β-Lactoglobulin A with *N*-Ethylmaleimide-Modified Sulfhydryl Residue, Polymerized through Intermolecular Disulfide Bridge on Heating in the Presence of Dithiothreitol

Ritsuko Wada and Naofumi Kitabatake*

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Roles of sulfhydryl groups on thermal aggregation of β-lactoglobulin A (βLG A) at pH 7.5 were investigated. It is known that βLG A modified at Cys^{121} with *N*-ethylmaleimide (NEM-βLG A) does not form an aggregate by heating and that dithiothreitol (DTT) reduces cystine residues and induces the intermolecular sulfhydryl/disulfide interchange reaction and/or oxidation. NEM-βLG A was heated in the presence of DTT. The molecules were linked together with an intermolecular disulfide bridge, and the polymer formed increased with increase in DTT concentration. The largest portion of polymer was formed when DTT was added at around the same molar concentration as that of NEM-βLG A. Then, polymer formation decreased with further increase in DTT concentration. The results suggest that sulfhydryl/disulfide residues other than Cys^{121}, generated from cysteine residues, can induce intermolecular sulfhydryl/disulfide interchange reactions to polymer and that thiol compounds, for example, added DTT, are capable of starting such reactions.

Keywords: *β-Lactoglobulin; molecular aggregation; sulfhydryl/disulfide interchange reaction; dithiothreitol (DTT)*

INTRODUCTION

Bovine β-lactoglobulin (βLG) is one of the major proteins in whey and contains ∼68% whey protein isolate (*1*). βLG is globular protein, and the molecular weight is ∼18350; it has one free sulfhydryl residue at position Cys^{121} and two disulfide bonds at Cys^{106}−Cys^{119} and Cys^{66}−Cys^{160} (*2*). Heating of βLG induces interchange reactions of sulfhydryl and disulfide residues, and it appears to play an important role in the molecular aggregation and gelation of βLG (*3*−*10*), whey protein (*11*−*13*), and mixtures of α-lactalbumin and βLG (*14*, *15*). Particularly βLG devoid of a free cysteine residue, such as porcine βLG, was unable to form a thermal gel (*16*), indicating the importance of the cysteine residue for thermal gelation.

We have already shown that βLG A (pH 7.5) was denatured irreversibly after heating at 80 °C for 1 h and that the conformational change caused by heating was due to the molecular aggregation through intermolecular disulfide bond (*17*). On the other hand, βLG A, which was modified at the free sulfhydryl residue of Cys^{121} with *N*-ethylmaleimide (NEM-βLG A), showed thermally reversible conformational change and no aggregates were formed. These results mean that one free cysteine residue in the βLG A molecule, that is Cys^{121}, was essential for the intermolecular sulfhydryl/disulfide interchange reaction. However, it is not clear that having a free sulfhydryl residue is the most important thing or that Cys^{121} is essential for causing the intermolecular disulfide reaction. Dithiothreitol (DTT) is one of the reducing reagents and is capable of maintaining monothiols completely in the reduced state and of reducing disulfides quantitatively because of its low redox potential, that is, −0.33 V at pH 7. If the other disulfide linkage(s) of NEM-βLG A at Cys^{106}−Cys^{119} and/or Cys^{66}−Cys^{160} was (were) reduced, newly produced sulfhydryl residues from Cys^{106}, Cys^{109}, Cys^{66}, or Cys^{160} might cause the sulfhydryl/disulfide interchange reaction. This seems to indicate that cysteine residues other than Cys^{121} have the ability to induce intermolecular sulfhydryl/disulfide interchange as does Cys^{121}.

We intended to cleave intramolecular disulfide bonds of NEM-βLG A, Cys^{106}−Cys^{119} and/or Cys^{66}−Cys^{160}, to make free cysteine residues by the addition of DTT and examined if such newly formed cysteine residues could induce the sulfhydryl/disulfide intermolecular interchange reaction and yield polymerized NEM-βLG A molecules.

MATERIALS AND METHODS

Materials. βLG A (variant A; L-7880) was obtained from Sigma Chemical Co. (St. Louis, MO). Porcine stomach pepsin (activity = 2500−3500 units/mg of protein; P-7012) was also obtained from Sigma. NEM, DTT, and 2-mercaptoethanol (2ME) were specially prepared reagent grade and purchased from Nacalai Tesque (Kyoto, Japan). Molecular weight marker (low molecular weight calibration kit) and Sephadex G-75 superfine were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). DEAE-Cellulofine A-500-m was purchased from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Other chemicals were of guaranteed reagent grade and obtained from Nacalai Tesque and Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Protein Concentration. Protein concentration was determined by spectrophotometry using values of $E^{1\%}_{1cm}$ at 280 nm of 9.7 for βLG A (*18*).

Purification of βLG A. βLG A (100 mg) was dissolved in 2 mL of ice-cold 20 mM sodium phosphate buffer, pH 7.5, containing 0.1 mM EDTA·2Na, 0.1 M NaCl, and 0.02% sodium

* Corresponding author (telephone +81-774-38-3742l; fax +81-774-38-3743; e-mail kitabata@food2.food.kyoto-u.ac.jp).

10.1021/jf001457v CCC: $20.00
Published on Web 09/11/2001

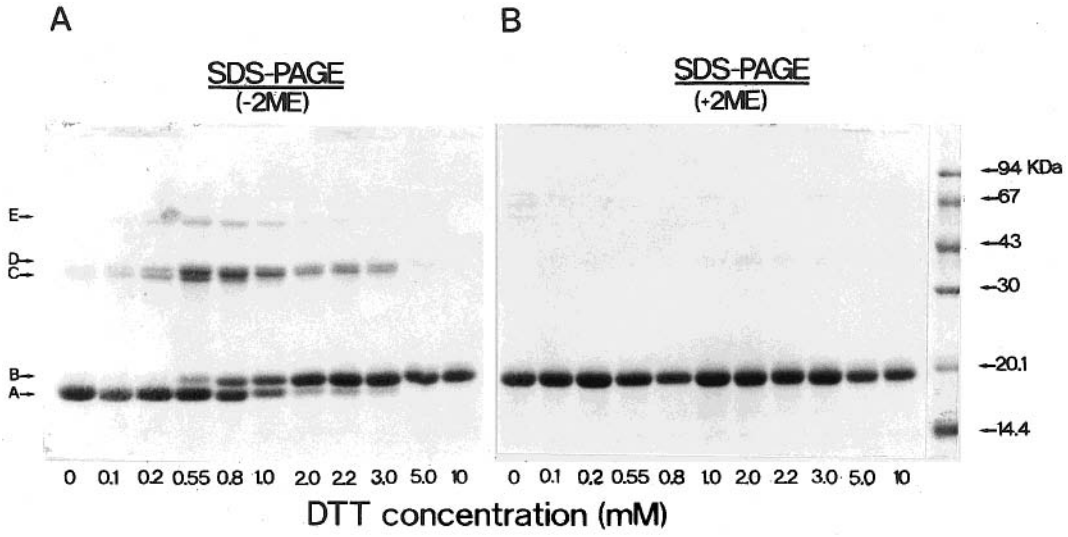

Figure 1. Nonreducing (A) and reducing (B) SDS-PAGE of NEM-βLG A after heating with DTT. βLG A modified with NEM (NEM-βLG A, 10 mg/mL; 0.55 mM, pH 7.5) was heated at 80 °C for 1 h with DTT (0, 0.1, 0.2, 0.55, 0.8, 1.0, 2.0, 2.2, 3.0, 5.0, or 10 mM) and electrophoresed. Molecular marker kit contained β-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase B (94 kDa).

azide. Contaminating proteins in βLG A sample were removed by chromatography using a Sephadex G-75 superfine column (2 cm diameter × 100 cm length) and the same buffer as above at 4 °C at a flow rate of 0.1 mL/min. The eluate was fractionated into 3 mL/tube. βLG A fractions that showed a single band on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) were collected and ammonium sulfate was added to yield 75% saturated solutions and kept at 4 °C until use.

Preparation and Purification of βLG A Modified with NEM (NEM-βLG A). NEM-βLG A was purified as follows. Purified βLG A 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 make a final concentration of 1.0 mg/mL (0.055 mM). After incubation, ammonium sulfate was added in the reaction mixture to be 75%. NEM-βLG A was purified with the DEAE chromatography to separate the unmodified βLG A. The precipitate collected by centrifugation at 8000*g* and 4 °C for 30 min 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, which was previously equilibrated with 125 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). NEM-βLG A fractions that showed a single band on native PAGE were collected, and the purity was also checked by counting the number of sulfhydryl residues of NEM-βLG A according to the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) method (*17*, *19*) and time-of-flight mass spectrometry (TOF-MS) (*17*). Purified NEM-βLG A (0.25−0.63 mg/mL) was mixed with DTNB (0.2 mM), SDS (0.5%), EDTA·2Na (1 mM), and sodium phosphate buffer (40 mM, pH 8.0) and heated 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, and the value at 3 min was used for calculation. The molar extinction of DTNB at 412 nm used was 13.6 (*19*, *20*). The number of sulfhydryl residues was zero (*17*), and it showed the purity of NEM-βLG A. The molecular size of βLG A and NEM-βLG A was 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 and checked that only 1 mol of NEM was introduced into 1 mol of βLG A. This purified NEM-βLG A was stored in 75% ammonium sulfate at 4 °C until use.

Heat Treatment of NEM-βLG A in the Presence of DTT. Ammonium sulfate suspension of purified NEM-βLG A was dialyzed against 5 mM sodium phosphate buffer (pH 7.5) to remove ammonium sulfate and the protein concentration adjusted [10 mg/mL (0.55 mM) or 40 mg/mL (2.2 mM)] in the various concentrations of DTT (0−20 mM). After heating at 80 °C for 1 h, the sample was rapidly cooled to 25 °C and electrophoresed.

Incubation of Heated NEM-βLG A in the Presence of NaCl. Heated NEM-βLG A in the presence of 0.55 mM DTT was incubated with NaCl to provide final protein and NaCl concentrations of 0.2 and 0.2 M, respectively.

PAGE. SDS-PAGE was performed according to the method of Laemmli (*21*), and acrylamide running gel (13.5%) and acrylamide stacking gel (3.0%) were prepared [90 mm (W) × 80 mm (H), 1 mm thickness]. A protein sample (0.25 mg/mL) with or without reducing agent 2ME (+2ME or −2ME) was mixed with an equal volume of sample buffer (2% SDS, 20% glycerol, 0.125 M Tris-HCl, pH 7.0), heated at 100 °C for 5 min, and rapidly cooled. The same volume of sample solution (20 μL, 2.5 μg of protein) was loaded in each lane and electrophoresed at 20 mA/plate for ∼70 min by using ATTO RAPIDAS rectangle electrophoresis apparatus AE-6050A and Crosspower 1000 (ATTO, Tokyo, Japan). Native (nondenaturing) PAGE was carried out according to the method of Davis (*22*) with acrylamide running gel (10%) and acrylamide stacking gel (4.5%). An aliquot of the sample solution was mixed with glycerol. Protein and glycerol concentrations were adjusted to 0.125 mg/mL and 20% (v/v), respectively. To each lane was loaded 20 μL of sample (protein; 2.5 μg), and electrophoresis at 20 mA/plate was carried out for ∼60 min by using the same apparatus as for SDS-PAGE. The bands were stained with 0.1% (w/v) Coomassie brilliant blue R250 in 50% methanol. The results were scanned with a CCD video camera module (ATTO) connected to image freezer AE-6905 (ATTO) and analyzed by using the densitograph software library lane and spot analyzer for Macintosh, version 5.0 (ATTO).

RESULTS AND DISCUSSION

Heating of NEM-βLG A in the Presence of DTT. NEM-βLG A, the sulfhydryl residue of which was blocked by NEM, did not polymerize through intermolecular disulfide linkage by heating and remained a clear solution (*17*). NEM-βLG A (10 mg/mL; 0.55 mM) was heated with DTT and electrophoresed (Figure 1).

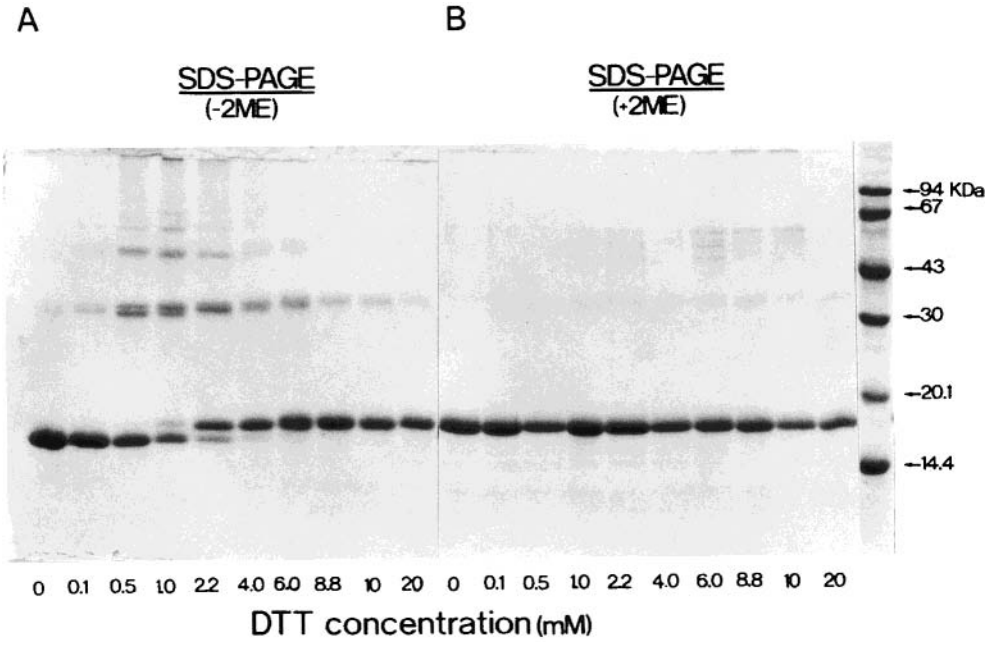

Figure 2. Nonreducing (A) and reducing (B) SDS-PAGE of NEM-βLG A after heating with DTT. NEM-βLG A (40 mg/mL; 2.2 mM, pH 7.5) was heated at 80 °C for 1 h with DTT (0, 0.1, 0.5, 1.0, 2.2, 4.0, 6.0, 8.8, 10, or 20 mM) and electrophoresed. Marker proteins are the same as in Figure 1.

When the concentration of DTT was increased, the color intensity of band A decreased. On the contrary, the intensity of the band at position B was increased. There were several bands of lower mobility than monomer NEM-βLG A in SDS-PAGE without 2ME (Figure 1A): two bands at monomer (bands A and B) and dimer (bands C and D) positions and one band at trimer (band E) position estimated by molecular size marker. The intensity of the bands for dimer, trimer, and higher polymerized NEM-βLG A molecules increased gradually at ~0.55 mM DTT, which was the same concentration of NEM-βLG A, and above this concentration, they were reduced gradually. At 10 mM DTT, a single band was observed at position B, and it had the same mobility as βLG A on SDS-PAGE with 2ME (Figure 1B). In this PAGE, disulfide bridges were completely reduced by 2ME; hence, it could be said that band B shows a completely reduced βLG A dimer. In the SDS-PAGE with 2ME (Figure 1B), only a single band was observed in any case. These results suggest that addition of DTT to NEM-βLG A induced the polymerization of NEM-βLG A through intermolecular disulfide linkages and that polymerization effectively occurred when the stoichiometric relation was about 1/1 between DTT and NEM-βLG A. The newly formed cysteine residues induce the sulfhydryl/disulfide interchange reaction and/or were oxidized to result in intramolecular and/or intermolecular disulfide linkages giving dimer, trimer, and polymer of NEM-βLG A molecules. Densitographic analysis revealed that the color intensity of the band A at 0.55 mM DTT was 47% of the total color intensity. This means about half of the NEM-βLG A molecule was reduced by DTT, indicating that the intermolecular sulfhydryl/disulfide interchange reaction and/or oxidation reaction occurred effectively at 0.55 mM.

Heating of Higher Concentrations of NEM-βLG A in DTT. Intramolecular sulfhydryl/disulfide interchange and oxidation reactions of sulfhydryl residues do not depend on protein concentration, whereas those of the intermolecular reaction must depend on the protein concentration. Thus, a similar experiment was performed using a higher protein concentration, 2.2 mM instead of 0.55 mM, to confirm whether the intermolecular reaction occurred. DTT concentration varied from 0 to 20 mM. Figure 2A shows that 2.2 mM DTT was the most effective concentration to induce polymer formation through intermolecular disulfide bridges, which was the same concentration as for βLG A. This indicates that the DTT added reduced one cystine residue in NEM-βLG A molecules and that then the cysteine residues react to form molecular aggregates through disulfide bridges. Only 50% of cystine residues per one NEM-βLG A molecule could be reduced by oxidation to give two cysteine residues at 2.2 mM DTT if no sulfhydryl/disulfide interchange reaction occurred. However, densitographic analysis of the band at position A, which is considered to be a monomer of NEM-βLG A, at 2.2 mM DTT was 18% of the total color intensity of the bands on the lane. This implies that 82% of the NEM-βLG A molecule was actually reduced. This result provides evidence that the free sulfhydryl residues in NEM-βLG A, which were newly formed after NEM-βLG A reduction with DTT, induced the reduction of cystine residues of other NEM-βLG A monomers and caused the sulfhydryl/disulfide interchange reaction leading to polymerization of NEM-βLG A molecules. The newly formed cysteine residues other than Cys^{121} could have behaved in the same way as the cysteine residue (Cys^{121}) of native βLG A. At higher concentrations of DTT, inter- and intramolecular disulfide bonds were completely reduced; hence, no polymers were formed and NEM-βLG A maintained the monomer form. DTT can reduce a disulfide bridge in NEM-βLG A; free sulfhydryl residues in NEM-βLG A newly arose, and intermolecular disulfide linkages between NEM-βLG A molecules by sulfhydryl/disulfide interchange reaction and/or oxidation reaction were formed.

Reheating of the Heated-βLG A Treated with NEM (NEM-Heated βLG A) in the Presence of DTT. From the results described above, it follows that free sulfhydryl residues other than Cys^{121} were also available for sulfhydryl/disulfide intermolecular interchange reac-

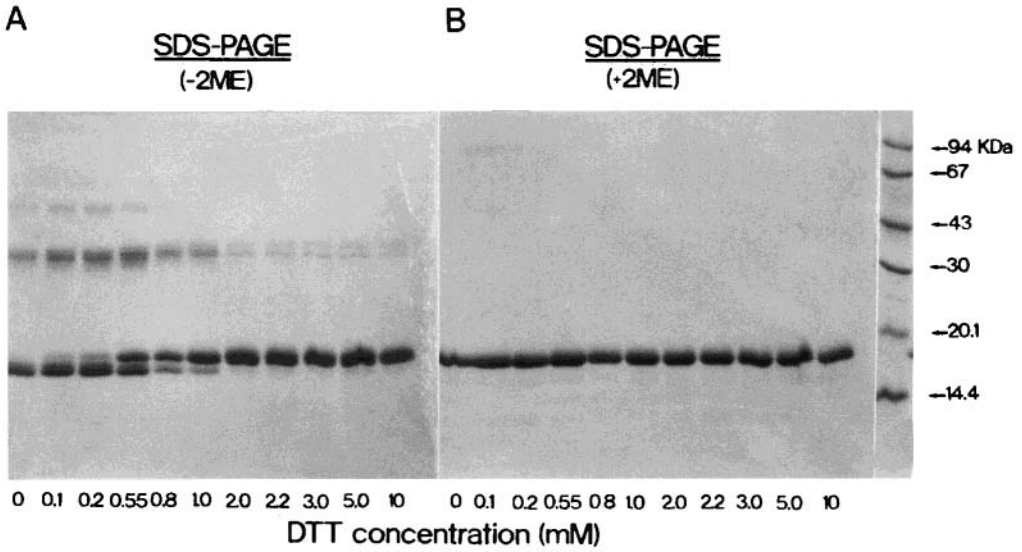

Figure 3. Nonreducing (A) and reducing (B) SDS-PAGE of NEM-heated βLG A after heating with DTT. βLG A (10 mg/mL; 0.55 mM, pH 7.5) was heated at 80 °C for 1 h, then incubated with NEM at 37 °C for 4 h, purified with Sephadex G-25, and then heated with DTT (0, 0.1, 0.2, 0.55, 0.8, 1.0, 2.0, 2.2, 3.0, 5.0, or 10 mM) at 80 °C for 1 h. After removal of unreacted DTT with Sephadex G-25, samples were electrophoresed. Marker proteins are the same as in Figure 1.

tion and making aggregates through intermolecular disulfide bridge. Newly formed sulfhydryl residues may contribute to enlargement of the aggregate by prolonged reaction. In the following experiments βLG A (10 mg/mL) was heated at 80 °C for 1 h, and the obtained heated βLG A was treated with NEM to block the remaining sulfhydryl residues including newly formed sulfhydryl residues as well as Cys^{121}. After treatment with NEM, excess NEM was removed by Sephadex G-25 and the heated βLG A treated with NEM (NEM-heated βLG A) was heated again in the presence of DTT (0−10 mM) to examine whether DTT can further enlarge the aggregates of the NEM-heated βLG A by sulfhydryl/disulfide interchange reaction similar to that in the case of heating of NEM-βLG A with DTT. SDS-PAGE without 2ME (Figure 3A) showed that there was an increase in the amount of aggregates of βLG A through intermolecular disulfide bridges up to 0.55 mM DTT and a decrease in the amount of polymerized βLG A for concentrations of DTT >0.55 mM. Similar to the results in Figures 1 and 2, there appeared double bands at the position of monomer by the incubation with DTT. The intensity of the band corresponding to monomer of nonreduced βLG A decreased as the concentration of DTT increased, a reduced monomer with a slightly lower mobility appeared (upper side of the double bands), and the intensity increased with increase in DTT concentration (Figure 3A). A dimer and other polymers also appeared, and the band intensity increased with increase in DTT concentration. However, on the SDS-PAGE (+2ME), only single bands were observed in all cases (Figure 3B).

Effects of Incubation with NaCl (0.2 M) on the NEM-βLG A Preheated with DTT (0.55 mM). Salt strongly affects the aggregation of heat-denatured protein (*23*−*27*). When protein is heated, amino acid residues that were once buried inside the molecule are exposed to the surface of the protein molecule. Most of these regions consist of hydrophobic amino acid residues, interacting to form aggregates by hydrophobic effects. High concentrations of salt can induce the aggregation of heat-denatured protein molecules. This is because salts tend to shield the charges on the surface of protein molecules, hence repressing a repulsive force among denatured molecules. Salt also disturbs the formation of tetrahedral structure constructed by water molecules. Therefore, the addition of salts accelerates the aggregation of the denatured protein. The effects of NaCl on aggregation of NEM-βLG A after heating with the same molar concentration of DTT were investigated.

NEM-βLG A (10 mg/mL; 0.55 mM) was heated with DTT (0.55 mM) at 80 °C for 1 h. After cooling, DTT was removed and the product incubated with 0.2 M NaCl at 37 °C for 0−120 min. Figure 4 shows native PAGE and SDS-PAGE patterns. Addition of salt to the NEM-βLG A treated with DTT showed no change regardless of incubation time. This observation suggests that the DTT-treated NEM-βLG A could not form aggregates or polymers by addition of NaCl, as there were no available hydrophobic areas on the surface of the NEM-βLG A molecule to allow intermolecular interaction. In this experiment DTT was removed from the sample after heating, and then the remaining NEM-βLG A was incubated with NaCl. It is possible that DTT promoted the conformational change of DTT-treated NEM-βLG A incubated with NaCl. To elucidate this, NEM-βLG A after heating in the presence of DTT was incubated with both NaCl and DTT and with only DTT.

Effects of Incubation with both DTT and NaCl on NEM-βLG A Preheated with DTT (0.55 mM). The NEM-βLG A preheated at 0.55 mM DTT was incubated in the presence of both NaCl and DTT. In Figure 5, SDS-PAGE without 2ME (−2ME) did not show any increase in further intermolecular aggregation, and the intensities of dimer and polymer bands were weakened. Double bands at the position of the monomer indicated that the inter- and/or intramolecular disulfide bridge in DTT-treated NEM-βLG A molecule was reduced by DTT (0.55 mM) even in the presence of NaCl. The DTT-treated NEM-βLG A molecules were already aggregated through intermolecular disulfide bridges. Further addition of DTT could not alter the form of aggregate and molecules. However, addition of DTT to NEM-βLG A in the presence of NaCl reduced the intermolecular disulfide bridge, indicating that disulfide bridges were available for reduction with DTT in the presence of NaCl, which

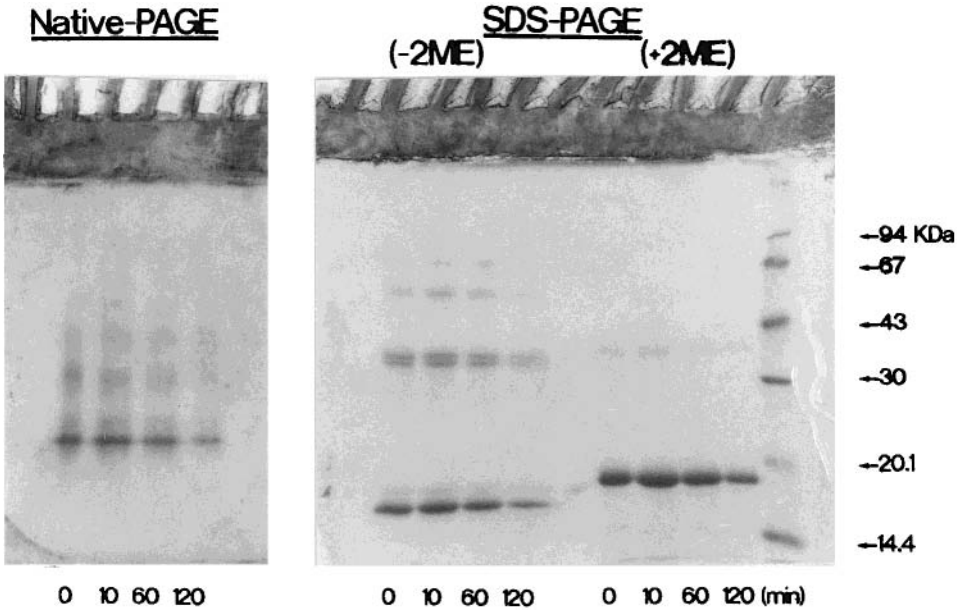

Figure 4. Non-denaturing (native) PAGE and nonreducing and reducing SDS-PAGE of DTT-treated NEM-βLG A after incubation with 0.2 M NaCl. NEM-βLG A (10 mg/mL; 0.55 mM, pH 7.5) was heated at 80 °C for 1 h with DTT (0.55 mM), and NEM-βLG A was purified with Sephadex G-25 and then incubated with 0.2 M NaCl at 37 °C for 0, 10, 60, or 120 min. After removal of NaCl with Sephadex G-25, samples were electrophoresed. Marker proteins are the same as in Figure 1.

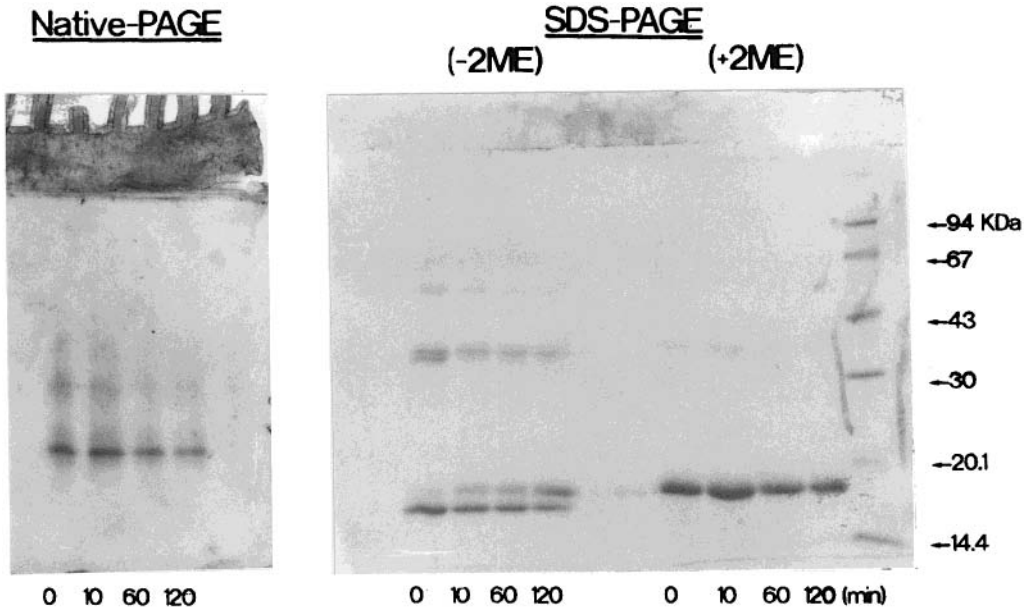

Figure 5. Non-denaturing (native) PAGE and nonreducing and reducing SDS-PAGE of DTT-treated NEM-βLG A after incubation with 0.2 M NaCl and 0.55 mM DTT. NEM-βLG A (10 mg/mL; 0.55 mM, pH 7.5) was heated at 80 °C for 1 h with DTT (0.55 mM), and NEM-βLG A was purified with Sephadex G-25 and then incubated with 0.2 M NaCl and 0.55 mM DTT at 37 °C for 0, 10, 60, or 120 min. After removal of NaCl and DTT with Sephadex G-25, samples were electrophoresed. Marker proteins are the same as in Figure 1.

altered the conformation of the NEM-βLG A molecule and its aggregate. This suggests that ionic interaction in and between NEM-βLG A molecules led to a tight structure that could not be attacked by DTT. Addition of NaCl released the ionic force, and disulfide linkages were reduced by DTT. Hoffmann and Van Mil (*8*) reported that the addition of NEM to βLG solution repressed the polymerization of βLG by heat through intermolecular disulfide bridge. Although they did not purify the NEM-modified βLG from the mixture, the importance of the free sulfhydryl residue for heat-induced polymerization was strongly suggested and is consistent with the result obtained in the present study. Liu et al. (*28*) investigated the effects of DTT on the heat-induced gelation time of βLG. They observed the delay of gelation by the addition of DTT, suggesting that blockage of disulfide formation inhibits the gelation. These findings also show the importance of intermolecular disulfide bridge formation during the gelation of βLG.

In this study, it was shown that the purified NEM-βLG A without a free sulfhydryl residue polymerized on heating in the presence of low concentrations of DTT. This result suggests that the newly formed sulfhydryl residue, probably from the intramolecular disulfide bond at Cys^{66}–Cys^{160} or Cys^{106}–Cys^{119}, induced the sulfhy-

dryl/disulfide interchange reaction resulting in intermolecular disulfide bridges (*15*, *29*−*31*).

ABBREVIATIONS USED

βLG A, β-lactoglobulin A; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2ME, 2-mercaptoethanol.

LITERATURE CITED

(1) Morr, C. V.; Foegeding, E. A. Composition and functionality of commercial whey and milk protein concentrates and isolates: a status report. *Food Technol*. **1990**, *44* (4), 100−112.

(2) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383−385.

(3) Lee, S.-P.; Cho, Y.; Batt, C. A. Enhancing the gelation of β-lactoglobulin. *J. Agric. Food Chem*. **1993**, *41*, 1343−1348.

(4) Xiong, Y. L.; Dawson, K. A.; Wan, L. Thermal aggregation of β-lactoglobulin: Effect of pH, ionic environment, and thiol reagent. *J. Dairy Sci*. **1993**, *76*, 70−77.

(5) Mcswinney, M.; Singh, H.; Campanella, O. H. Thermal aggregation and gelation of bovine β-lactoglobulin. *Food Hydrocolloids* **1994**, *8* (5), 441−453.

(6) Iametti, S.; Cairoli, S.; De Gregori, B.; Bonomi, F. Modifications of high-order structures upon heating of β-lactoglobulin: dependence on the protein concentration. *J. Agric. Food Chem*. **1995**, *43*, 53−58.

(7) Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin. *Eur. J. Biochem*. **1996**, *237*, 106−112.

(8) Hoffmann, M. A. M.; Van Mil, P. J. J. M. Heat-induced aggregation of β-lactoglobulin: Role of the free thiol group and disulfide bonds. *J. Agric. Food Chem*. **1997**, *45*, 2942−2948.

(9) Hoffmann, M. A. M.; Sala, G.; Olieman, C.; De Kruif, K. G. Molecular mass distributions of heat-induced β-lactoglobulin aggregates. *J. Agric. Food Chem*. **1997**, *45*, 2949−2957.

(10) Galani, D.; Apenten, R. K. O. Heat-induced denaturation and aggregation of β-lactoglobulin: kinetics of formation of hydrophobic and disulfide-linked aggregates. *Int. J. Food Sci. Technol*. **1999**, *34*, 467−476.

(11) Shimada, K.; Cheftel, J. C. Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate. *J. Agric. Food Chem*. **1989**, *37*, 161−168.

(12) Monahan, F. J.; German, J. B.; Kinsella, J. E. Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *J. Agric. Food Chem*. **1995**, *43*, 46−52.

(13) Bazinet, L.; Lamarche, F.; Boulet, M.; Amiot, J. Combined effect of pH and temperature during electroreduction of whey proteins. *J. Agric. Food Chem*. **1997**, *45*, 101−107.

(14) Dalgleish, D. G.; Senaratne, V.; Francois, S. Interaction between β-lactalbumin and β-lactoglobulin in the early stages of heat denaturation. *J. Agric. Food Chem*. **1997**, *45*, 3459−3464.

(15) Gezimati, J.; Creamer, L. K.; Singh, H. Heat-induced interactions and gelation of mixtures of β-lactoglobulin and β-lactalbumin. *J. Agric. Food Chem*. **1997**, *45*, 1130−1136.

(16) Gallagher, D. P.; Lynch, M. G.; Mulvihill, D. M. Porcine β-lactoglobulin does not undergo thermally induced gelation. *J. Dairy Res*. **1996**, *63*, 479−482.

(17) Kitabatake, N.; Wada, R.; Fujita, Y. Reversible conformational change in the β-lactoglobulin A modified with *N*-ethylmaleimide and resistance to molecular aggregation on heating. *J. Agric. Food Chem*. **2001**, submitted for publication.

(18) Davie, E. W.; Newman, C. R.; Wilcox, P. E. Action of carboxypeptidase on β-lactoglobulin. *J. Biol. Chem*. **1959**, *234*, 2635−2641.

(19) Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys*. **1959**, *82*, 70−77.

(20) Janatova, J.; Fuller, J. K.; Hunter, M. J. The heterogeneity of bovine albumin with respect to sulfhydryl and dimer content. *J. Biol. Chem*. **1968**, *243*, 3612−3622.

(21) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriopharge T4. *Nature* **1970**, *227*, 680−685.

(22) Davis, B. J. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci*. **1964**, *121*, 404−427.

(23) Kitabatake, N.; Tahara, M.; Doi, E. Thermal denaturation of soybean protein at low water contents. *Agric. Biol. Chem*. **1990**, *54*, 2205−2212.

(24) Kinekawa, Y.; Kitabatake, N. Turbidity and rheological properties of gels and sols prepared by heating process whey protein. *Biosci., Biotechnol., Biochem*. **1995**, *59* (5), 834−840.

(25) Kitabatake, N.; Fujita, Y.; Kinekawa, Y. Viscous sol and gel formation from process whey protein below 25 °C. *J. Food Sci*. **1996**, *61*, 500−503.

(26) Verheul, M.; Roefs, S. P. F. M.; De Kruif, K. G. Kinetics of heat-induced aggregation of β-lactoglobulin. *J. Agric. Food Chem*. **1998**, *46*, 896−903.

(27) Ju, Z. Y.; Kilara, A. Gelation of pH-aggregated whey protein isolate solution induced by heat, protease, calcium salt, and acidulant. *J. Agric. Food Chem*. **1998**, *46*, 1830−1835.

(28) Liu, T. X.; Relkin, P.; Launay, B. Thermal denaturation and heat-induced gelation properties of β-lactoglobulin. Effects of some chemical parameters. *Thermochim. Acta* **1994**, *246*, 387−403.

(29) Molinari, H.; Ragona, L.; Varani, L.; Musco, G.; Consonni, R.; Zetta, L.; Monaco, H. L. Partially folded structure of monomeric bovine β-lactoglobulin. *FEBS Lett*. **1996**, *381*, 237−243.

(30) Manderson, G. A.; Hardman, M. J.; Creamer, L. K. Effect of heat treatment on the conformation and aggregation of β-lactoglobulin A, B, and C. *J. Agric. Food Chem*. **1998**, *46*, 5052−5061.

(31) Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, H. M.; Baker, E. N.; Jameson, G. B. Structural basis of the tanford transition of bovine β-lactoglobulin. *Biochemistry* **1998**, *37*, 14014−14023.

Received for review December 6, 2000. Revised manuscript received July 16, 2001. Accepted August 2, 2001.

JF001457V