Reversible Conformational Change in β-Lactoglobulin A Modified with *N*-Ethylmaleimide and Resistance to Molecular Aggregation on Heating

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β-Lactoglobulin A (βLG A) modified with *N*-ethylmaleimide (NEM−βLG A) was purified by ion exchange chromatography, and modification of βLG A by NEM was confirmed by time of flight mass spectrometry and 5,5′-dithiobis(2-nitrobenzoic acid) methods. The fluorescent spectrum of NEM-βLG A was slightly different from that of native βLG A. NEM−βLG A gave no polymerization after heating at 80 °C and pH 7.5, as shown by polyacrylamide gel electrophoresis. Conformational change of NEM−βLG A was observed at 80 °C by ultraviolet differential spectra, whereas after cooling it recovered to its original state as before heating, indicating apparent reversible thermal denaturation. Native βLG A is resistant to pepsin hydrolysis, whereas heated βLG A was easily hydrolyzed by pepsin. NEM−βLG A before heating was also resistant to pepsin hydrolysis, and after heating NEM−βLG A was still resistant to pepsin hydrolysis. These results indicate that NEM−βLG A maintained a conformation similar to its native form even after heating. Addition of 0.2 M NaCl to the βLG A heated under salt-free condition induced polymerization of heated βLG A molecules, but not that of heated NEM−βLG A. This seemed to indicate that the formation of inter- or intramolecular disulfide linkage made the heat-induced conformational change of βLG A irreversible.

Keywords: *β-Lactoglobulin A; N-ethylmaleimide; heat-induced aggregation; intermolecular disulfide bridge; sulfhydryl−disulfide interchange reaction*

INTRODUCTION

Milk whey protein consists of globular proteins such as β-lactoglobulin (βLG), α-lactalbumin, and serum albumin. βLG, the major protein component, is largely responsible for the aggregation and gelation of whey protein on heating. A βLG molecule consists of 162 amino acid residues and has two disulfide (S−S) bonds, at Cys^{66}−Cys^{160} and Cys^{106}−Cys^{119}, and a free sulfhydryl group at Cys^{121}. Under physiological conditions, this globular protein exists as a dimer consisting of antiparallel β-sheets formed by nine β-strands (*1*). βLG has seven variants, and most of them are variants A and B (*2*, *3*). βLG variant A (βLG A) was used throughout this study.

βLG A molecules aggregate when they are heated, and intermolecular disulfide bonds seem to play an important role in such molecular aggregation (*4*). Aggregation of βLG A depends on pH (*5*−*7*), salt concentration (*8*, *9*), and heating temperature (*10*). Although the importance of free sulfhydryl residues is accepted for molecular aggregation of βLG on heating, the molecular events occurring on the thermal denaturation and aggregation via intermolecular disulfide formation have not been revealed in detail. To reveal the mechanism of heat-induced molecular aggregation of βLG, it is important to clarify the participation of the sulfhydryl residue.

Milk whey protein is widely used as a food ingredient due to its high gelation and emulsification abilities. Changes in the conformation of the protein at the molecular level strongly influence the functional properties of the food protein. Gel formation is usually induced by the formation of a network structure with protein molecules when heated in the food system. Heating induces the conformational change in a protein molecule. Molecular interaction among the denatured protein molecules is necessary for the construction of the three-dimensional network structure.

The objective of the present study was to examine the conformational change of βLG A by heating, particularly its reversibility and pH dependency. In addition, the molecular interactions through the disulfide bridge and other noncovalent forces have been analyzed to reveal the mechanism of molecular polymerization and gelation.

MATERIALS AND METHODS

Materials. βLG A obtained from Sigma Chemical Co. (variant A; L-7880 St. Louis, MO) was dissolved in 5 mM sodium phosphate buffer (pH 7.5) and applied onto a G-75 Sephadex (Pharmacia, Uppsala, Sweden) column previously equilibrated with 20 mM sodium phosphate buffer (pH 7.5) and containing 0.1 M sodium chloride (NaCl) and 0.02% sodium azide. To understand the aggregation mechanism correctly, dimers of βLG A were removed. βLG A fractions were collected and stored in a 75% ammonium sulfate suspension at 4 °C until use. Porcine stomach pepsin (activity = 2500−3500 units/mg of protein; P-7012) was also obtained from Sigma Chemical Co. Other chemicals were of reagent grade and obtained from Nacalai Tesque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan). *N*-Ethylmaleimide (special reagent for the research of sulfur-containing protein, NEM) was purchased from Nacalai Tesque.

Protein Concentration. The protein concentration of βLG A and porcine pepsin was determined spectrophotometrically on the basis of molar extinction coefficients of ϵ_{280nm} for βLG

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A and porcine pepsin of 0.96 and 1.32 M^{-1} cm^{-1}, respectively (*11*, *12*).

Determination of the Number of Sulfhydryl Residues by 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB). The number of sulfhydryl residues of the protein sample was measured according to the DTNB method (*13*). The reaction mixture contained protein (0.25−0.63 mg/mL), DTNB (0.2 mM), SDS (0.5%), EDTA·2Na (1 mM), and sodium phosphate buffer (40 mM, pH 8.0). The reaction was carried out at 50 °C for 3 min. Absorbance at 412 nm was continuously measured using a spectrophotometer (UV-160A, Shimadzu Co., Kyoto, Japan) for a duration of 10 min; the value at 3 min was used for calculation. The molar extinction coefficient of ϵ_{412nm} for TNB was 13.6 M^{-1} cm^{-1} (*13*, *14*).

Native (Non-denaturant) Polyacrylamide Gel Electrophoresis (Native PAGE). Native PAGE was carried out with a 10% gel. A protein sample (2.5 μg) was loaded in each lane, and the bands were stained with 0.1% (w/v) Coomassie Brilliant Blue R250 in 50% methanol.

SDS—Polyacrylamide Gel Electrophoresis. SDS-PAGE was carried out with a 15% gel. A protein sample (2.5 μg) with or without reducing agent 2-mercaptoethanol (+2ME or −2ME) was loaded on each lane, and the bands were stained with 0.1% (w/v) Coomassie Brilliant Blue R250 in 50% methanol.

Time-of-Flight Mass Spectrometry (TOF-MS). The molecular sizes of βLG A and NEM−βLG A were determined by matrix-assisted laser desorption ionization (MALDI) TOF-MS (Voyager RP, Biospectrometry Workstation, Perseptive Biosystems Vestec Mass Spectrometry Products, Boston, MA). Protein solution (0.25 mg/mL) was mixed with matrix solution (saturated sinapinic acid in 30% acetonitrile containing 0.1% TFA) and analyzed.

Purification of the βLG A Modified with NEM. The βLG A modified with NEM (NEM−βLG A) was purified as follows. βLG A (1.0 mg/mL; 0.055 mM) was incubated with NEM (1.0 mM) in 5 mM sodium phosphate buffer (pH 7.5) at 37 °C for 4 h in the dark. To the reaction mixture was added ammonium sulfate (75% saturation), and the precipitate was collected by centrifugation at 8000*g* at 4 °C for 30 min. Precipitate was dissolved in 10 mM Tris-HCl buffer (pH 8.0) and dialyzed against 10 mM Tris-HCl buffer (pH 8.0). The dialysate was applied onto DEAE-Cellulofine A-500-m (Seikagaku Co., Tokyo, Japan), which was previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0). Elution was carried out by linear pH gradient from pH 8.0 (125 mM Tris-HCl buffer) to pH 7.2 (125 mM Tris-HCl buffer). The purity of the NEM−βLG A was determined by native-PAGE. NEM−βLG A was stored in 75% ammonium sulfate at 4 °C until use.

UV Absorption Difference Spectra. UV absorption difference spectra were obtained with a UV-160A spectrophotometer. Protein sample was desalted by using Sephadex G-25 column (PD-10, Pharmacia) equilibrated with 5 mM sodium phosphate buffer (pH 7.5). The desalted protein solution (1.0 mg/mL, 1.2 mL) was placed in two cuvettes and used as sample and reference. The quartz cuvette (10 × 10 mm) containing a sample solution was placed in a cuvette holder with a water jacket in which temperature-regulated water was circulated. A small magnetic stirrer bar (rod shape, 2 mm length × 1 mm diameter) was placed in the cuvette, and the protein solution was stirred. The cuvette was covered with a silicon plug through which a wire thermistor was inserted into the protein solution directly. The solution was heated using a programmed heater from 25 to 80 °C at the rate of 2 °C/min, maintained at 80 °C for 10 min, and cooled to 25 °C at the rate of 2 °C/min. The spectrum was read at 25, 60, and 80 °C. The process took 45 s.

Measurements of Fluorescence Spectra. Fluorescence spectra of the samples were measured using a fluorescence spectrophotometer (F3000, Hitachi Ltd., Tokyo, Japan) equipped with a water jacket to keep the sample at a constant temperature (25 °C). The samples analyzed were βLG A or modified βLG A (1.0 mg/mL) in 5 mM sodium phosphate buffer (pH 7.5). The excitation wavelength was 295 nm, and the emission was scanned from 305 to 400 nm.

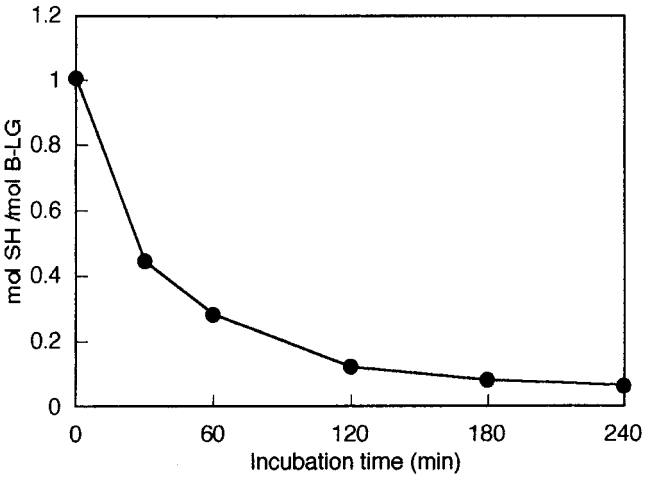

Figure 1. Number of free sulfhydryl residues per βLG A monomer as a function of incubation time with NEM. βLG A (1.0 mg/mL; 0.055 mM) was incubated with NEM (1.0 mM) in 5 mM sodium phosphate buffer, pH 7.5, at 37 °C in the dark. At each time the sample was taken and passed through Sephadex G-25 column to remove free NEM. The number of free sulfhydryls in each sample was measured by reactivity to DTNB.

Digestion of βLG A and NEM—βLG A by Pepsin. βLG A and NEM−βLG A (1.0 mg/mL, pH 7.5, 100 μL) were heated at 80 °C for 1 h. Sodium acetate buffer (500 mM, pH 2.0, 300 μL) and porcine pepsin (1.0 μg/mL, pH 2.0, 100 μL) were added to each sample. The final concentration of βLG A and NEM−βLG A was 0.5 mg/mL, and porcine pepsin concentration was 0.2 μg/mL (100 mM sodium acetate buffer, pH 2.0). The reaction mixtures were incubated at 37 °C for 0, 5, 10, and 30 min. After incubation, the reaction was stopped by adding the SDS sample buffer (pH 7.0), containing 125 mM Tris, 2% SDS, 20% glycerol, and analyzed by SDS-PAGE.

Effects of NaCl on Heated NEM—βLG A. βLG A was first dissolved in 5 mM sodium phosphate buffer (pH 7.5). The protein concentration was then adjusted to 10 mg/mL and heated at 80 °C for 1 h. The heated sample was then immediately cooled and NEM added. The reaction mixture containing βLG A (1.0 mg/mL; 0.055 mM) and NEM (1.0 mM) in 5 mM sodium phosphate buffer (pH 7.5) was incubated at 37 °C for 4 h in the dark. Then, free NEM was removed from the reaction mixture by using a Sephadex G-25 column (PD-10), previously equilibrated with 5 mM sodium phosphate buffer (pH 7.5). Protein fractions were pooled and dialyzed against 5 mM sodium phosphate buffer (pH 7.5) at 4 °C overnight to completely remove free NEM. The dialysate was concentrated to ∼12 mg/mL by centrifugation at 2500*g* and 4 °C using a centrifugal concentrator (Centriplus-10, Amicon, Inc., Beverly, MA). Then NaCl was added to the concentrated sample. The final concentrations of protein and NaCl for incubation were 10 mg/mL and 0.2 M, respectively. The mixture was incubated at 37 °C for 0, 10, 60, and 120 min. After the incubation, the mixture was applied to Sephadex G-25 gel column chromatography (PD-10) to remove NaCl. The sample was analyzed by native PAGE.

Effects of NaCl on the Aggregation of Heated Protein. Two types of experiments were performed. The first experiment is as follows. βLG A and NEM−βLG A were heated at 80 °C for 1 h, and then after cooling they were incubated at 37 °C with 0.2 M NaCl for 120 min. For the second experiment, the heated βLG A was modified with NEM and then the NEM-modified heated βLG A was incubated at 37 °C with 0.2 M NaCl for 120 min.

RESULTS AND DISCUSSION

Figure 1 shows the number of free sulfhydryl residues per βLG A monomer as a function of incubation time with NEM. The number of free sulfhydryl residues

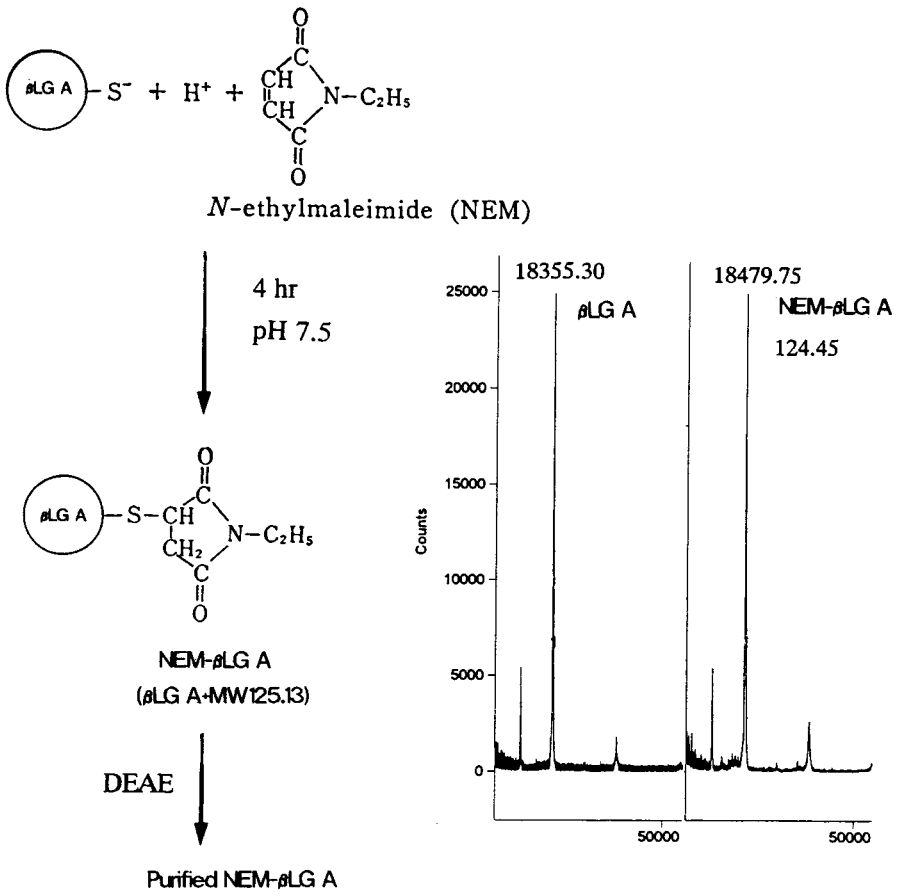

Figure 2. Modification of βLG A with NEM and TOF-MS spectra of βLG A and NEM−βLG A.

decreased with time due to the modification of the free sulfhydryl residues of βLG A by NEM. More than 90% of the free sulfhydryl residues per βLG A monomer was modified by incubation with NEM for 4 h. Modified βLG A was purified by DEAE chromatography. Because it is known that NEM reacts with not only sulfhydryl residues but also other residues (*15*, *16*), the molecular weight of the purified NEM−βLG A was measured with TOF-MS. The scheme in Figure 2 shows the reaction of βLG A with NEM. From this scheme, the increase in molecular weight of βLG A seemed to be 125.13 if only one residue of cysteine was modified. The MS fragmentation pattern showed that the molecular mass of NEM−βLG A was 124.45. Although there was a slight difference between the two values, it could be presumed that only 1 mol of NEM was introduced into 1 mol of βLG A. Purified NEM−βLG A had no sulfhydryl residue, as measured by the DTNB method (Figure 1). Therefore, this indicates that 1 mol of NEM modified 1 mol of sulfhydryl residue of one βLG A molecule.

To demonstrate the role of sulfhydryl residues in βLG A for the heat-induced aggregation, both βLG A and purified NEM−βLG A were heated at different protein concentrations, and the products were analyzed by native PAGE and SDS-PAGE as shown in Figure 3. Heated βLG A gave many bands in native PAGE, including a band between the separating gel and the stacking gel at a protein concentration >10 mg/mL. As protein concentration increased, the number of bands corresponding to higher molecular size also increased; the band for huge aggregates, which could not enter the separating gel, was observed at >10 mg/mL, and a band on the stacking gel was found at >30 mg/mL. Although polymerization of βLG A molecules by heating was not observed on SDS-PAGE under any reducing conditions, SDS-PAGE in the presence of reducing agent gave no bands except the original monomer band. These results indicate that polymerization occurred through intermolecular disulfide linkage.

NEM−βLG A showed only one band in SDS-PAGE with reducing agent. This was also the case for nonmodified βLG A. Each native PAGE and SDS-PAGE without reducing agent also gave a main single band with the same mobility as that for the unheated βLG A, indicating that NEM−βLG A was not polymerized by heat. This means that only one sulfhydryl residue in the βLG A molecule is enough and essential for polymerization of βLG A molecules by heat.

Heating induced the polymerization of the βLG A molecule through an intermolecular disulfide bridge as shown above. Prior to polymerization of βLG A the conformation of βLG A seemed to be changed by heating, whereas the conformation of NEM−βLG A during heating at 80 °C was not known. The conformational change of βLG A and NEM−βLG A during heating was verified by UV absorption difference spectra. In the case of βLG A, the change of the difference spectra appeared with an increase in the temperature (Figure 4). Although the difference absorbance was reduced with a decrease in temperature, clear differences remained after cooling, meaning that the conformational changes of βLG A by heat were irreversible. In the case of NEM−βLG A, difference spectra appeared at 60 and 80 °C; however, the difference disappeared after cooling. This clearly indicates that NEM−βLG A showed reversible conformational change through heating and cooling and that the irreversibility of thermal denaturation depends on the reactivity of the sulfhydryl residues of the βLG

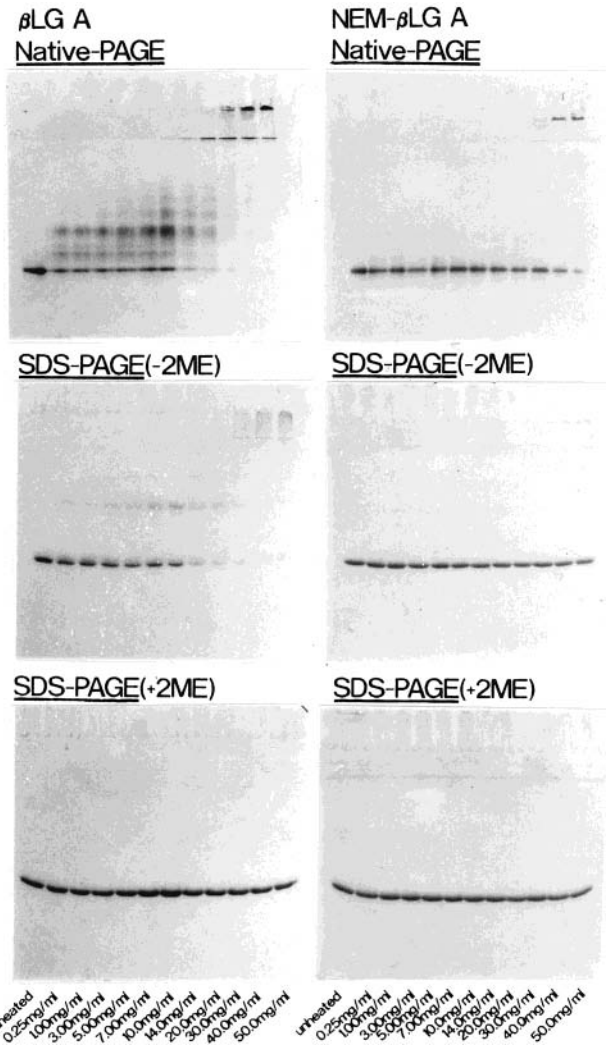

Figure 3. Nonreducing PAGE (native PAGE) and SDS-PAGE of heated βLG A and NEM$-\beta$LG A. βLG A and NEM$-\beta$LG A (0.25−50 mg/mL, 5 mM sodium phosphate buffer, pH 7.5) were heated at 80 °C for 1 h, and 2.5 μg of the samples was electrophoresed.

A molecule. When protein denatures by heating, the chromophore interacts with the solvent, that is, water molecules. This interaction leads the blue shift of the spectrum. In the case of ribonuclease the blue shift at 287 nm was clear, and its conformational changes by heating could be followed by the difference absorbance at 287 nm (*17*). This change seems to result from the microenvironmental change around the tyrosine residue in the ribonuclease molecule. In Figure 4, the difference spectrum peak at 287 nm in both cases of βLG A and NEM$-\beta$LG A are distinct, suggesting the conformational change of βLG A and NEM$-\beta$LG A by heating. A distinct difference spectrum was obtained at 80 °C. Difference absorption was reduced with a decrease in temperature in the case of NEM$-\beta$LG A, and a significant difference absorption was not observed after cooling, whereas such difference remained after cooling in the case of βLG A.

When the conformation of protein changes on heating, the microenvironment around the tyrosine and tryptophan residues is often changed, which is reflected in the alteration of the UV spectrum: a shift to the short-wavelength region, that is, a blue shift. This shift can be detected sensitively by UV absorption difference spectra. A difference absorption spectrum is useful to

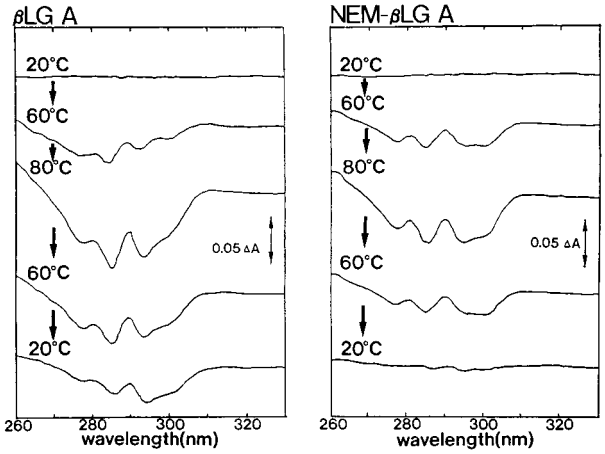

Figure 4. UV difference spectra of βLG A and NEM$-\beta$LG A at pH 7.5. βLG A and NEM$-\beta$LG A (1.0 mg/mL, 5 mM sodium phosphate buffer, pH 7.5) were heated from 25 to 80 °C and cooled at the rate of 2 °C/min. The spectra were measured between 260 and 330 nm.

detect the denaturation and conformational change of a protein molecule. In both cases of βLG and NEM$-\beta$LG the difference absorption at 277 nm (tyrosine) and 285 and 294 nm (tryptophan) appeared with an increase in temperature, meaning that the microenvironment around the tyrosine and tryptophan residues changed by heating, indicating the conformational change occurred in βLG and NEM$-\beta$LG by heating. In the case of βLG the difference spectrum that occurred by heating did not return to the original state after cooling, meaning that the heat-induced conformational change, including the alteration of the microenvironment of these residues, was thermally irreversible. On the other hand, the spectrum of NEM$-\beta$LG returned to the original state after cooling, showing a reversible conformational change of NEM$-\beta$LG by heating and cooling.

Native βLG A is known to be resistant to pepsins even at acid pH values, and the heat-denatured βLG A is easily susceptible to pepsin (*18*). Proteinase susceptibility sensitively reflects the change in the conformation of the protein molecule. Then the susceptibility of NEM$-\beta$LG A after heating to porcine pepsin was investigated. Figure 5 shows the SDS-PAGE pattern of βLG A and NEM$-\beta$LG A heated at 80 °C for 1 h after treatment with porcine pepsin. Heated βLG A gave several bands with higher mobility than that of unheated βLG A, indicating that the heated βLG A was hydrolyzed by pepsin as shown before (*18*). The SDS-PAGE of NEM$-\beta$LG A without a reducing agent gave nearly a single band with a mobility similar to that of the unheated βLG A. The SDS-PAGE of NEM$-\beta$LG A with a reducing agent (+2ME) gave in addition other quite faint bands that have higher mobility than βLG A. This shows that NEM$-\beta$LG A was resistant to pepsin even after heating at 80 °C for 1 h. Nevertheless, some slight faint bands with lower molecular sizes were observed, which seemed to be due to a few nicks in NEM$-\beta$LG A (Figure 5). This means that the conformation of NEM$-\beta$LG A was not so different from that of the native βLG A and that NEM$-\beta$LG A showed a reversible conformational change on heating and cooling.

The change in the conformation of NEM$-\beta$LG A on heating and cooling was investigated further by fluorescence spectrum measurement. Figure 6 shows the

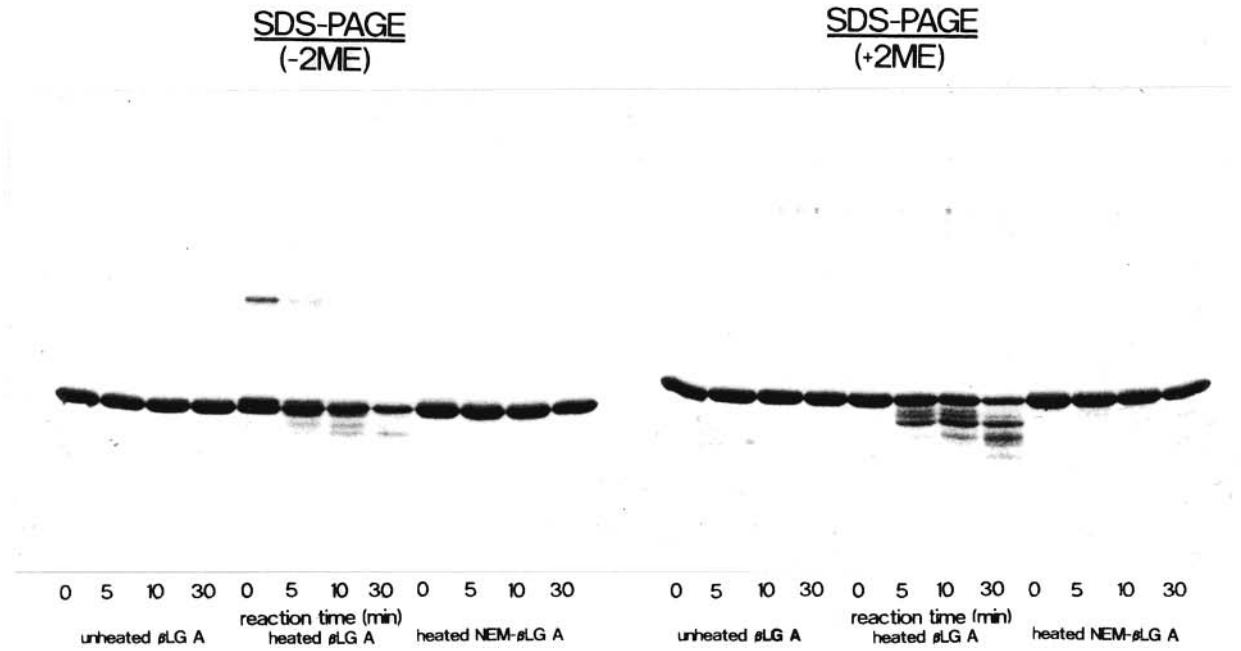

Figure 5. Pepsin treatment of βLG A and NEM−βLG A at pH 7.5. βLG A and NEM−βLG A (0.2 mg/mL, 5 mM sodium phosphate buffer, pH 7.5) were heated at 80 °C for 1 h, and the pH was adjusted to 2.0 with sodium acetate buffer (100 mM, pH 2.0). Porcine pepsin was added to make a final concentration of 0.2 μg/mL and incubated at 37 °C for 0, 5, 10, and 30 min. Reaction was stopped by the addition of SDS-PAGE sample buffer, and 2.5 μg of the samples was electrophoresed.

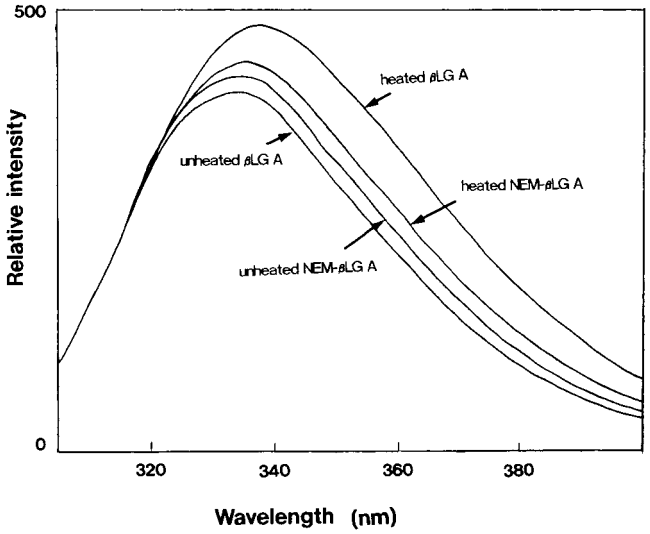

Figure 6. Fluorescence spectra of βLG A and NEM−βLG A. Fluorescence spectra of βLG A or NEM−βLG A (1.0 mg/mL, 5 mM sodium phosphate buffer, pH 7.5) were measured at 25 °C. The excitation wavelength was 295 nm, and the emission was scanned from 305 to 400 nm.

fluorescence spectra of unheated βLG A, heated βLG A, unheated NEM−βLG A, and heated NEM−βLG A. Upon heating and cooling, the fluorescence spectrum of βLG A showed a red shift and the fluorescence intensity increased. This suggests that the microenvironment of some tryptophan and/or tyrosine residues in βLG A changed and probably these amino acid residues were exposed and interacted with water molecules; hence, the spectrum was shifted. That is, the shift of spectrum indicates the change in the conformation of protein. The spectrum of unheated NEM−βLG A was different from that of unheated βLG A. For this change in spectrum there may be two interpretations. The NEM introduced to βLG A interacted with some amino acid residues in the βLG A molecules, which induced the change in the fluorescence spectrum, although the conformation of βLG A was not altered. Another interpretation is that the modification by NEM induced a conformational change in the βLG A molecule that caused the alteration of its fluorescence spectrum. According to the results of a differential scanning calorimetry (DSC) study (*19*), it is presumed that the latter interpretation is more plausible. After cooling of NEM−βLG A, a slightly different spectrum from that of unheated NEM−βLG A was obtained; however, the difference is much smaller than that of the heated βLG A from the unheated βLG A. This indicates that NEM−βLG A changed its conformation with heating at 80 °C for 1 h, and the change in conformation was reversible and almost returned to that of unheated NEM−βLG A. However, the reversed conformation was not completely the same as those of the unheated βLG A and unheated NEM−βLG A, from the observation of the pepsin study and the fluorescence spectra.

It should be noted that in all of the experiments above heating was done under salt-free conditions and no salt was added. Ionic strength is known to produce a significant effect on the aggregation of heated protein molecules (*8*, *9*, *18*, *20*, *21*). Therefore, to examine the effects of salt on the aggregation of βLG A and NEM−βLG A the following experiments were performed. For the first experiment, Figure 7 shows that the heated βLG A giving polymers aggregated by incubation with 0.2 M NaCl at 37 °C and larger sizes of molecular polymers was formed through intermolecular disulfide bonds. On the other hand, the heated NEM−βLG A gave almost a single or only a few bands on native PAGE and SDS-PAGE, and no change in electrophoretic pattern was observed after incubation with NaCl. These results indicate that the heated βLG A molecules and/or the aggregates of heated βLG A interact by noncovalent forces to give larger aggregates by the incubation with NaCl. In this case, intermolecular disulfide linkages among heated βLG A molecules were newly formed during incubation with NaCl. It might be that

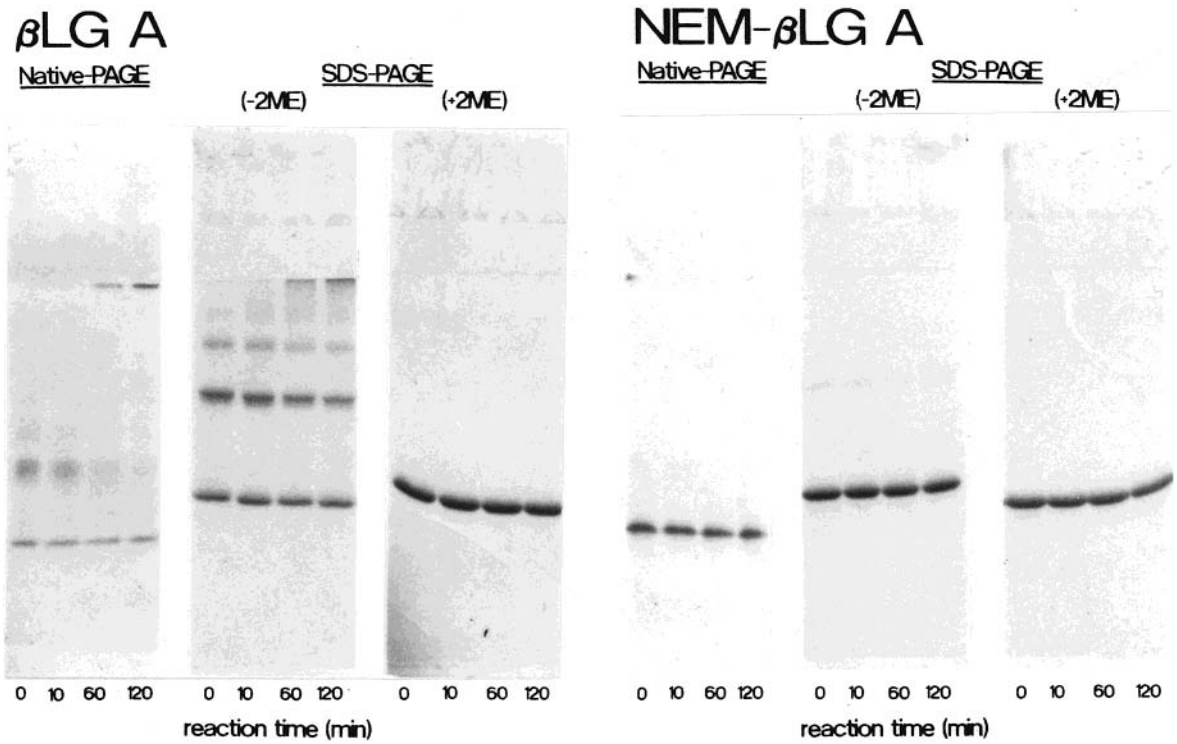

Figure 7. Native PAGE and SDS-PAGE of heated βLG A and heated NEM−βLG A after incubation with NaCl at 37 °C. βLG A and NEM−βLG A (10 mg/mL, pH 7.5) were heated at 80 °C for 1 h, and NaCl was added to bring the final concentration to 0.2 M; 2.5 μg of the samples was electrophoresed.

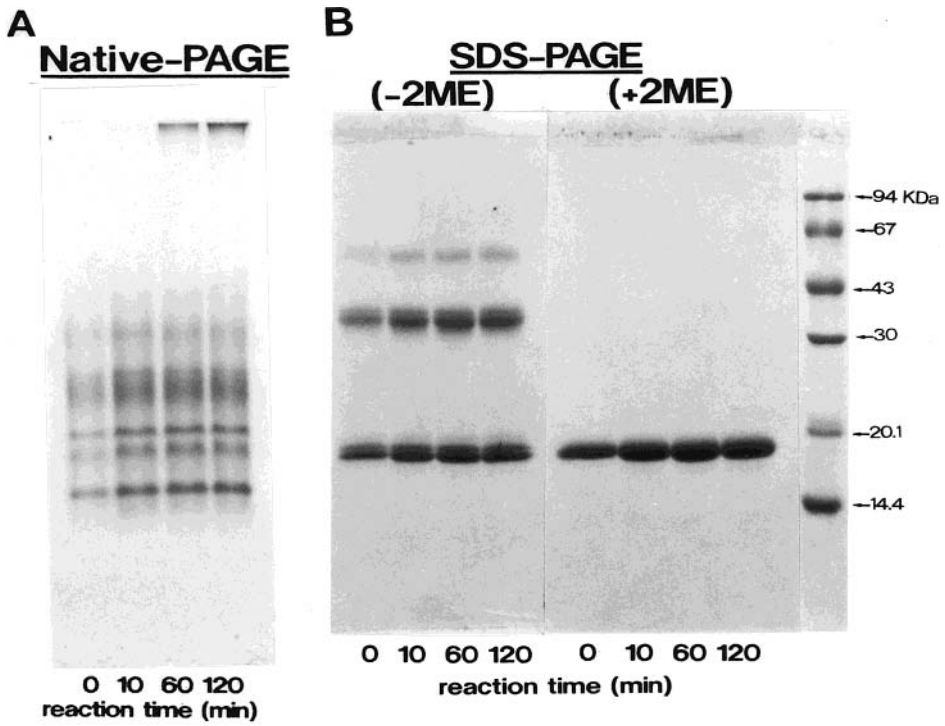

Figure 8. Effects of NaCl on aggregation of NEM-heated βLG A: native PAGE (A) and SDS-PAGE (B) of NEM-heated βLG A. Details are shown in the text.

the hydrophobic amino acid residues in the βLG A molecule, exposed from inside the βLG A molecule to the surface of the βLG A molecule by heating, induced the aggregation of βLG A molecules with hydrophobic interaction, which increased with an increase in ionic strength because of the addition of NaCl (*15*), resulting in the formation of intermolecular disulfide bridges. For heated NEM−βLG A the electrophoretic patterns were not changed by incubation with NaCl at 37 °C, as it seemed that the conformation of NEM−βLG A had already returned almost to the native state. Thus, the hydrophobic area on the NEM−βLG A, which should be exposed by heating, was hidden again after cooling. For the second experiment, the heated βLG A was modified with NEM and then the NEM-modified and heated βLG A (NEM-heated−βLG A) was incubated with 0.2 M NaCl at 37 °C for 120 min. The electrophoretic pattern of NEM-heated βLG A in native PAGE

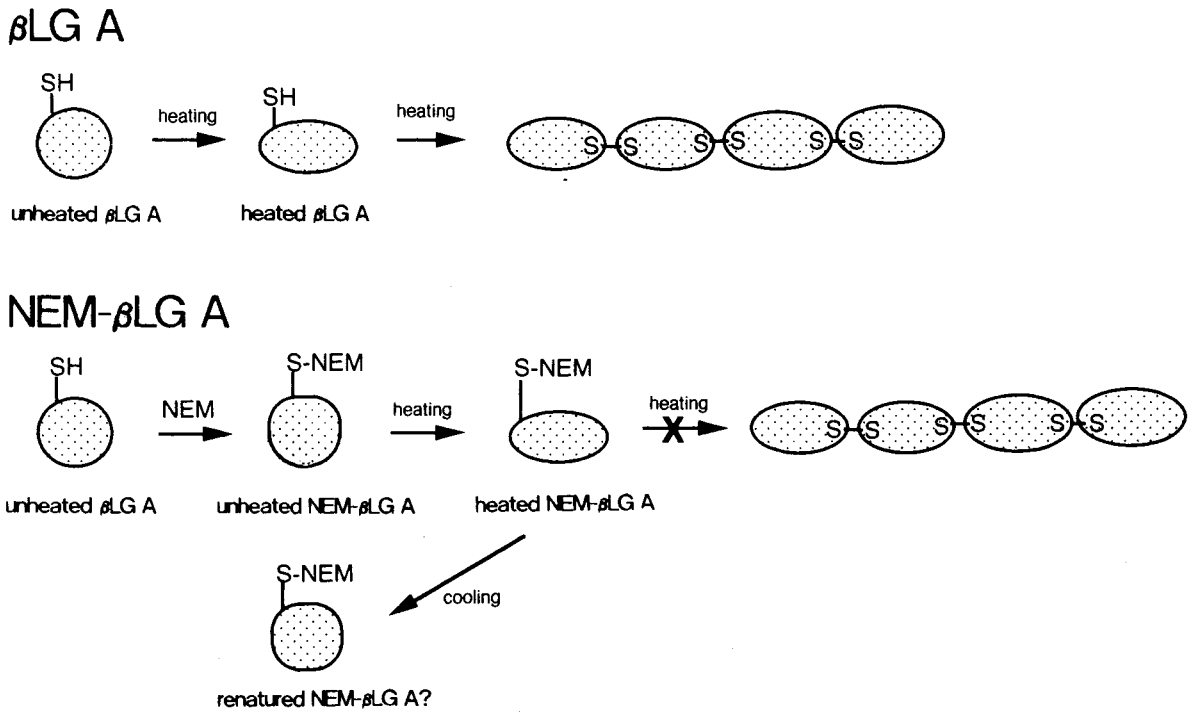

Figure 9. Scheme of the change in the conformation and polymerization of βLG A and NEM−βLG A.

showed further aggregation by incubation with NaCl, and huge aggregates, which could not enter the separation gel, were observed after long incubations, that is, 60 and 120 min (Figure 8A). SDS-PAGE of NEM-heated βLG A (Figure 8B) showed no change in the electrophoretic pattern after incubation with NaCl. This indicates that the size of the aggregates, which was formed by the first heating under salt-free condition, was not changed by the addition of NaCl. Both heated βLG A and NEM-heated−βLG A showed an increase in the size of aggregate by the addition of NaCl. Native PAGE in Figures 7 and 8A demonstrates this. Although huge aggregates were also observed by SDS-PAGE in the case of heated βLG A, such aggregates could not be found in NEM-heated−βLG A. This means that the aggregates of NEM-heated−βLG A, in which the sulfhydryl residue was blocked with NEM, associated together by hydrophobic interaction. Interaggregate disulfide linkage did not occur. On the other hand, the aggregates of heated βLG A associated by incubation with NaCl and linked together through disulfide bridges.

CONCLUSION

Figure 9 summarizes the results in this study. The conformational change in βLG A occurred on heating and the denatured βLG A molecules interacted and linked together by an intermolecular disulfide bridge that was formed by sulfhydryl−disulfide interchange reaction. Prior to the formation of the intermolecular disulfide, some noncovalent forces among heated βLG A molecules seemed to contribute to the aggregation, particularly hydrophobic interaction. Because of the intermolecular disulfide linkage between βLG A molecules, the change in the conformation was irreversible as sulfhydryl-blocked βLG A, that is, NEM−βLG A, could retain its original conformation after cooling. Intramolecular disulfide linkage as well as intermolecular linkage may be formed. Noncovalent forces, mainly hydrophobic interaction, contributed to the formation of the aggregates. In addition, intermolecular disulfide linkage formation in the aggregates could occur to give even huge aggregates when incubated with NaCl.

ABBREVIATIONS USED

βLG A, β-lactoglobulin A; NEM, *N*-ethylmaleimide; TOF-MS, time-of-flight mass spectrometry; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2ME, 2-mercaptoethanol; DSC, differential scanning calorimetry.

LITERATURE CITED

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