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Received for review August 27, 1987. Revised manuscript received May 25, 1988. Accepted July 13, 1988. Mention of a trademark or proprietary product is for identification only and does not imply a warranty or guarantee of the product by the U.S. Department of Agriculture over other products that may also be suitable.

Sulfhydryl Group/Disulfide Bond Interchange Reactions during Heat-Induced Gelation of Whey Protein Isolate

Kazuko Shimada[1] and Jean Claude Cheftel*

The kinetics of reaction between the SH group of β-lactoglobulin and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was used to detect the SH/S–S interchange reactions taking place when dispersions of whey protein isolate (1 or 9% protein) were heated at 85 °C at pH 7.5 or 2.5. The method is based on the assumption that the reactivity of the SH^{121} group adjacent to the $S–S^{106–119}$ bond (native state) is low in the presence of sodium dodecyl sulfate (SDS). However, the new SH group formed in position 66 or 160 through an SH/S–S interchange reaction reacts rapidly with DTNB in the presence of SDS. The data of reaction kinetics were compared to those of protein solubility, gel texture, and SDS–polyacrylamide gel electrophoresis. Heating a 9% protein dispersion caused (1) the formation of a highly elastic gel at pH 7.5 [intermolecular S–S bonds due essentially to SH/S–S interchange reactions are predominant in the gel network and partly responsible for the high elasticity] and (2) the formation of a nonelastic gel at pH 2.5.

It is known that disulfide (S–S) bonds and sulfhydryl (SH) groups play an important role in the heat-induced gelation of proteins. Covalent cross-linking of protein molecules can be brought by SH oxidation into S–S bonds and/or by SH-induced S–S interchange reactions. This has been reported by Yasuda et al. (1986) for bovine serum albumin (BSA), by Jiang et al. (1986) for fish proteins, by Mori et al. (1982), Utsumi and Kinsella (1985a,b), Mori et al. (1986), and Shimada and Cheftel (1988a) for soy proteins, by Shimada and Matsushita (1980) and Hayakawa and Nakai (1985) for egg albumin, by Beveridge et al. (1984) for egg albumin and whey protein concentrate, and by Schmidt et al. (1978, 1979), Dunkerley and Zadow (1984), and To et al. (1985) for whey protein concentrate. However, none of these studies has indicated whether intermolecular S–S bonds in the network of protein gels depend mainly on SH oxidation into additional S–S bonds or on SH/S–S interchange reactions.

The changes in SH group/S–S bond contents have been recently investigated in heat-induced gels of whey protein isolate (WPI) (Shimada and Cheftel, 1988b). It was observed that the rate of reaction between SH groups and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (in a Tris buffer containing 8 M urea and 0.5% sodium dodecyl sulfate) was lower for unheated WPI than for heat-processed WPI. It has also been demonstrated that β-lactoglobulin (β-LG), the major protein of WPI, reacts about 150 times slower with *N*-ethylmaleimide (NEM) in 1% sodium dodecyl sulfate (SDS) than in 8 M urea (Franklin and Leslie, 1968). These results lead to the hypothesis that SDS may inhibit the reaction between the SH group of native β-LG and DTNB and that the inhibition by SDS decreases after heat processing of β-LG.

In the present study, it is attempted to determine changes in the contents of slow-reacting (with DTNB) SH groups (mainly the SH group in position 121 adjacent to the S–S bond between Cys^{106} and Cys^{119}; native β-LG) as a function of protein concentration and of pH during the heating of WPI dispersions. The method takes advantage of differences in kinetics and is based on the assumption that the SH^{121} group of β-LG reacts slowly with DTNB in the presence of SDS as compared to SH groups in other positions, especially those formed through SH/S–S interchange reactions. Correlations between the texture of WPI gels and SH/S–S interchange reactions have also been investigated.

MATERIALS AND METHODS

Materials. Bovine β-lactoglobulin-1 (β-LG-1; lyophilized, Lot No. 52F-8035) and bovine β-lactoglobulin-2 (β-LG-2; 3× crystallized and lyophilized; Lot No. 36F-8085) were obtained from Sigma Chemical Co., St. Louis. Both β-LG contained genetic variants A and B, and β-LG-1 also contained about 2% NaCl.

Whey protein isolate (WPI) was obtained as indicated previously (Shimada and Cheftel, 1988b). It contained about 72 g of native β-LG and 5 g of native α-lactalbumin (α-LA) per 100 g of protein.

Heat Treatment of WPI Dispersions and Preparation of Gels. The aqueous dispersions of WPI (1 or 9% protein) were adjusted to pH 7.5 or 2.5 with 6 NaOH or 6 N HCl, treated for partial deaeration (to avoid air bubbles in the subsequent gels) with a water pump for 2 min, and placed in glass tubes (2.2-cm i.d. × 4.5-cm height) with tightly closed stoppers. The tubes were heated at 85 °C for varying periods of time (0–45 min) in a water bath and then cooled rapidly in ice water. After being allowed to

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0021-8561/89/1437-0161$01.50/0

stand at 0 °C for 1 h, the heat-processed WPI samples were kept at room temperature for 1–3 h before all analyses.

Determination of Protein Solubility, Total SH Groups (SHt), and Slow-Reacting SH Groups (SHs). WPI gels or heated solutions were solubilized either in 0.086 M Tris–0.09 M glycine–4 mM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), pH 8.0 buffer (standard buffer) (analysis of protein solubility), or in the same standard buffer containing also 8 M urea and 0.5% (17.3 mM) SDS (analyses of protein solubility, of total SH groups, of slow-reacting SH groups, and SDS–polyacrylamide gel electrophoresis (SDS-PAGE)). The protein solutions were adjusted to 0.1% (0.05 g of protein/50 mL of buffer), homogenized with an Ultra-Turrax for 3 min below 25 °C, and then centrifuged at 20000*g* for 15 min. Protein solubility was calculated as 100 × protein content of the supernatant/total protein content.

Total SH groups and slow-reacting SH groups were determined with use of DTNB according to Ellman (1959). To a 3-mL aliquot of the protein supernatant, of unheated β-LG-1 or -2, or of unheated WPI solution (each at a 0.1% proteion concentration) in the standard buffer containing 8 M urea and 0.5% SDS (unless otherwise noted) was added 0.03 mL of Ellman's reagent solution (40 mg of DTNB/10 mL of standard buffer) at zero time. Unheated β-LG and WPI were dissolved in the standard buffer containing denaturant(s) 1 h before the addition of DTNB. After the solutions were mixed rapidly, the absorbance was recorded at 412 nm on a Beckman Model DB-GT spectrophotometer at 23 °C, against a reagent blank. A molar extinction coefficient of 1.36×10^4 $M^{-1} \cdot cm^{-1}$ was used for calculating micromoles of SH/gram of protein.

SDS-PAGE. β-LG (1 and 2) and WPI were dissolved in 50 mM Tris-HCl buffer (pH 6.8) containing 7.5% glycerol and 2% SDS with 0.02% NEM or 5% 2-mercaptoethanol (2-ME) (1 mg of protein/mL) and the resultant mixtures left overnight at room temperature before electrophoresis. Protein solutions extracted from WPI gels or heated 1% protein dispersions were treated with 0.02% NEM for 5 h and then dialyzed against 50 mM Tris-HCl buffer (pH 6.8) containing 7.5% glycerol and 2% SDS. Protein contents of dialyzed protein solutions were measured by the method of Lowry et al. (1951) using BSA as a standard. The protein concentrations were adjusted to 0.80 mg/mL. A part of the dialyzed protein solution was treated overnight with 2-ME at room temperature. Portions of 25 μL of protein solutions with or without 2-ME were used for SDS-PAGE.

SDS-PAGE was performed according to Laemmli (1970). Linear-gradient separating gel was prepared with equal volumes of 8 and 20% acrylamide solutions. The stacking gel contained 4.4% acrylamide. Electrophoresis was performed with constant power (35 W) at 15 °C for 4 h. The gel was stained with 0.1% Coomassie Brilliant Blue R-250 for 1 h and destained with 7.5% acetic acid plus 5% methanol. The relative intensity of the stained bands was determined by scanning the gel sheet with a densitometer, Model GS 300, Hoefer Scientific Instruments. The molecular weight of each protein band was checked with known protein standards.

Determination of Gel Texture. Texture analysis (firmness and elasticity) was carried out on gel sections (2.2-cm diameter × 1.0-cm height) at 25 °C with a Stevens-LFRA texture analyzer as indicated previously (Shimada and Cheftel, 1988b).

Protein Determination. Protein concentration was determined by spectrophotometry using values of $E^{1\%}_{1cm}$ at 280 nm of 9.5 for β-LG (Boyer, 1954) and of 10.2 for WPI (Shimada and Cheftel, 1988b).

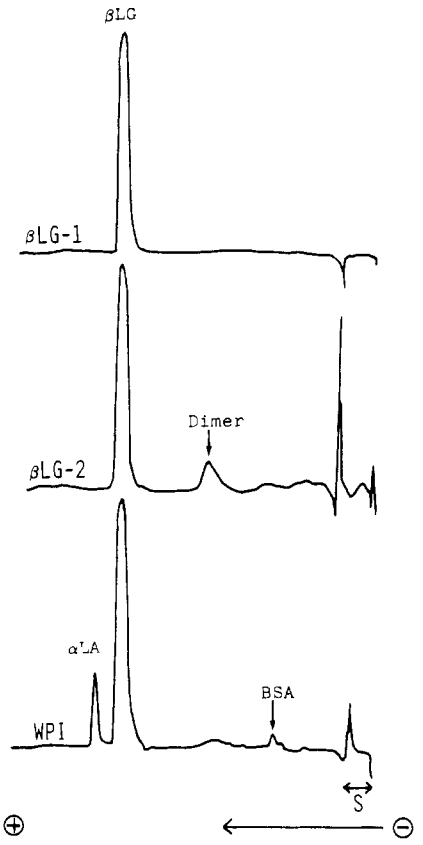

Figure 1. SDS-PAGE patterns of β-LG-1, β-LG-2, and WPI. Migration is from right to left. S = stacking gel.

RESULTS AND DISCUSSION

Reaction of β-LG Preparations and of WPI with DTNB. SDS-PAGE (without 2-ME) patterns of unheated β-LG-1, β-LG-2, and WPI are shown in Figure 1. β-LG-1 showed a single protein band, while β-LG-2 and WPI contained additional protein species. SDS-PAGE in the presence of 2-ME (data not shown) plus identification of molecular weights with standard proteins revealed that the additional protein species of β-LG-2 were composed of β-LG dimer and polymers, while WPI contained α-LA, BSA, dimers (2 β-LG and/or β-LG–α-LA), and higher molecular weight fractions that could not enter the separation gel [immunoglobulins (IG) and/or polymers].

The reaction rates of β-LG (1 and 2) and WPI with DTNB were compared in the standard buffer containing 8 M urea with or without 0.5% SDS (Figure 2A). Reaction in 8 M urea alone was completed before the first reading, 30 s after mixing, while reaction in 8 M urea plus 0.5% SDS was completed only after about 30 min. The total SH group contents of β-LG-1, β-LG-2, and WPI were 54.1, 43.0, and 40.6 μmol/g of protein, respectively. The SH group content of β-LG-1 is consistent with the value for pure β-LG (54.6 μmol/g of protein; Brunner, 1977). Some of the SH groups in β-LG-2 may have been previously oxidized to S–S bonds and/or to other oxidation products. The SH groups of WPI probably come mainly from β-LG because WPI contains only little amounts of BSA and of IG, and α-LA does not contain any SH group.

Although the reaction between the SH groups of protein and DTNB is known to follow a second-order kinetics (Friedman, 1973), pseudo-first-order reaction is apparent under conditions where DTNB is in excess (Srere, 1965; Phillips et al., 1967; Okabe et al., 1970). In the present study, a 2-fold excess of DTNB was used (a larger excess

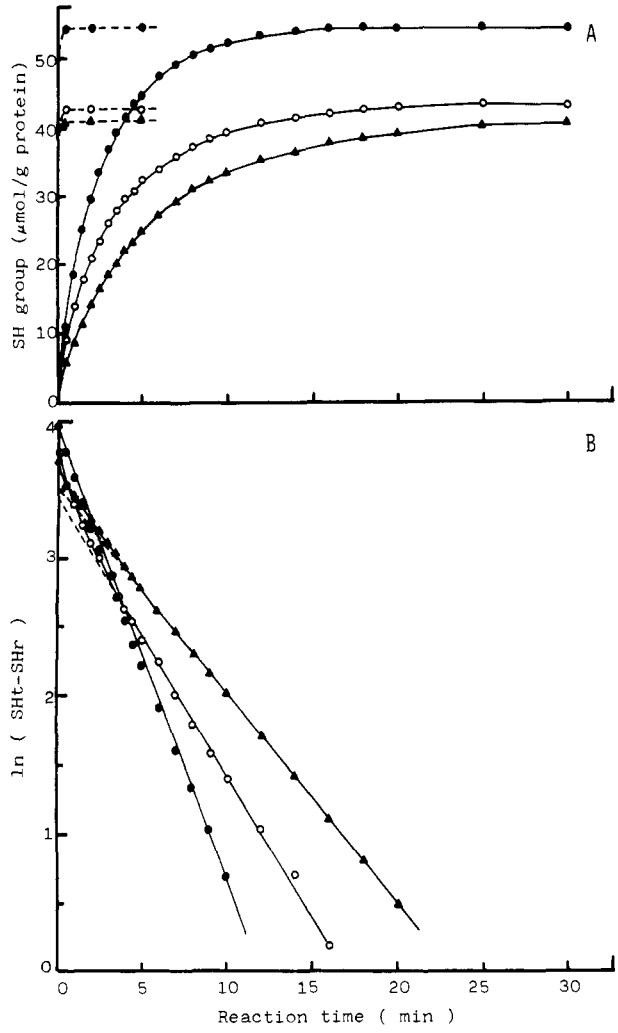

Figure 2. Reaction of β-LG-1, β-LG-2, and WPI with DTNB in the standard buffer containing 8 M urea with or without 0.5% SDS (A) and corresponding pseudo-first-order plots in the standard buffer containing 8 M urea and 0.5% SDS (B). The reaction was carried out with 0.1 g of protein/100 mL and 99 μM DTNB. Key: (●) β-LG-1, (○) β-LG-2, (▲) WPI. A: (—) standard buffer containing 8 M urea and 0.5% SDS; (---) standard buffer containing 8 M urea. B: SHt = total SH group content obtained from maximum value; SHr = content of SH groups having reacted at time t.

was avoided in order to prevent an increase in the reaction rate). The equation of pseudo first order is

$$\ln [SHt-SHr] = -k_{obs}t + \ln [SHt] \quad (1)$$

where SHt is the total SH group content obtained from the maximum value, SHr is the content of SH groups having reacted at time t, and k_{obs} is the observed reaction rate constant. The reaction rate constant is known to be greatly influenced by the initial concentration of DTNB (Okabe et al., 1970).

It can be seen from Figure 2B that the reaction between DTNB and the SH groups of β-LG-1, β-LG-2, or WPI in 8 M urea and 0.5% SDS followed a pseudo-first-order kinetics up to at least 90% of the total reaction (in 8 M urea alone the reaction was too fast to obtain kinetic data). The pseudo-first-order plot was linear with β-LG-1, but minor deviations were observed near zero time for β-LG-2 and WPI. This indicates that all the SH groups of β-LG-1 have the same reactivity to DTNB, while those of β-LG-2 or WPI may exhibit different reactivities. Phillips et al. (1967) have shown that the genetic variants A and B of β-LG react with DTNB at identical rates in potassium phosphate buffer, pH 7.6.

The SH group of native β-LG is located in position 121, near the S-S bond between Cys^{106} and Cys^{119} (Creamer et al., 1983; Papiz et al., 1986). The sequence of β-LG reveals that many hydrophobic amino acids are located near the SH^{121} group and the $S-S^{106-119}$ bond (Leu^{103}, Leu^{104}, Phe^{105}, Leu^{117}, Val^{118} (A variant), Ala^{118} (B variant), Leu^{122}, Val^{123}), while fewer hydrophobic residues are located near the other $S-S^{66-160}$ bond (Creamer et al., 1983).

The experimental observation (Figure 2) that the SH group of β-LG reacts quickly or slowly with DTNB in urea depending on the absence or presence of SDS may be explained by a reversible interaction between SDS and the hydrophobic region near the SH^{121} group. The bound SDS molecules may block the accessibility of DTNB to the SH^{121} group. The decreased reactivity of SH groups in the presence of SDS was not observed with soy proteins or BSA (Shimada and Cheftel, 1988a).

If the SH^{121} group reacts with the $S-S^{66-160}$ bond through an interchange reaction, as SH group will be formed at position 66 or 160, in a nonhydrophobic environment. This new SH group may then react quickly with DTNB even in the presence of SDS.

If a β-LG sample contains both native and displaced SH groups, the reaction with DTNB (in the presence of SDS) will represent the sum of slow and fast reactions. Once the fast reaction is completed, only the slow reaction will proceed. The pseudo-first-order equation for the slow reaction is

$$\ln [SHt - SHr] = -k_{obs_s}t + \ln [SHs] \quad (2)$$

where k_{obs_s} is the observed reaction rate constant for the slow reaction and [SHs] is the total content of SH groups reacting slowly with DTNB. These SH groups are located in the hydrophobic environment that binds SDS. From the known sequence of β-LG, it can be speculated that these SH groups are mainly in the 121-position adjacent to the $S-S^{106-119}$ bond (native state) and possibly also in position 119 adjacent to a $S-S^{106-121}$ bond. An intramolecular interchange reaction between SH^{121} and $S-S^{106-119}$ may easily take place when the β-LG conformation is distorted (McKenzie et al., 1972; Papiz et al., 1986), but it is likely that the environment near the newly formed SH^{119} group remains similar to that near the native SH^{121} group. Thus, any decrease in the initial content of slow-reacting SH groups (SHs) may reflect approximately the number of previous SH/S-S interchange reactions involving the $S-S^{66-160}$ bond.

From eq 2, one can obtain the total SH content (SHt; intercepted value at zero time), the content of slow-reacting SH (SHs; extrapolated value at zero time of the linear ln [SHt - SHr] versus t plot), and the rate constant for slow reaction (k_{obs_s}; slope of straight line). The SHs contents of β-LG-1, β-LG-2, and WPI were obtained graphically from Figure 2B and found to be equal to 54.1, 31.2, and 33.1 μmol/g of protein, respectively. The SHt contents have already been indicated. The SHs to SHt ratios in β-LG-1, β-LG-2, and WPI are therefore 100, 72.5, and 81.5%, respectively. This indicates that the SH groups in the commercial β-LG-2 and the industrial WPI preparations have partly undergone SH/S-S interchange reactions.

Reaction of WPI with DTNB as a Function of the Concentration of SDS, Urea, and NaCl. The time course and the observed rate constants of the reaction between WPI and DTNB are shown in Figures 3 and 4 as a function of the concentration of various chemicals. In 8 M urea plus various concentrations of SDS (0.1–1.0%), the rate of the slow SH–DTNB reaction first decreased with increasing concentration of SDS (up to 0.2% SDS),

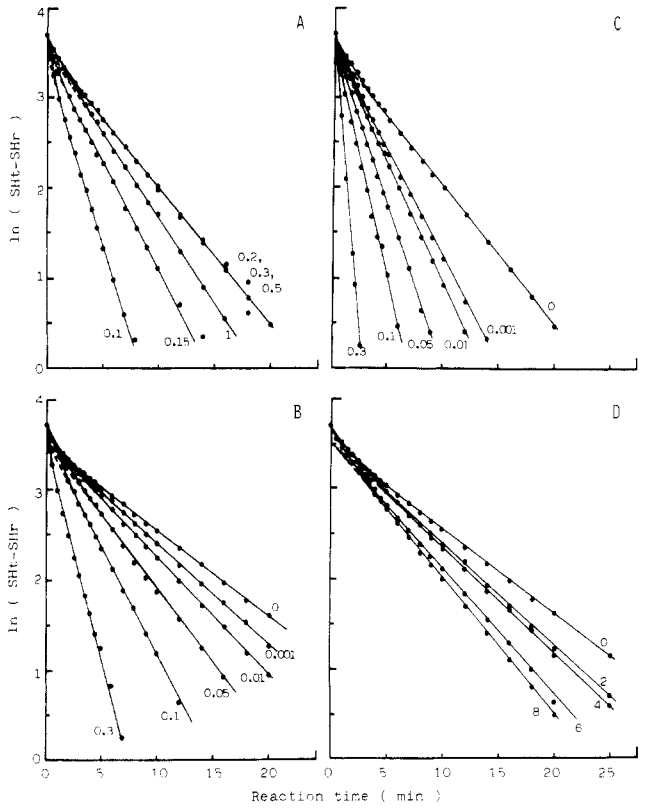

Figure 3. Pseudo-first-order plots for the reaction of DTNB with WPI: A, reaction in the standard buffer containing 8 M urea plus increasing SDS concentrations (0.1–1.0%); B, reaction in the standard buffer containing 0.5% SDS plus increasing NaCl concentrations (0–0.3 M); C, reaction in the standard buffer containing 8 M urea, 0.5% SDS plus increasing NaCl concentrations (0–0.3 M); D, reaction in the standard buffer containing 0.5% SDS plus increasing urea concentrations (0–8 M). Reactions were carried out with 0.1 g of protein/100 mL and 99 μM DTNB. Figures on each graph represent concentration of SDS, NaCl, or urea.

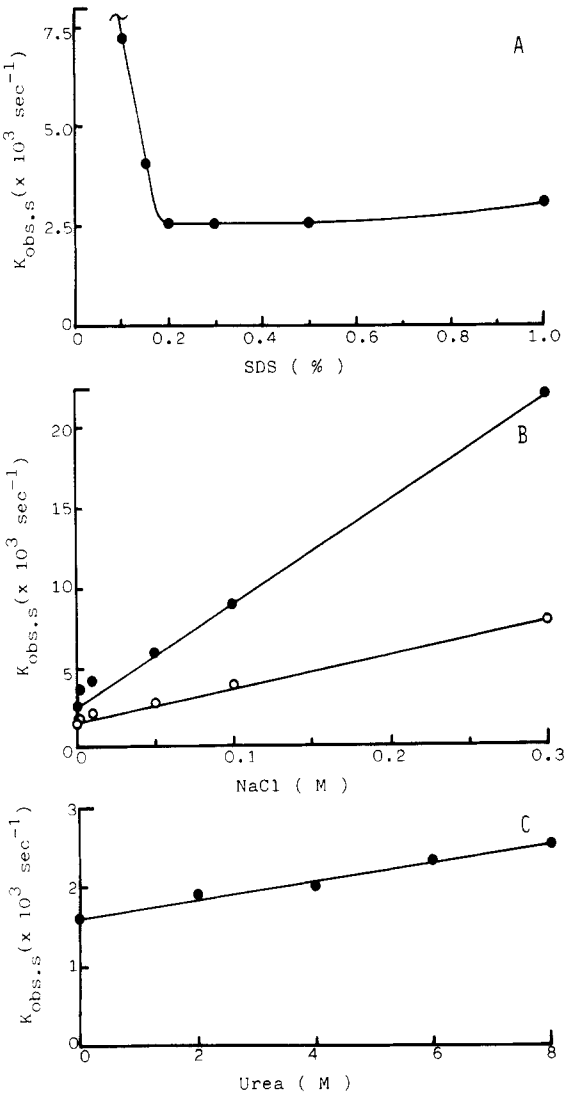

Figure 4. Influence of the concentration of SDS (A), NaCl (B), or urea (C) on the observed rate constant of the WPI–DTNB reaction. Reactions were carried out with 0.1 g of protein/100 mL and 99 μM DTNB. Key: A, reaction in the standard buffer containing 8 M urea plus increasing SDS concentrations (0.1–1.0%); B, reaction in the standard buffer (○) containing 0.5% SDS plus increasing NaCl concentrations (0–0.3 M) and (●) containing 8 M urea and 0.5% SDS plus increasing NaCl concentrations (0–0.3 M); C, reaction in the standard buffer containing 0.5% SDS plus increasing urea concentrations (0–8 M).

was constant from 0.2 to 0.5%, and then increased slightly at 1.0% (Figures 3A and 4A). It is known that the critical micellar concentration (CMC) of SDS is 0.24% (8.2 mM) in distilled water and 0.015% (0.52 mM) in a 0.5 M NaCl solution, at room temperature (Helenius and Simons, 1975). The CMC of SDS in the standard buffer containing 8 M urea is unknown but may be close to 0.2%, since the rate of reaction does not decrease further when the SDS concentration exceeds 0.2%. It is likely that the inhibition of the slow SH–DTNB reaction depends on the free (nonmicellar) SDS concentration. In order to check this point, the rate constant of reaction was measured in the presence of NaCl (0–0.3 M) (Figures 3B,C and 4B). The CMC of SDS is reduced in the presence of NaCl, as indicated above. NaCl may also affect eventual electrostatic interactions between SDS and β-LG by neutralizing electrical charges. The observed rate constant increased as the NaCl concentration of the standard buffer containing 0.5% SDS (with or without 8 M urea) increased. This may result from reduced SDS binding to β-LG at lower free SDS concentrations and/or to the neutralization of electric charges by NaCl. Since the rate constant is greatly influenced by NaCl, the different rates of reaction (k_{obs}) between DTNB and β-LG-1, β-LG-2, and WPI (Figure 2B) may be due to salt contaminants present in these protein preparations.

In the presence of 8 M urea, the reaction rate constant increased very markedly with increasing NaCl concentrations (Figure 4B). The interaction between SDS and β-LG may decrease with increasing NaCl concentration because the free SDS concentration decreases. The rate constant also increased with increasing urea concentrations (in the presence of 0.5% SDS; Figures 3D and 4C). Urea may therefore also reduce the hydrophobic binding of SDS to β-LG. Phillips et al. (1967) speculated that the slower DTNB–β-LG reaction rate in SDS solutions may be caused both by the steric effect of bound SDS molecules and by the compactness of the β-LG–SDS complex. Damon and Kresheck (1982) observed that α-helix and β-sheet structures partly persisted in β-LG in the presence of SDS. In the presence of urea, unfolding of the β-LG–SDS complex into unordered open forms may lead to increased reaction rates with DTNB.

Although the observed rate constant depended on the concentrations of SDS, NaCl, and urea, the SHs values remained constant in all experiments (Figure 3). The standard buffer containing 8 M urea plus 0.5% SDS was selected for the next experiments in order to achieve an extensive solubilization of heat-induced WPI gels.

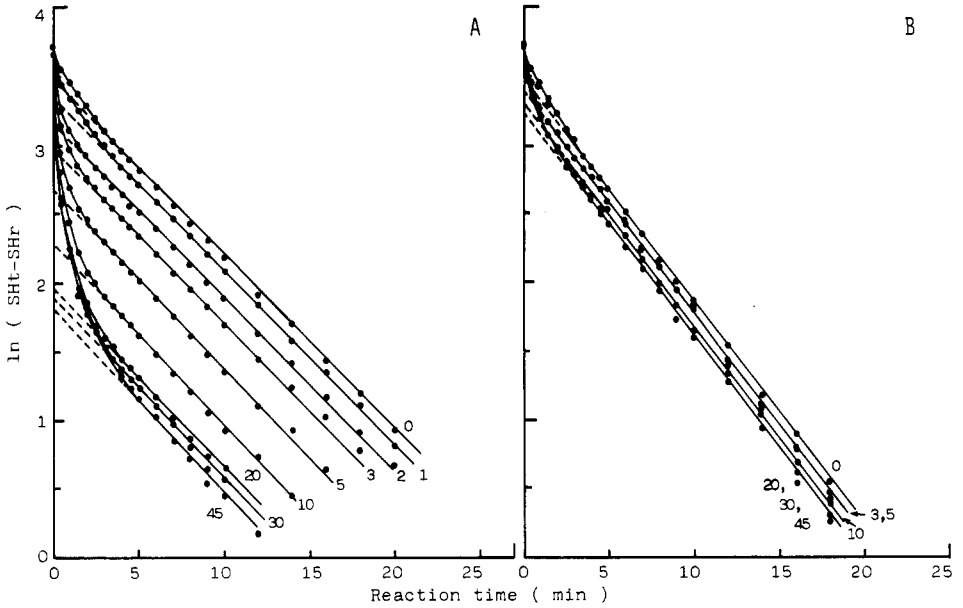

Figure 5. Pseudo-first-order plots for the reaction of DTNB with heated WPI dispersions. Reactions were carried out with 0.1 g of protein/100 mL and 99 μM DTNB in the standard buffer containing 8 M urea and 0.5% SDS. WPI dispersions (1 g of protein/100 mL) were previously heated at 85 °C for various times (0–45 min, as indicated on the figure) at pH 7.5 (A) or at pH 2.5 (B).

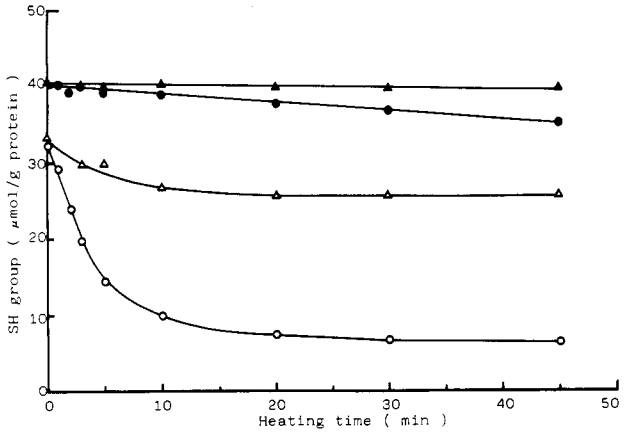

Figure 6. Total SH group content and content of slow-reacting SH groups as a function of the heating time (0–45 min) at 85 °C of a WPI dispersion (1 g of protein/100 mL) at pH 7.5 or at pH 2.5: (●) total SH groups (pH 7.5); (○) slow-reacting SH groups (pH 7.5); (▲) total SH groups (pH 2.5); (△) slow-reacting SH groups (pH 2.5).

SH/S–S Interchange Reactions during Heat Processing of 1% WPI Dispersions. At pH 7.5 or 2.5, WPI dispersions containing 1 g of protein/100 mL were heated at 85 °C for 0–45 min. The heated dispersions were dissolved completely in the standard buffer containing 8 M urea plus 0.5% SDS. The kinetics of reaction between heated WPI and DTNB are shown in Figures 5 and 6. At pH 7.5, the SHt content decreased slightly with heating time, while the SHs content decreased markedly (although the rate of slow reaction remained constant as expected) (Figures 5A and 6). At pH 2.5, the SHt content remained constant during the 45-min heating period while the SHs content decreased moderately (and the rate of reaction remained constant) (Figures 5B and 6). This appears to reflect the low reactivity of SH groups at acid pHs.

SDS-PAGE (without 2-ME) patterns of the heated WPI dispersion are shown in Figure 7. When heating was carried out at pH 7.5, β-LG, α-LA, and BSA fractions decreased and the dimer fraction increased with heating time. Trimer and tetramer fractions appeared after a heating period of 10 min (Figure 7A). SDS-PAGE (with 2-ME) of WPI heated for 45 min revealed that these

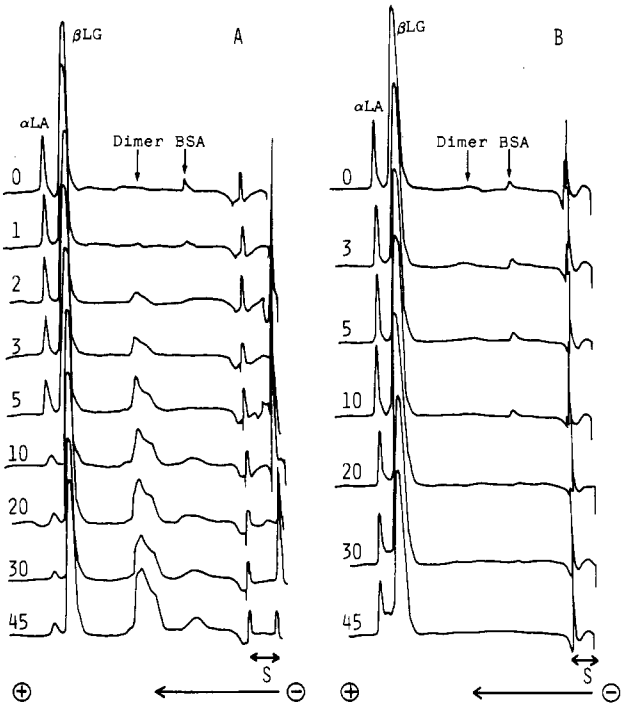

Figure 7. SDS-PAGE patterns of WPI dispersions (1 g of protein/100 mL) heated at 85 °C for various times (0–45 min, as indicated on the figure) at pH 7.5 (A) or at pH 2.5 (B). Migration is from right to left. S = stacking gel.

polymers were heterogeneous fractions composed of β-LG and α-LA linked by S–S bonds (data not given here). When heating was carried out at pH 2.5, β-LG and α-LA fractions decreased slightly with heating time, but polymers were not formed (Figure 7B). Harwalkar (1980, 1986) has shown that protein constituents resisted aggregation at pH 2.5 (but not at pH 4.5 or 6.5) when acid whey or β-LG (1% protein concentration) was heated at 90 °C for 30 or 60 min. An additional protein band appeared between α-LA dn β-LG after 20-min heating at pH 2.5, possibly corresponding to an acid hydrolysis product of β-LG (Figure 7B).

The decrease in slow-reacting SH groups during heating at pH 7.5 was roughly parallel to the increase in protein

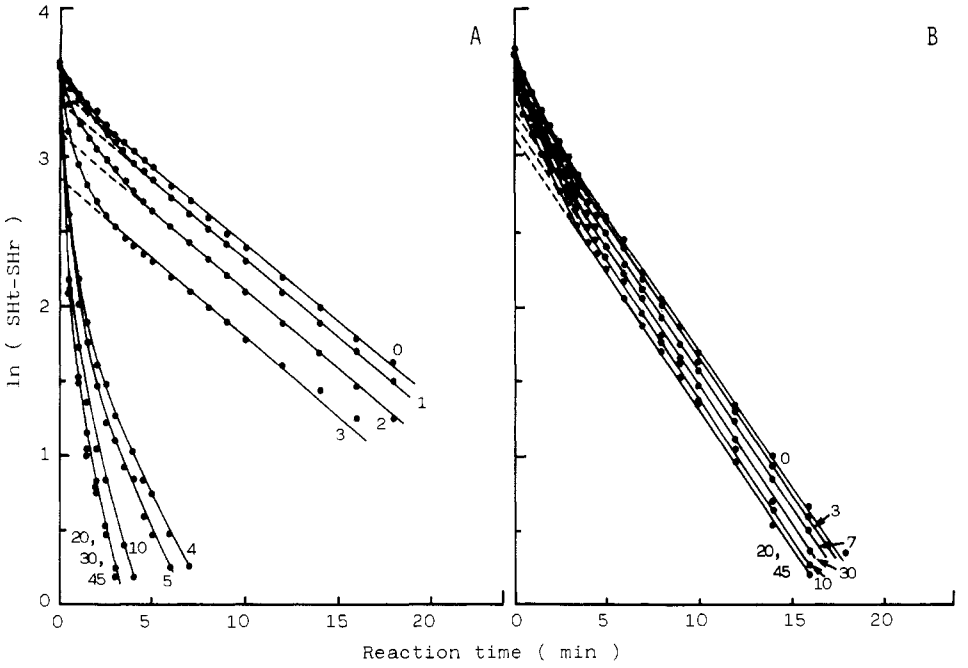

Figure 8. Pseudo-first-order plots for the reaction of DTNB with heated WPI dispersions. Reactions were carried out with 0.1 g of protein/100 mL and 99 μM DTNB in the standard buffer containing 8 M urea and 0.5% SDS. WPI dispersions (9 g of protein/100 mL) were previously heated at 85 °C for various times (0–45 min, as indicated on the figure) at pH 7.5 (A) or at pH 2.5 (B).

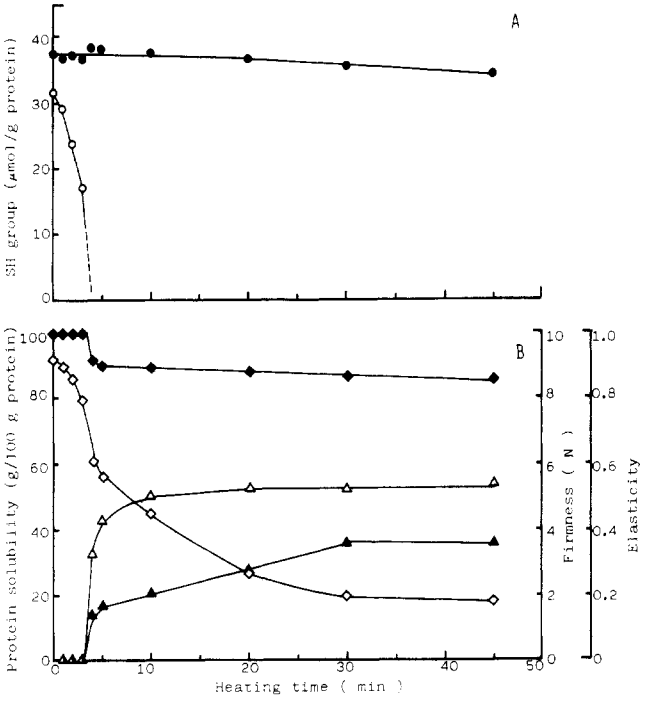

Figure 9. Total SH group content, content of slow-reacting SH group (A), gel texture, and protein solubility (B) as a function of the heating time (0–45 min) at 85 °C of WPI dispersions (9 g of protein/100 mL) at pH 7.5: A, (●) total SH groups, (○) slow-reacting SH groups; B, (▲) gel firmness, (△) gel elasticity, (♦) protein solubility of dispersion or gel in the standard buffer containing 8 M urea and 0.5% SDS, (◇) protein solubility of dispersion or gel in the standard buffer.

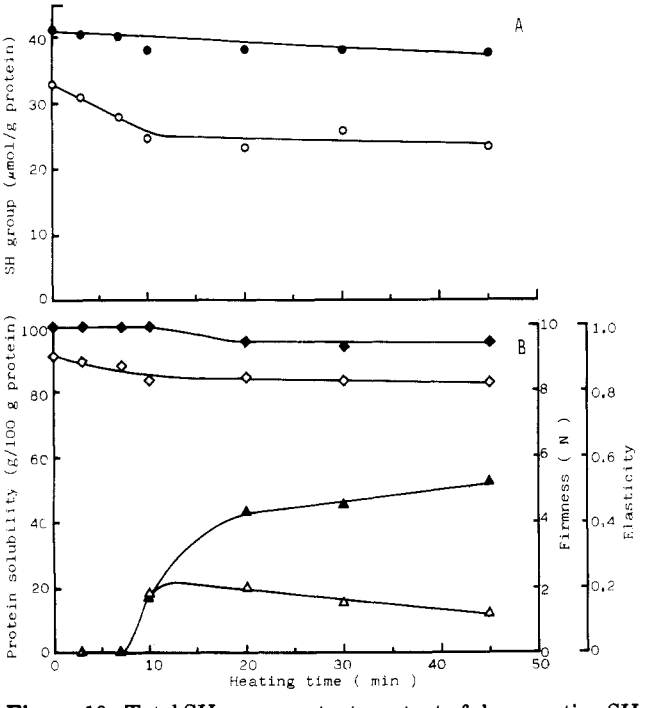

Figure 10. Total SH group content, content of slow-reacting SH groups (A), gel texture, and protein solubility (B) as a function of the heating time (0–45 min) at 85 °C of WPI dispersions (9 g of protein/100 mL) at pH 2.5: A, (●) total SH groups, (○) slow-reacting SH groups; B, (▲) gel firmness, (△) gel elasticity, (♦) protein solubility of dispersion or gel in the standard buffer containing 8 M urea and 0.5% SDS, (◇) protein solubility of dispersion or gel in the standard buffer.

polymers (Figures 6 and 7). Because the content in total SH groups decreased only slightly, it clearly appears that the polymers are formed mainly through intermolecular SH/S–S interchange reactions. Since no polymer could be detected as a result of heating at pH 2.5, the slight decrease in the SHs content may reflect mainly intramolecular SH/S–S interchange reactions.

SH/S–S Interchange Reactions during the Heat-Induced Gelation of 9% WPI Dispersions. When WPI dispersions containing 9 g of protein/100 mL were heated at 85 °C, at pH 7.5 or 2.5, gels were formed. The kinetics of reaction between heated WPI and DTNB are shown in Figures 8–10. The SH group contents (SHt, SHs) are expressed per gram of protein soluble in the buffer containing 8 M urea plus 0.5% SDS. At pH 7.5, the rate of

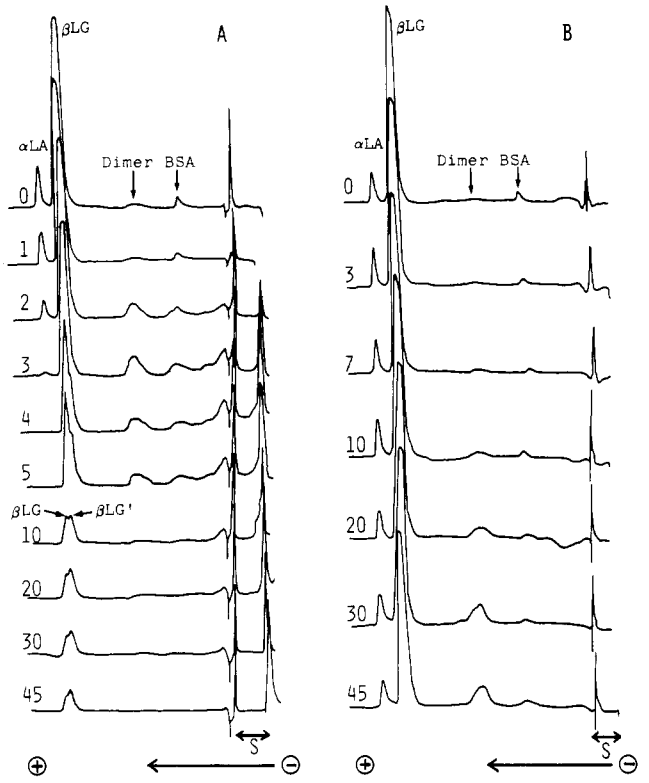

Figure 11. SDS-PAGE patterns of WPI dispersions (9 g of protein/100 mL) heated at 85 °C for various times (0–45 min, as indicated on the figure) at pH 7.5 (A) or at pH 2.5 (B). Migration is from right to left. S = stacking gel.

reaction of the slow-reacting SH groups remained constant until a heating period of 3 min (Figure 8A). For longer heating times the reaction rate could not be determined graphically with precision. As a consequence, the SHs contents of WPI gels could not be determined after 4-min heating. The SHt content at zero time (pH 7.5) was found to be 37.2 μmol/g of protein (Figure 9A). This decreased value (SHt of WPI powder was equal to 40.6 μmol/g of protein) may reflect partial air oxidation of SH groups during the preparation of the 9% WPI dispersion at pH 7.5. The SHt content decreased slightly during the 45-min heating period. The SHs content decreased drastically during the first 3 min of heating. This probably indicates that numerous SH/S–S interchange reactions take place during heating. After 4 min at 85 °C, the gel network structure began to form and the contents of SHs groups could not be determined anymore (Figure 9A,B). Gel firmness increased with heating time and reached a plateau after 30 min. The pH 7.5 gels were also highly elastic, the maximum elasticity being obtained after 20 min.

The solubility of protein constituents in the buffer containing 8 M urea and 0.5% SDS remained equal to 100% during the first 3 min of heating, decreased suddenly to 90% upon formation of the gel network, and then decreased slightly to 85% at 45 min (Figure 9B). The decrease in protein solubility parallels the increase in gel elasticity. This suggests that protein solubility (in the presence of denaturants) and gel elasticity are both closely related to the formation of intermolecular S–S bonds. The solubility of protein constituents in the standard buffer (without denaturants) decreased markedly during the first 30 min of heating. A parallelism can be observed between this decrease and the increase in gel firmness. It is therefore likely that the increase in gel firmness taking place after the formation of the initial gel network is mainly due to intermolecular hydrophobic interactions.

SDS-PAGE (without 2-ME) patterns of WPI heated at pH 7.5 are shown in Figure 11A. β-LG and α-LA fractions decreased drastically during heating as compared to the situation with 1% protein WPI dispersions (Figure 7A). An additional protein band (β-LG′; slower migration) appeared after 4-min heating. This new fraction is poorly separated from the β-LG A plus B fraction (faster migration), as seen by scanning densitometry, although both fractions can be detected visually. The new fraction (β-LG′) may represent partially denatured monomeric β-LG with different pairs of S–S bonds due to intramolecular SH/S–S interchange reactions. Dimer, trimer, and tetramer fractions were detected after 2-min heating but disappeared after 10-min heating. Higher molecular weight aggregates (that could not enter the separation and stacking gels) were observed at increasing heating times. These protein aggregates were formed by intermolecular S–S bonds since SDS-PAGE in the presence of 2-ME gave identical patterns with WPI heated for 45 min and with unheated WPI (data not shown here). Such bonds were caused by SH/S–S interchange reactions, as demonstrated by the drastic decrease in SHs content and the very slight decrease in SHt content. The predominance of intermolecular over intramolecular SH/S–S interchange reactions may be due both to β-LG unfolding during heating and to the relatively high protein concentration. It would be of interest to determine precisely which cysteine residues are involved in the newly formed S–S bonds.

These data indicate that the pH 7.5 gel network is formed in two stages as heating progresses; the initial junction zones include intermolecular S–S bonds due to SH/S–S interchange reactions. The gel network is then strengthened mainly by intermolecular hydrophobic interactions, although the involvement of hydrogen bonds and of electrostatic interactions is not excluded.

The kinetics of reaction between WPI heated at pH 2.5 and DTNB was similar to that observed with the 1% protein WPI dispersion heated at pH 2.5 (Figure 8B). The SHt content decreased slightly during heating while the SHs content decreased more consistently (Figure 10A). At pH 2.5, the gel network was formed after 10-min heating, and gel firmness increased with heating time (Figure 10B). Gel elasticity was lower at pH 2.5 than at pH 7.5 and decreased gradually with heating time. Solubility of protein constituents in the standard buffer (with or without denaturants) decreased during heating but remained high as compared to that at pH 7.5 (Figure 10B).

SDS-PAGE (without 2-ME) patterns of WPI heated at pH 2.5 are shown in Figure 11B. β-LG and α-LA fractions decreased slightly during heating. A dimer fraction appeared after 10-min heating and did not resist treatment with 2-ME (data not shown here). Higher molecular weight polymers were not detected even after heating for 45 min. Since the decrease in SHs groups was large as compared to that in SHt groups, it is likely that the intermolecular S–S bonds resulting in dimer formation were due to SH/S–S interchange reactions. The protein fraction located between α-LA and β-LG, which had been observed after heating the 1% protein WPI dispersion for 20 min at pH 2.5, was again detected, but very slightly and after 45-min heating. The results of SDS-PAGE revealed that intermolecular S–S bonds did not predominate in the gel network at pH 2.5, because high molecular weight polymers were not formed. This results from low SH reactivity at acid pH.

The low elasticity of the pH 2.5 gels may reflect a very small number of intermolecular S–S bonds in the gel network. Since the pH 2.5 gels are easily solubilized in the

standard buffer, it also appears that intermolecular hydrophobic interactions do not play an important role at low pHs. The major forces usually involved in the network of protein gels are van der Waals interactions, hydrogen bonds, electrostatic interactions, hydrophobic interactions, and S–S bonds. Since the contribution of electrostatic interactions, hydrophobic interactions, and S–S bonds to the pH 2.5 gel network is negligible, it is likely that hydrogen bonds and van der Waals interactions are the main forces stabilizing the gel at low pHs [see also Shimada and Cheftel (1988b)].

In the present study, the kinetics of reaction between DTNB and the SH groups of β-LG (in the presence of SDS) was used to detect SH/S–S interchange reactions caused by the heat processing of WPI dispersions. This method confirms that intermolecular S–S bonds due to SH/S–S interchange reactions are responsible for the gel network formed at pH 7.5 and for the high gel elasticity. The contribution of intermolecular S–S bonds to the gel network formation at pH 2.5 is negligible.

ACKNOWLEDGMENT

This study was supported in part by a grant from the French Ministry of Foreign Affairs (to K.S.).

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Received for review November 24, 1987. Accepted June 21, 1988.