme activity was completely and irreversibly abolished upon heating for 20 min (Figure 1D). Although the rate of inactivation was very fast under the denaturation condition employed, we were able to obtain various forms of lysozyme with different degrees of denaturation.

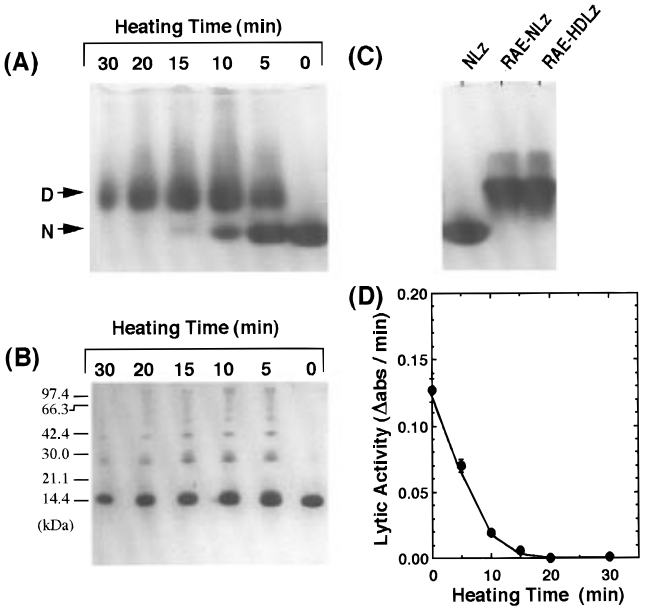

Figure 1. Time course of irreversible denaturation of lysozyme. Samples of lysozyme heated at 80 °C for various lengths of time were applied to 15% polyacrylamide nondenaturing acid−PAGE (A) or nonreducing SDS−PAGE (B). (C) Nondenaturing acid−PAGE of native lysozyme (NLz) or RAE-NLz and RAE-HDLz, whose disulfide bonds were eliminated. (D) Loss of the enzymatic activity of lysozyme as a function of the extent of heat denaturation. Experiments were performed in replicate measurements on multiple days.

Structural Changes Accompanying Heat Denaturation. Lysozyme has compact tertiary structure

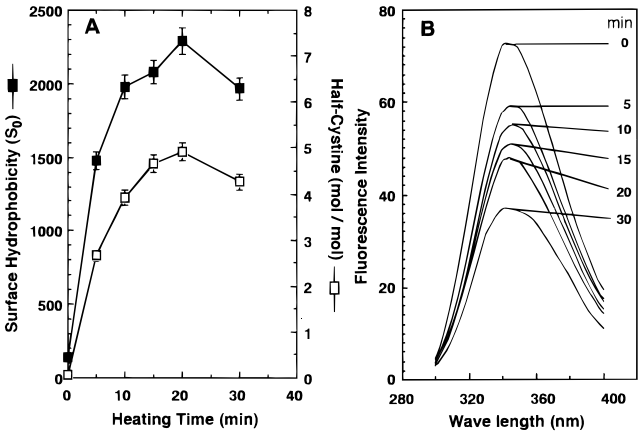

Figure 2. Changes of structural properties of lysozyme as a function of heating time: (A) heat-induced changes in surface hydrophobicity (solid squares) and free sulfhydryl groups (open squares) of lysozyme; (B) fluorescence spectral changes of lysozyme as a function of heating time at 80 °C. Emission was recorded upon excitation at 280 nm. Heating time (minutes) is indicated on the right of each spectrum.

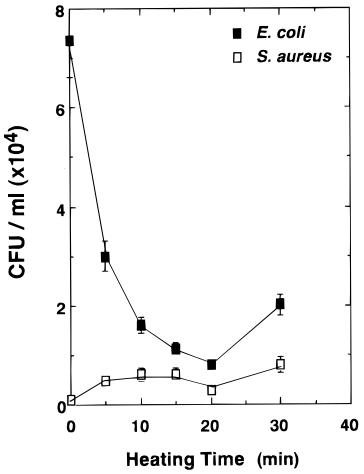

Figure 3. Bactericidal action of lysozyme to *E. coli* K12 and *S. aureus* as a function of heating time at 80 °C. The protein (50 μg/mL) was incubated with *E. coli* (solid squares) or *S. aureus* (open squares) in 2 mL of 10 mM sodium phosphate buffer (pH 7.2) for 1 h at 37 °C. CFU values were obtained on nutrient agar plates after incubation for 24 h. The initial levels of viability were 10^5 CFU/mL (*E. coli*) and 2×10^5 CFU/mL (*S. aureus*). The vertical bars indicate the mean values of triplicate counts.

maintained by four disulfide bridges (Cys6−Cys127, Cys30−Cys115, Cys64−Cys80, and Cys76−Cys94) cross-linking the polypeptide chain (Blake et al., 1965). Denaturation of lysozyme at temperatures higher than its denaturation temperature (72 °C) would result in cleavage and/or isomerization of the intramolecular disulfide bonds. To investigate the possible cleavage of disulfide bonds of lysozyme by heating, free sulfhydryl groups were determined. There has been progressive exposure of free cysteines with an increase in the heating time of lysozyme up to 20 min (Figure 2A, open squares). Heating of lysozyme for 10 min was sufficient to cleave two disulfide bonds (exposure of four cysteines per monomer), where no further release of cysteines was detected with increasing heating time. Similar results were obtained by measuring the increase in surface hydrophobicity (Figure 2A, solid squares) of lysozyme heated for different periods of time.

Intrinsic fluorescence emission spectra of lysozyme as a function of heating time are shown in Figure 2B. Quenching of fluorescence emission intensity (F_{max}) with increasing heating time was observed. An increase (red shift) in the wavelength of maximum emission (λ_{max}) was observed with heated lysozyme for 5, 10, and 15 min, while λ_{max} remained unchanged at 340 nm for the heated lysozyme for 20 and 30 min. Quenching of F_{max} of protein is attributed to conformational changes leading to the exposure of tryptophan groups to the polar environment (Mills, 1976). The spectral changes associated with heating of lysozyme indicate substantial conformational changes in heat-denatured lysozyme. Since this measurement was carried out in aqueous solution, the spectral changes in Figure 2B indicate the irreversibility of denaturation. If the denaturation was reversible, in aqueous solution the exposed hydrophobic tryptophan residues would attempt to bury in the interior of molecule, leading to refolding. It has been reported (Walker et al., 1967) that although a fluorophore may be buried in the hydrophobic interior of the molecule, it may still form an excited-state complex "exciplex" with a polar residue, which has a λ_{max} of the order of 350 nm. The red shift in the λ_{max} from 340 to 350 nm observed with an increase in the heating time up to 15 min of lysozyme could be due to the formation of an exciplex. Heating for more than 15 min may result in complete exposure of fluorophore (Trp) to the surface of HDLz molecule.

Antimicrobial Activities of Denatured Forms of Lysozyme. Having obtained various forms of lysozyme with different enzyme activities, we investigated the antimicrobial activities against Gram-negative (rough strain, *E. coli* K12) and Gram-positive (*S. aureus*) bacteria. As shown in Figure 3, severe reduction in the CFU of *E. coli* was observed with increases in heating time up to 20 min. Although heating resulted in marginal decrease in the antimicrobial activity of lysozyme against *S. aureus*, strong activity is retained at any heating time up to 30 min. Interestingly, lytic activity of lysozyme is lost by heating for 20 min or more, but antimicrobial activity against Gram-positive bacteria does not parallel enzyme inactivation, indicating that the conformationally altered lysozyme exerts antimicrobial action regardless of its enzymatic activity. Increasing the degree of denaturation of lysozyme consistently increased the antimicrobial effect to Gram-negative *E. coli*.

To further verify the antimicrobial potency of the heat-denatured lysozyme, the antimicrobial activity against *E. coli* and *S. aureus* was evaluated as a function of protein concentration of lysozyme heated for 20 min (HDLz). As shown in Figure 4A, the native lysozyme (solid circles) caused dramatic decrease in the CFU of *S. aureus* with increasing protein concentration up to 50 μg/mL, where it became constant. HDLz showed approximately similar activity, but more pronounced at low protein concentrations (Figure 4A, open circles). On the other hand, HDLz was substantially bactericidal to *E. coli* (Figure 4B, open circles) compared with the activity of the native lysozyme (Figure 4B, solid circles). Additionally, HDLz showed a dose-dependent bactericidal action. These results clearly indicate that the inactivated lysozyme is a potent bactericide to Gram-negative *E. coli* without loss in its inherent bactericidal activity to Gram-positive bacteria. To pursue this intriguing fact, we tested the HDLz against different bacteria. As shown in Table 1, 100 μg/mL HDLz was very potent in killing Gram-negative *E. coli*, *P. mirabilis*, and *S. enteritidis* and Gram-positive *S.*

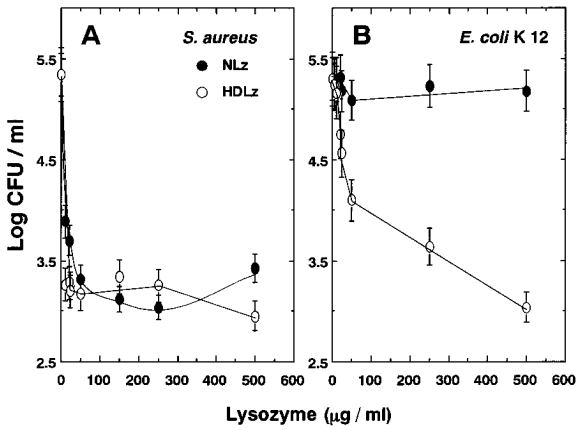

Figure 4. Effect of HDLz concentration on bacterial viability. Bacteria, *S. aureus* (A) and *E. coli* K12 (B), were exposed to native (NLz) and HDLz lysozymes. The lysozyme concentration varied from 5 to 500 μg/mL, and incubation was for 1 h at 37 °C in 10 mM sodium phosphate buffer (pH 7.2). CFU values were obtained on nutrient agar plates after incubation for 24 h. The vertical bars indicate the means of quadruplicate counts.

Table 1. Bactericidal Action of Native Lysozyme (NLz) and Lysozyme Heat-Denatured for 20 min at 80 °C (HDLz)

bacteria	% survival of bacteria[a] NLz	HDLz
Gram-positive		
S. aureus	0.85 ± 0.10	0.76 ± 0.10
S. mutans	18.60 ± 2.80	0.56 ± 0.05
B. cereus	0.01 ± 0.05	0.01 ± 0.06
Gram-negative		
E. coli	72.70 ± 5.40	13.0 ± 1.82
P. mirabilis	61.20 ± 3.40	25.4 ± 2.27
S. enteritidis	97.50 ± 2.10	59.2 ± 1.75

[a] Data are expressed as percent survival (± SE) to the control bacteria (no lysozyme added). Lysozymes at a concentration of 100 μg/mL were incubated with bacteria in the mid-log phase for 1 h at 37 °C in 0.65% peptone broth (pH 7.4). Initial levels of viability for test suspensions ranged from 1.0×10^5 to 5.0×105 CFU/mL.

aureus, *B. cereus*, and *S. mutans*. Surprisingly, HDLz (devoid of enzymatic activity) showed much stronger bactericidal activity to *S. mutans* than the native lysozyme.

Binding of HDLz to *E. coli* Membranes. To study the ability of HDLz to interact with bacterial membranes, HDLz was allowed to interact with microtiter plates coated with purified IM or OM from *E. coli* K12. The results of this experiment are shown in Figure 5. The HDLz showed markedly promoted binding capacity to both membrane fractions, in a dose-dependent manner, compared with native lysozyme. It should be noted that the capacity of HDLz to bind the inner membrane was greater than its capacity to interact with the outer membrane. The interaction profile of HDLz with the IM fraction exhibits linear and progressive increase up to the maximum protein concentration used in this experiment (25 μg/mL). These results demonstrate that the promoted antimicrobial activity of the denatured lysozyme may be attributed to its enhanced affinity to the bacterial membrane, in particular, the IM which is known to be the killing site of bacteria. HDLz also showed very high reactivity with purified lipopolysaccharides of *E. coli* compared to NLz, as demonstrated by ELISA (data not shown).

Permeabilization of *E. coli* Phospholipid Liposomes by HDLz. The strong binding affinity of HDLz

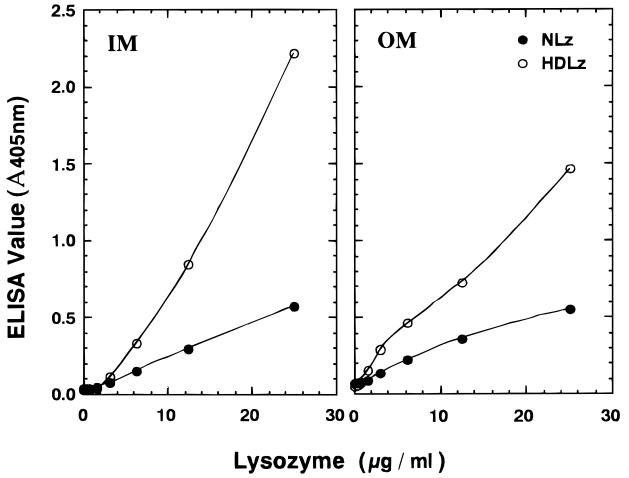

Figure 5. Binding of HDLz to the isolated outer (OM) and inner (IM) membranes of *E. coli* K12. The indicated concentrations of NLz or HDLz were allowed to interact with microtiter plates coated with purified membrane fractions for 1 h at 37 °C. The binding was measured by ELISA using mouse anti-lysozyme monoclonal IgG and goat anti-mouse serum polyclonal IgG coupled to alkaline phosphatase. *y*-axis scales refer to color developed at 405 nm. Solid circles designate native lysozyme; open circles designate HDLz.

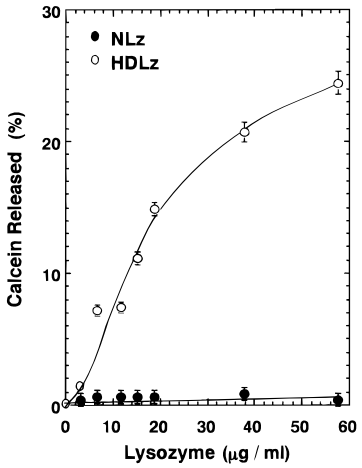

Figure 6. HDLz-induced permeability of liposomes made from *E. coli* phospholipids in 50 mM sodium phosphate buffer (pH 7.2) at 25 °C. Percent leakage of calcein after 1 min is plotted as a function of lysozyme (NLz, solid circles; HDLz, open circles) concentration. Leakage of calcein was monitored in a fluorescence spectrophotometer at an excitation wavelength of 495 nm, and emission was recorded at 520 nm. One hundred percent leakage refers to the release obtained by addition of Triton X-100 (final 0.1%).

to the bacterial membranes suggests that this interaction may distort the integrity of the lipid bilayer, thus inhibiting many biosynthetic reactions occurring at this site. To test this hypothesis, leakage of calcein loaded in the inner aqueous space of liposomes made from *E. coli* phospholipids was measured as a function of HDLz concentration. Extent of leakage after 1 min was determined in this study. Figure 6 shows a typical profile of HDLz-induced leakage of calcein. Addition of the native lysozyme did not cause any detectable leakage (solid circles), and results were similar to those for untreated liposomes (controls). The HDLz induced substantial leakage of calcein from the liposomes (open circles). Calcein efflux caused by HDLz implies the formation of a structural defect or a pore through which the relatively large dye molecule can pass. It appears, therefore, that during heating for denaturation certain

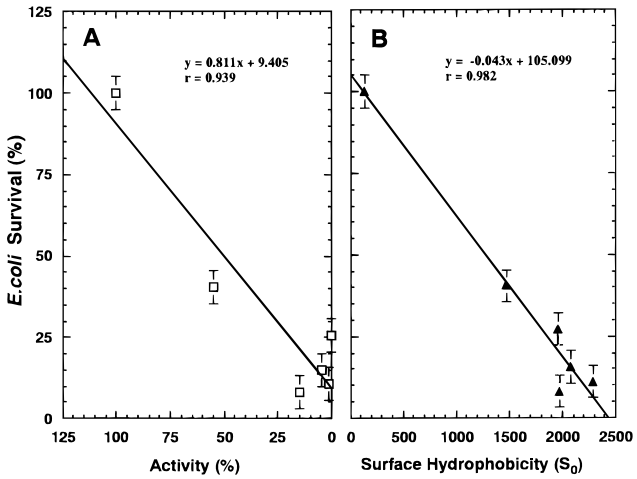

Figure 7. Viability of *E. coli* versus residual activity (A) and change of surface hydrophobicity (B) of lysozyme heat denatured to various degrees at 80 °C. Symbols represent different heating times (0−30 min). Viability of *E. coli* was determined in 10 mM phosphate buffer (pH 7.2) in the presence of 50 μg/mL lysozyme type. Straight lines were determined by least-squares analysis. Correlation coefficients and standard error bars are shown. Each point represents the average of duplicates of three independent experiments.

conformational changes occur in the lysozyme molecule that result in a strong interaction between HDLz and the lipid bilayer. Most probably the conformation (such as dimerization with increased exposure of hydrophobic tryptophan residues) of HDLz is important for pore formation in the lipid bilayer.

Figure 7 shows the relationships between lytic activity or surface hydrophobicity and antimicrobial potency against *E. coli* K12 of lysozyme heated for various periods of time. A good positive correlation between the bactericidal effects and the surface hydrophobicity was obtained. Consistently, lytic activity inversely correlated with the bactericidal effects of lysozyme. The results clearly indicate that, as the enzyme activity decreases, the bactericidal effects to *E. coli* increase.

DISCUSSION

The mechanism by which lysozyme kills Gram-positive bacteria is known (peptidoglycan degradation). The results obtained in the present study demonstrate, first of all, that heat-denatured lysozyme, devoid of enzymatic activity, retains antimicrobial activity against Gram-positive bacteria and exhibits enhanced bactericidal effects to Gram-negative bacteria. The native lysozyme molecule is ovoid and consists of two domains or lobes, between which lies the active site. The first domain consists of residues 40−88 and is mostly antiparallel β-sheet. The second domain is made up of residues 1−39 and 89−129, and its secondary structure is largely α-helical (Young et al., 1994). The hydrophobic core of hen egg white lysozyme contains a set of spatially arranged hydrophobic amino acid residues (Trp62, Trp63, Leu83, Leu84, Trp108, Leu17, Tyr 20, Tyr23, Trp28, Ile98, Met105, and Trp111). Trp 62, Trp63, and Trp108 are spatially close to the disulfide bonds of Cys64−Cys80 and Cys76−Cys94 and are involved in the catalytic function of lysozyme. Therefore, cleavage of such disulfide cross-links would expose more hydrophobic Trp residues to the surface of lysozyme molecule. There are two Trp residues adjacent to the S−S linkage 64/80, and cleavage of this bond would expose more buried Trp to the solvent. Fluorescence quenching at 340−350 nm (Figure 2B) is attributed to increased exposure of a portion of the three buried Trp residues of lysozyme (Yu, 1974). The exposed Trp (Figure 2B) leading to increased surface hydrophobicity (Figure 2A) and with the presence of high surface basicity of lysozyme (Blake et al., 1965) can be attributed to increased membrane interaction (Figure 5) and the subsequent disruption (Figure 6), membrane permeabilization.

HDLz exists mainly as a dimer with some high molecular weight oligomer (Figure 1A). The secondary structures of the HDLz were found to consist mainly of β-sheets, as revealed by circular dichroism (CD) analysis (data not shown). It is conceivable that intermolecular β-sheets are the major structures holding the two denatured monomers in the dimeric form. In this conformation, the structural integrity of the active site falls apart and enzymatic activity becomes zero. This conformation of inactive lysozyme was found to be potently bactericidal against Gram-negative and Gram-positive bacteria (Figure 4; Table 1). The HDLz permeabilized the liposomal membranes while the native lysozyme did not. This activity coincides with the stronger antimicrobial activity to Gram-negative bacteria (Figures 4 and 6). The increased hydrophobicity and half-cystines at the surface of the dimer may contribute in the bactericidal action of HDLz, which apparently involves increasing the permeability of the bacterial membrane.

Membrane permeabilizing peptides, including melittin, cecropin, magainin, and α-hemolysin, are all amphiphilic α-helices that contain no β-sheet and have no disulfide bonds. The few membrane-permeabilizing proteins that do contain large amounts of antiparallel β-sheets are either cyclic peptides, e.g., gramicidin S and tachyplesin (Matsuzaki et al., 1993), or dimeric ellipsoidal proteins such as defensin (Hill et al., 1991) and the trimeric form of tumor necrosis factor α (Kagan et al., 1992). The emerging structural properties of HDLz mimic the structure of dimeric defensin. The amphiphilic dimeric structure suggests two ways in which denatured lysozyme molecules might interact with and permeabilize the lipid bilayer of bacteria. One mechanism is the permeabilization of membranes resulting from the amphiphilicity of the dimer. A cluster of hydrophobic side chains on the surface of the dimer (Figure 2A) provides a hydrophobic patch of solvent-accessible surface area that is surrounded by a set of positively charged Arg and Lys side chains (Imoto et al., 1972; Blake et al., 1965). Dimers of denatured lysozyme disrupt membranes by distorting lipid−lipid interactions. The dimer does this by burying the hydrophobic surface into the bilayer while the positively charged residues interact with lipid phosphate groups. This is consistent with the restored epitope accessibility of the membrane-bound HDLz to the anti-lysozyme antibodies in the membrane binding assay (Figure 5). Additionally, the enhanced affinity of denatured lysozyme to bacterial membrane (Figure 5) and its disruptive effects on the liposome integrity (Figure 6) agree well with this hypothesis. Since HDLz exhibits antimicrobial effects on both Gram-negative and Gram-positive bacteria, it may interfere with the function of cytoplasmic membrane, such as inhibition of the transport of macromolecular precursors which are necessary for cell growth and division.

The second plausible mechanism of action is the stimulation of cell autolysis. Although evidence is still

lacking, heat-denatured lysozyme devoid of enzymatic activity may kill both Gram-negative and Gram-positive bacteria by triggering autolysin activity. Autolysins are a subset of peptidoglycan-synthesizing enzymes, the activation of which causes bacteria to form faulty peptidoglycan and lyse (Kagan et al., 1992). Low molecular weight surfactants, amphiphilic molecules (Cho et al., 1990), and other nonlytic antimicrobial agents (Hakenbeck, 1987) have been reported to induce the activation of autolysins. SEM observation of HDLz-treated *E. coli* K12 revealed an altered cell morphology, with collapsed membrane structure and the appearance of membrane blisters (data not shown), suggesting possible involvement of autolysin stimulation. Investigation of HDLz mediation of autolysin activity is now in progress.

The following concluding remarks may be made from the data of the present study: (i) Broadening the antimicrobial activity of the unfolded lysozyme may shed light on the importance of the rigid, stable conformation of the native lysozyme molecule for defining its antimicrobial specificity *in vivo*. (ii) The results that denatured lysozyme, devoid of any enzymatic activity, retains its antimicrobial activity against Gram-positive bacteria suggests that the inactive conformation of lysozyme may have a role in stimulating autolysins, most likely by interacting with activators or suppressers of autolysins. (iii) Thiol−disulfide exchange reactions for isomerization of lysozyme could occur *in vivo* for the switching of the antimicrobial specificity of lysozyme under certain conditions, such as in the vacuole of phagocytosis of bacteria. In this regard, it has recently been reported that a portion of lysozyme exists as dimers in the secretory lamellar bodies of the lung alveolar type II epithelial cells (Gibson and Phadke, 1994). (iv) The variable sensitivity of certain bacteria to the antimicrobial action of lysozyme may be ascribed to the known pH- and temperature-dependent phase transition of the molecule (Jolles and Jolles, 1984).

ABBREVIATIONS USED

CFU, colony forming unit; CPA, *cis*-parinaric acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; HDLz, lysozyme derivative that is heat-denatured at 80 °C for 20 min; NLz, native lysozyme; RAE-NLz, reduced and S-aminoethylated NLz; RAE-HDLz, reduced S-aminoethylated HDLz; IM and OM, inner and outer membrane, respectively; PBS-Tw, phosphate-buffered saline, containing 0.05% Tween 20, wash buffer for ELISA assay; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEM, scanning electron microscopy; S_0, surface hydrophobicity; Trp, tryptophan residue.

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