Interactions Involved in the Gelation of Bovine Serum Albumin

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The thermal and structural properties of bovine serum albumin (BSA) were studied at different pH values, at different NaCl, lactose, sucrose, and glucose concentrations, and in the presence of cysteine, urea, NEM, and SDS. Maximum thermal stability was observed at pH 5. Glucose had a greater stabilizing effect on the thermal denaturation of BSA than sucrose. Denaturation of BSA resulted in the loss of the 1654 cm^{-1} band attributed to α-helical structure and the rise of two bands at 1616 and 1684 cm^{-1} attributed to the formation of ordered non-native β-sheet structure associated with aggregation. SDS markedly increased the thermal stability of BSA and prevented aggregate formation. The greatest unfolding on heat treatment was observed in the presence of cysteine and the least in SDS.

Keywords: *Bovine serum albumin; differential scanning calorimetry; Fourier transform infrared spectroscopy; denaturation; gelation*

INTRODUCTION

Bovine serum albumin (BSA) accounts for approximately 10% of the proteins of whey (Fox, 1989); although it is a relatively minor protein component, it is considered to be an important gelling protein of whey (Hines and Foegeding, 1993). The other important gelling protein, β-lactoglobulin, is the major protein component. Together, these two proteins contribute to the whey protein gelling characteristics (Zeigler and Foegeding, 1990), which can range from viscous fluids to soft, smooth pastes or curds to stiff, rubbery gels which vary in hardness, cohesiveness, stickiness, color, and mouthfeel (Hillier and Cheeseman, 1979; Schmidt et al., 1979; Hillier et al., 1980).

BSA is a large globular protein (66 000 Da) consisting of 580 amino acid residues with 17 intrachain disulfide bonds and 1 free thiol group at residue 34 (Kinsella and Whitehead, 1989). The protein consists of approximately 54% α-helix and 40% β-structure (β-sheet plus β-turns) (Kinsella et al., 1989) and contains three domains specific for metal-ion binding, lipid binding, and nucleotide binding (Peters and Reed, 1977). McLachlan and Walker (1978) have reported an α-helix content of over 75% for BSA.

The mechanism by which gels are formed when proteins such as BSA are heated has been established (Ferry, 1948; Edwards et al., 1987). This thermally induced gelation is a two-stage sequential process; the first phase involves heat-induced conformational changes in the protein with unfolding of some polypeptide segments followed by a subsequent phase of protein−protein interactions resulting in a progressive buildup of a network structure (Bernal and Jelen, 1985). Protein gels can therefore be represented as consisting of intermolecular cage-like unit structures, with the solvent continuous throughout the matrix (Foegeding, 1989). The formation of this matrix is dependent on a balance between attractive and repulsive forces (Schmidt, 1981), which has been shown to vary with the physical and chemical properties of the protein.

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During the first phase of gelation, there is a transition from the native state to the progel state; this is associated with dissociation and denaturation of the protein (Damodaran, 1989). During this process functional groups engaged in intramolecular hydrogen bonding and electrostatic interactions in the native state become available for intermolecular interactions (Damodaran, 1994). Exposure of hydrophobic groups results in hydrophobic interactions which are necessary in the aggregation and cross-linking of gel networks (Clark, 1992; Damodaran, 1994). Buried sulfhydryl groups can initiate disulfide−sulfhydryl interchange reactions which contribute to cross-linking (Nakamura et al., 1984; Mori et al., 1986; Shimada and Cheftel, 1989).

The particular changes that occur in the secondary structure of proteins and the mechanism by which networks build from individual molecules affect gel texture (Kinsella and Whitehead, 1989). The β-sheet content of native BSA, for example, is relatively low in comparison to that of other proteins; when heated, a decrease in α-helix content is observed, with a concomitant increase in β-sheet prior to gelation (Clark et al., 1981; Byler and Purcell, 1989). This led to the proposition that β-sheet hydrogen bonding may be important for aggregate formation during the gelation of BSA. Non-native, ordered structures of hydrogen-bonded β-sheets have been observed when BSA was heated above its transition temperature by infrared spectroscopy (Clark et al., 1981). These authors further correlated the formation of bands at 1620 and 1680 cm^{-1} with the formation of antiparallel hydrogen-bonded β-sheet and protein aggregation.

Proteins must be heated above their denaturation temperature to form gels (Damodaran, 1989). In addition, factors such as pH, ionic strength, protein concentration, and the nature and concentration of other solids (for, e.g., sugars) affect gel formation and texture (Yasuda et al., 1986; Zeigler and Foegeding, 1990). The secondary structure of denatured proteins is altered by these environmental conditions, which affects the temperature of denaturation (Privalov, 1979), an important parameter in protein gelation. Previous work monitored the effects of these environmental factors on the thermal properties and secondary structure of whey protein

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concentrate, α-lactalbumin, and β-lactoglobulin (Boye et al., 1995a,b).

In this work, the effects of pH, NaCl concentration, sucrose, glucose, and lactose on the secondary structure and denaturation temperature of BSA were studied. The effects of sodium dodecyl sulfate (SDS), cysteine, urea, and *N*-ethylmaleimide (NEM), which are known to interfere with hydrophobic, disulfide, hydrogen bonding, and sulfhydryl oxidation, respectively, were determined to establish the involvement of these interactions in BSA gelation.

MATERIALS AND METHODS

Materials. Bovine albumin (BSA) (product A-2153) was obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Deuterium oxide (product 15,188-2, minimum 99.9 atom % D) was purchased from Aldrich (Milwaukee, WI).

Sample Preparation. *Effect of pH.* Deuterium oxide solutions of BSA (20% w/v) were prepared by dissolving the protein in deuterated phosphate buffer at pH 3, 5, 7, and 9 (ionic strength 0.2). In this work, pD = pH + 0.4. For the sake of clarity, pH is employed in place of pD. (Phosphate buffers were used only for pH studies.)

Effect of NaCl and Sugars. To study the effect of ionic strength and sugars, the protein was dissolved in D_2O solutions containing 0.5−2 M NaCl, 10 and 50% (w/v) sucrose and glucose, and 20% (w/v) lactose. The pH of the protein dispersions was ∼6.8.

Effect of Denaturing Agents. To study the effect of denaturing agents by FTIR, 50 mM solutions of cysteine, urea, SDS, and NEM in D_2O (pH ∼7.4) were used as solvents for dissolving the protein; 50 mM and 2 M urea solutions in D_2O were used to study the effect of urea concentrations. For the DSC studies, H_2O was used in place of D_2O. The protein concentration used for all experiments was 20% (w/v).

Differential Scanning Calorimetry (DSC). The method for DSC analysis has been described previously (Boye et al., 1995a). Twenty-five microliters of each solution was placed in preweighed DSC pans, which were hermetically sealed and weighed accurately. The samples were placed in the DSC (TA3000, Mettler Instrument Corp., Greifensee, Switzerland) and scanned from 15 to 100 °C at a programmed heating rate of 5 °C/min. For each run, a sample pan containing the deuterated buffer used for dissolving BSA was used as reference. After heating, the samples were allowed to cool to room temperature in the DSC, and the heating cycle was repeated under the same experimental conditions to check for reversibility. The DSC was calibrated by use of indium standards. All DSC experiments were done in triplicate.

After scanning, the DSC pans were opened and the BSA solutions were visually checked for gelation (Clark and Lee-Tuffnel, 1986; Patel and Fry, 1987). A white nonflowable firm texture was classified as an opaque gel. A clear nonflowable firm texture was classified as a translucent gel.

Fourier Transform Infrared Spectroscopy. The method for FTIR analysis has been described previously (Boye et al., 1995a). BSA (20% w/v) dissolved in deuterium oxide solutions in the presence of the reagents described above was subjected to infrared spectroscopy. Deuterium oxide was used for all FTIR experiments because of its transparency in the region of interest (1600−1700 cm^{-1}). Infrared spectra were recorded with a 8210E Nicolet FTIR spectrometer equipped with a deuterated triglycine sulfate detector. A total of 256 scans were averaged at 4 cm^{-1} resolution. Wavenumber accuracy was within ±0.01 cm^{-1}. The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA). The samples were held in an IR cell with a 25 μm path length and CaF_2 windows. The temperature of the sample was regulated by placing the cell in a holder employing an Omega temperature controller (Omega Engineering, Laval, QC). The temperature was increased in 5 °C increments and the cell allowed to equilibrate for 3 min prior to data acquisition. The reported temperatures are accurate to within ±0.5 °C. Deconvolution of the observed spectra was performed using the Nicolet FTIR software, Omnic 1.2a. The deconvolution of the infrared spectra was done as described by Kauppinen et al. (1981). The signal to noise ratio was >20000:1, and the bandwidth used for deconvolution was 13 cm^{-1} with a narrowing factor of 2.6. All FTIR experiments were done in duplicate.

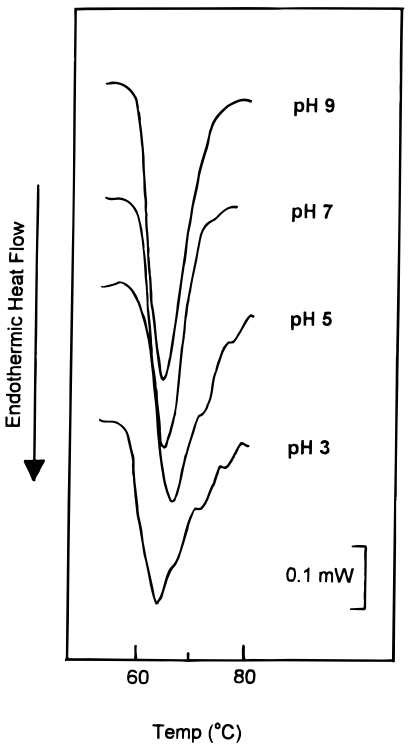

Figure 1. Effect of pH on the DSC characteristics of BSA (20% w/v in deuterated phosphate buffer; ionic strength 0.2). Heating rate was 5 °C/min.

Table 1. DSC Characteristics of BSA: Effect of pH and NaCl

treatment	T_d[a] (°C)	ΔH[b] (J/g)	T_w[c]
pH			
3	62.7 ± 0.3	1.28 ± 0.4	11
5	65.0 ± 0.2	1.45 ± 0.05	8.1
7	63.4 ± 0.1	1.75 ± 0.02	5.7
9	60.8 ± 0.1	1.37 ± 0.04	5.7
NaCl			
0 M	63.1 ± 0.2	1.43 ± 0.3	8
0.5 M	70.7 ± 0.1	2.08 ± 0.04	5
1.0 M	72.2 ± 0.3	2.05 ± 0.05	5
1.5 M	72.8 ± 0.2	1.85 ± 0.01	5
2.0 M	72.9 ± 0.3	1.77 ± 0.04	5

[a] T_d, peak temperature of denaturation. [b] ΔH, change in enthalpy. [c] T_w, peak width at half-height.

RESULTS AND DISCUSSION

Effect of pH. The effect of pH on the DSC characteristics of BSA is shown in Figure 1. At pH 3, the peak temperature of denaturation (T_d) was observed at 62.7 ± 0.3 °C. The T_d increased to a maximum of 65 ± 0.2 °C at pH 5 and then decreased to 63.4 ± 0.1 at pH 7 and to 60.8 ± 0.1 °C at pH 9 (Table 1). These data suggest that BSA has maximum thermal stability at pH 5 and is more thermally stable at acid pH than at alkaline pH. The highest enthalpies (calculated as the peak area under the peak) were 1.75 ± 0.02 J/g at pH 7 and 1.45 ± 0.05 J/g at pH 5. At pH 3 and 9, the enthalpies were 1.28 ± 0.04 and 1.37 ± 0.04 J/g, respectively. This suggests that the protein was more denatured at pH 3 and 9 prior to heat treatment, since

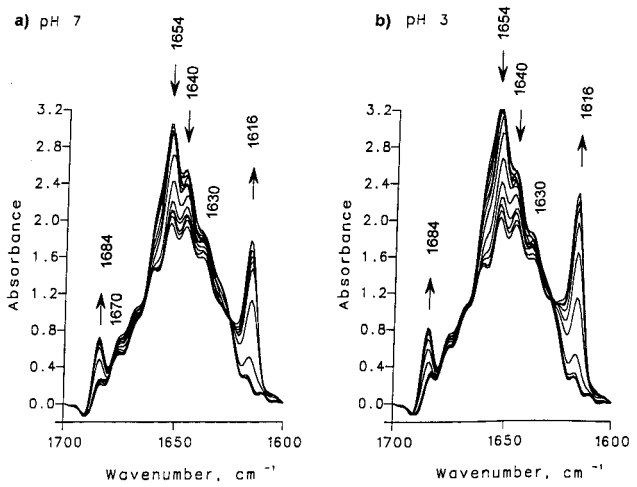

Figure 2. (a) Deconvoluted infrared spectra of BSA (20% w/v in D_2O, pH 7). The numbers refer to the wavenumber (cm^{-1}) of the main bands observed. (b) Superimposed deconvoluted infrared spectra of BSA (20% w/v in deuterated phosphate buffer, pH 3; ionic strength 0.2). Each overlapping spectrum was recorded at temperatures between 30 and 95 °C at 5 °C increments. Arrows indicate change in intensity of bands on heating.

partially denatured proteins require less energy for further denaturation (Ma and Harwalkar, 1988). The thermograms showed a broadening of the peaks with decreasing pH. The peak width at half-height (T_w) increased from 5.7 °C at pH 9 to 8.1 °C at pH 5 and then to 11 °C at pH 3. The T_w, which has been used as an indication of the cooperativity (ease) of denaturation (Myers, 1990), suggests that the unfolding and denaturation of BSA at acid pH were less cooperative than at alkaline pH. Two shoulders could be observed above the peak temperature in the BSA thermogram at pH 5 (Figure 1). At pH 3, three shoulders could be observed. These shoulders suggest that there are residual structures that continue to unfold (denature) as the heating temperature is increased and may reflect unfolding of the three domains in the tertiary structure of BSA reported by Peters and Reed (1977). The transition temperature of each domain and the order in which domains unfold within a protein have been reported to change with environmental conditions such as pH (Potekin and Privalov, 1982; Bertazzon and Tsong, 1990).

Figure 2 shows the FTIR spectra of BSA (20% w/v) in D_2O (pH 7) and in deuterated phosphate buffer at pH 3, heated from 30 to 95 °C. The spectra showed an intense peak at 1654 cm^{-1} (attributed to α-helical structure) and shoulders at 1670 cm^{-1} (attributed to turns) and 1640 and 1630 cm^{-1} (attributed to β-sheet) (Susi and Byler, 1988). At both pH values, heat treatment resulted in the rise of two new bands at 1684 and 1616 cm^{-1} and a decrease in the bands at 1654 and 1640 cm^{-1}. The 1618 and 1684 cm^{-1} bands have been attributed to the formation of intermolecular hydrogen-bonded β-sheet structures resulting in aggregate or gel formation (Clark et al., 1981). Similar transitions were observed at pH 9 (not shown).

To monitor the changes occurring in the 1645 cm^{-1} band, which have been attributed to random coil structures (Susi and Byler, 1988), the integrated intensity of the 1644−1646 cm^{-1} region was plotted as a function of temperature at pH 3 and 7 (Figure 3a). The plot shows an increase in random coil formation with increasing temperature from 30 °C to a maximum value

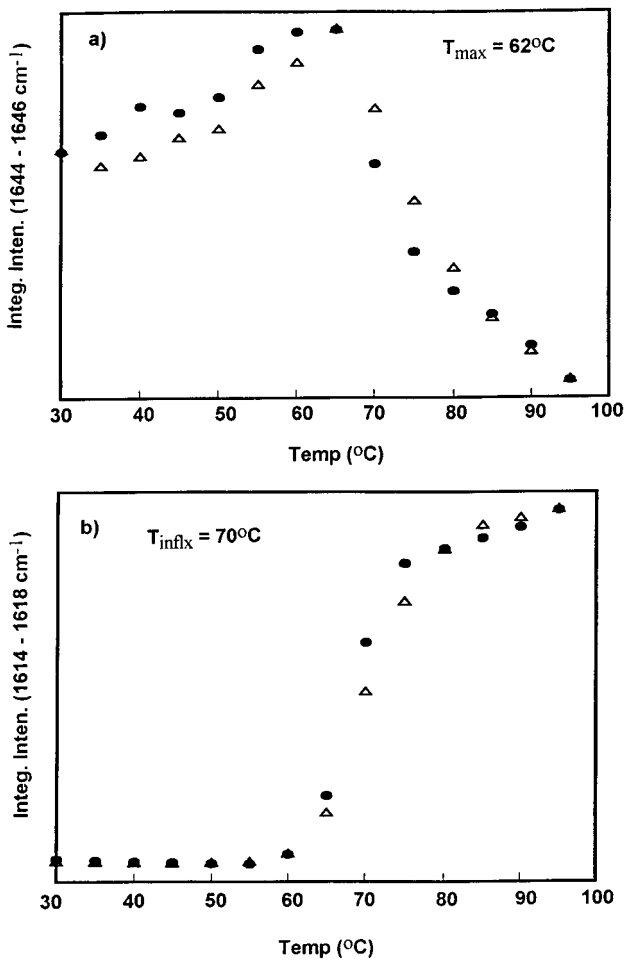

Figure 3. Integrated intensity of the (a) 1644−1646 and (b) 1614−1618 cm^{-1} regions in the amide I′ band of BSA (20% w/v): pH 3 (△) and pH 7 (●) as a function of temperature.

at 62 °C; this temperature was similar to the T_d observed from the DSC analysis of BSA (Figure 1) at pH 3 (62.7 °C) and pH 7 (63.4 °C). This suggests that, when heated, BSA unfolded into random coil structures. As the heating progressed above 62 °C, however, the intensity of the 1646 cm^{-1} band decreased to a minimum at 95 °C, suggesting a decrease in random coil formation. A plot of the integrated intensity of the 1614−1618 cm^{-1} band (attributed to intermolecular hydrogen-bonded β-sheet aggregation) as a function of temperature (Figure 3b) showed a marked increase in intensity above 62 °C which coincided with the temperature at which the intensity of the 1644−1646 cm^{-1} region decreased; this suggests that, after the initial unfolding, the protein lost most of its random structure and assumed a more compact and ordered β-sheet structure. Similar findings were reported by Clark et al. (1981) and Byler and Purcell (1989).

Visual evaluation (Schmidt, 1981; Clark and Lee-Tuffnel, 1986; Patel and Fry, 1987) of the gels formed at each pH value showed that at pH 3 the gel was firm and opaque, whereas the gel formed at pH 9 was translucent. According to Kinsella and Whitehead (1989), clear gels reflect the formation of uniform networks of fine filaments, greater linear aggregation, and frequent cross-linking. As network density becomes less regular, gels increase in turbidity. This may suggest that aggregate formation at pH 3 occurred in a more random and unordered fashion than at pH 9.

Protein−Salt Interactions. Figure 4 shows the thermogram of BSA in various concentrations of NaCl.

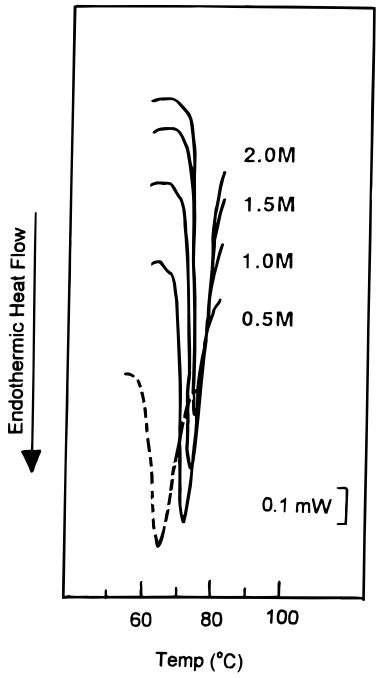

Figure 4. Effect of NaCl on the DSC characteristics of BSA (20% w/v). Heating rate was 5 °C/min.

The T_d increased from 63.1 ± 0.2 J/g (no NaCl) to 70.7 ± 0.1 J/g in 0.5 M NaCl (Table 1); at 2 M NaCl the T_d increased to 72.9 ± 0.3 J/g, suggesting a stabilizing influence of NaCl on the thermal denaturation of BSA. Similar findings were reported for β-lactoglobulin and α-lactalbumin in earlier studies (Boye et al., 1995b,c). The enthalpy of denaturation of BSA decreased from 2.08 ± 0.04 J/g in 0.5 M NaCl to 1.77 ± 0.04 J/g in 2 M NaCl. Our results suggest that at the higher NaCl concentration there was increased aggregation on heat treatment since aggregation of proteins is an exothermic reaction that has been shown to lower enthalpy values (Jackson and Brandts, 1970; Privalov and Khechinashvilli, 1974).

The peak width at half-height (T_w) in the absence of NaCl was 8 °C. In the presence of 0.5 M NaCl, T_w decreased to 5 °C and then remained constant with increasing NaCl concentration; this suggests that although NaCl increased the stability of BSA to thermal denaturation, the transitions that occur during unfolding are more cooperative in the presence of NaCl.

The FTIR spectra of BSA heated in the presence of 0.5 and 2 M NaCl showed the bands at 1616 and 1684 cm^{-1} attributed to aggregate formation (not shown). These bands were observed at 80 °C in 0.5 M NaCl and at 85 °C in 2 M NaCl, which further confirms that at the higher NaCl concentration higher temperatures were required to denature and aggregate BSA. These findings were similar to that observed for β-lactoglobulin (Boye et al., 1995b) but not for α-lactalbumin (Boye et al., 1995c), which showed the 1616 and 1684 cm^{-1} bands only in the presence of NaCl concentrations above 1 M.

Protein−Sugar Interactions. The effect of sugars on the T_d of BSA is shown in Figure 5. In the presence of 10 and 50% (w/v) sucrose, the T_d was observed at 64.6 ± 0.2 and 67.4 ± 0.1 °C, respectively (Table 2). In 50% (w/v) glucose, the T_d increased to 68.3 ± 0.3 °C, which suggests that glucose had a greater stabilizing effect on the denaturation of BSA than sucrose. In the presence of 20% (w/v) lactose, the T_d was at 65.5 ± 0.1 °C. Very little changes were observed in the width at half peak

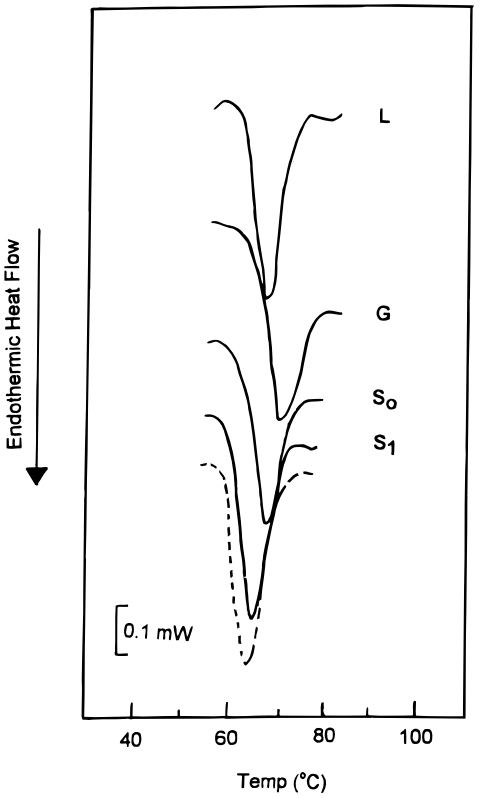

Figure 5. Effect of 20% (w/v) lactose (L), 50% (w/v) glucose (G), 10% (S_0), and 50% (S_1) (w/v) sucrose on the DSC characteristics of BSA (20% w/v). Heating rate was 5 °C/min.

Table 2. DSC Characteristics of BSA: Effect of Sugars and Denaturing Agents

treatment	T_d[a] (°C)	ΔH[b] (J/g)
no sugar	63.1 ± 0.2	1.43 ± 0.03
sucrose, 10% (w/v)	64.6 ± 0.2	1.56 ± 0.01
50%	67.4 ± 0.1	1.51 ± 0.07
glucose, 10%	64.8 ± 0.1	1.51 ± 0.03
50%	68.3 ± 0.3	1.34 ± 0.04
lactose, 20%	65.5 ± 0.1	1.44 ± 0.02
cysteine	60.1 ± 0.01	2.00 ± 0.1
urea, 50 mM	61.0 ± 0.2	1.90 ± 0.06
2 M	56.5 ± 0.3	1.15 ± 0.02
NEM	63.0 ± 0.1	2.50 ± 0.04
SDS	83.9 ± 0.3	2.8 ± 0.02

[a] T_d, peak temperature of denaturation. [b] ΔH, change in enthalpy.

height of the DSC thermograms in the presence of the three sugars studied, which may suggest that the sugars did not interfere with the cooperativity of unfolding. The stabilizing influence of glucose and sucrose on the thermal denaturation of BSA was also observed for β-lactoglobulin and apo- and holo-α-lactalbumin (Boye et al., 1995b,c); in each instance glucose had a greater stabilizing influence than sucrose.

Effect of Denaturing Agents. Figure 6 shows the thermograms of BSA in the presence of cysteine, urea, NEM, and SDS. In the presence of cysteine and urea (50 mM) the T_d values of BSA were 60.1 ± 0.1 and 61.0 ± 0.2 °C, respectively. In the presence of NEM the T_d was 63 ± 0.1 °C; this is similar to the T_d (63.1 °C) of BSA in D_2O (Table 2; Figure 4), suggesting that NEM had little effect on the T_d of BSA. In the presence of SDS, the T_d increased by 20.8 °C to 83.9 ± 0.3 °C. SDS has been shown to both increase and decrease the thermal stability of a number of proteins (Hegg et al., 1978; Hegg, 1980; Harwalkar and Ma, 1987). The higher temperature of denaturation observed in this

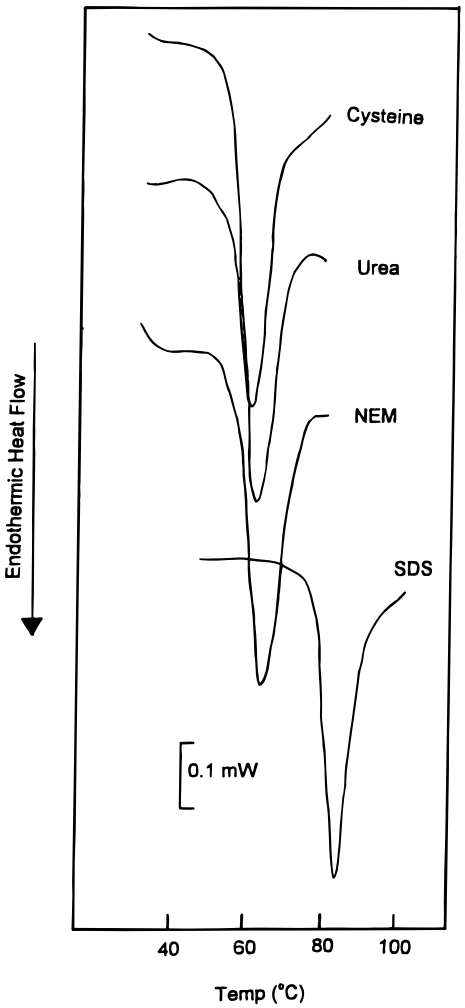

Figure 6. Effect of cysteine (50 mM), urea (50 mM), NEM (50 mM) and SDS (50 mM) on the DSC characteristics of BSA (20% w/v). Heating rate was 5 °C/min. (Solutions of denaturing agents were made in H_2O.)

study suggests that SDS increases the thermal stability of BSA. It has been suggested (Hegg and Löfqvist, 1974; Markus et al., 1964) that SDS can form a bridge between a positively charged group in one loop of a polypeptide chain and a hydrophobic region in another; this could explain its stabilizing effect on thermal denaturation. The thermogram (not shown) of BSA in 2 M urea showed a T_d at 56.5 ± 0.3 °C; this represents a decrease from 61.0 °C in the presence of 50 mM urea. The lower T_d value observed suggests that BSA was more denatured (unfolded) in 2 M urea prior to heat treatment. The highest enthalpy of 2.8 ± 0.02 J/g was obtained in the presence of SDS, and the lowest (2.0 ± 0.1 J/g) was in the presence of cysteine and urea. In 2 M urea, the enthalpy decreased to 1.15 ± 0.02 J/g. The higher enthalpy value observed in the presence of SDS reflects weaker hydrophobic interactions in the protein prior to thermal denaturation. Aggregation and disruption of hydrophobic interactions are both exothermic reactions which lower the observed enthalpy (Myers, 1990). In the absence of aggregation, a high enthalpy value is an indication of a reduction in the breakup of hydrophobic bonds.

The peak width at half-height (T_w) in the presence of 50 mM SDS was 4.8 °C and in the presence of NEM 9 °C; in urea and cysteine, the T_w values were 8.4 and 7.4 °C, respectively. These results suggest that although SDS stabilized the protein against thermal denaturation, the process of unfolding was extremely cooperative in its presence.

The FTIR spectra of BSA heated in the presence of cysteine, NEM, urea, and SDS (50 mM) is shown Figure 7. The spectra recorded at 25 °C show a shift in the band observed at 1630 cm^{-1} in the absence of any dissociating agent (Figure 2) to 1631 cm^{-1} in the presence of cysteine and 1634 cm^{-1} in urea, NEM, and SDS (Figure 7). This shift to higher wavenumbers represents a decrease in hydrogen bonding in the presence of the dissociating agents (Krimm and Bandekar, 1986). The band at 1640 cm^{-1}, observed in the absence of any dissociating agents (Figure 2) and also in the presence of cysteine, NEM, and urea (Figure 7), was not observed in the SDS spectra. The absence of this band, which has been attributed to β-sheet structure (Susi and Byler, 1988), suggests a decrease in β-sheet content in the presence of SDS; this suggests that SDS may have disrupted the bonds responsible for β-sheet formation. Above 65 °C, two bands at 1616 and 1684 cm^{-1} (attributed to intermolecular β-sheet aggregation) were observed in the presence of cysteine, NEM, and urea but not in the presence of SDS; this suggests that there was no aggregation on heating BSA to 90 °C in the presence of SDS.

The width of the amide I′ band (1600−1700 cm^{-1} region) as a function of temperature has been used as an indication of protein unfolding (Ismail et al., 1992). As the protein is heated, the amide I′ band broadens or blurs, which results in an increase of the bandwidth and is indicative of the loss or collapse of secondary structural integrity, possibly due to partial unfolding as the hydrogen bonds defining the protein structure are broken (Ismail et al., 1992). To study the extent of BSA unfolding in the presence of the four reagents studied, the width at half-height of the 1600−1700 cm^{-1} region was plotted as a function of temperature (Figure 8a). Below 60 °C, there was little broadening in the presence of all four denaturing agents. Above 60 °C, a marked broadening of the band was observed in cysteine followed by urea and NEM. This increase in width was not observed with SDS. Our results suggest that the greatest unfolding of BSA on heating was in the presence of cysteine and the least unfolding was in the presence of SDS.

The rate of hydrogen−deuterium exchange, which also gives an indication of the extent of protein unfolding (Hvidt and Nielsen, 1966), can be studied in the amide II′ region of the infrared spectrum (1549−1542 cm^{-1}). To observe the effect of the denaturing agents on the rate of H−D exchange, the integrated intensity of the 1549−1542 cm^{-1} region was plotted as a function of temperature (Figure 8b). In the presence of urea, cysteine, and NEM the intensity of the band decreased to a minimum at 75 °C, which suggests a completion of hydrogen−deuterium exchange. In SDS, however, the decrease in the intensity of the band was slow and a minimum level was not reached. The fastest rate of exchange was in the presence of urea followed by cysteine and NEM. The fast rate of hydrogen−deuterium exchange in the presence of urea might result from the breakup of hydrogen bonds, which exposed the hydrogen ions and made them more accessible to solvent. In the presence of cysteine, disruption of intramolecular disulfide bonds may have opened up the protein structure for deuterium exchange. NEM, however, did not contribute to the unfolding of the protein

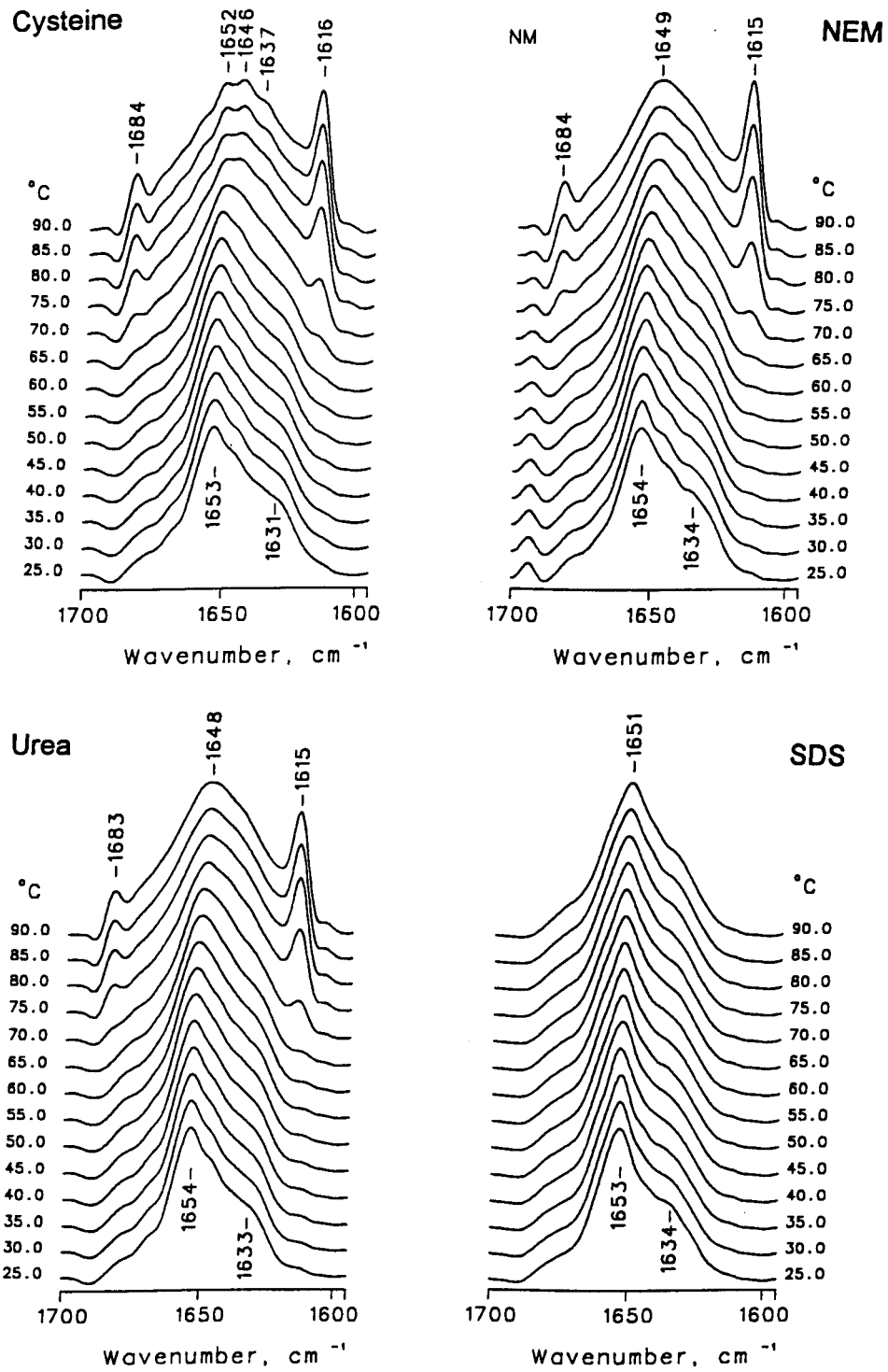

Figure 7. Stacked plot of deconvoluted infrared spectra of BSA (20% w/v) in 50 mM deuterated cysteine, NEM, urea, and SDS solutions at the specified temperatures.

below the temperature of denaturation, probably because the native conformation of the protein was not disrupted. In the presence of SDS (which induces a net negative charge on proteins) excessive repulsive forces would have been expected to result in an unfolding of the protein and thus enhance hydrogen−deuterium exchange. The slow rate of exchange observed in SDS may therefore suggest a shielding effect of the dodecyl ions on the hydrogen ions of the protein.

A plot of the integrated intensities of the 1654−1644 cm^{-1} bands showing the shift in these bands as BSA was heated is shown in Figure 8c. On heat treatment, the band observed at 1654 cm^{-1} (25 °C) shifted to 1648, 1651, and 1652 cm^{-1} in the presence of NEM and urea, SDS, and cysteine, respectively. In the presence of cysteine, a marked increase in the intensity of the 1646 cm^{-1} band was observed (Figures 7 and 8c). The shift of the 1654 cm^{-1} band to lower wavenumbers may have resulted from an increase in the intensity of the band at 1646 cm^{-1} attributed to random coil structures, which suggests increased unfolding of the protein on heating. The greatest increase in the intensity of the 1646 cm^{-1} band was observed in the presence of cysteine, suggesting that it had greater effect in unfolding BSA.

A plot of the integrated intensity of the 1621−1608 cm^{-1} region (which represents the area under the 1616

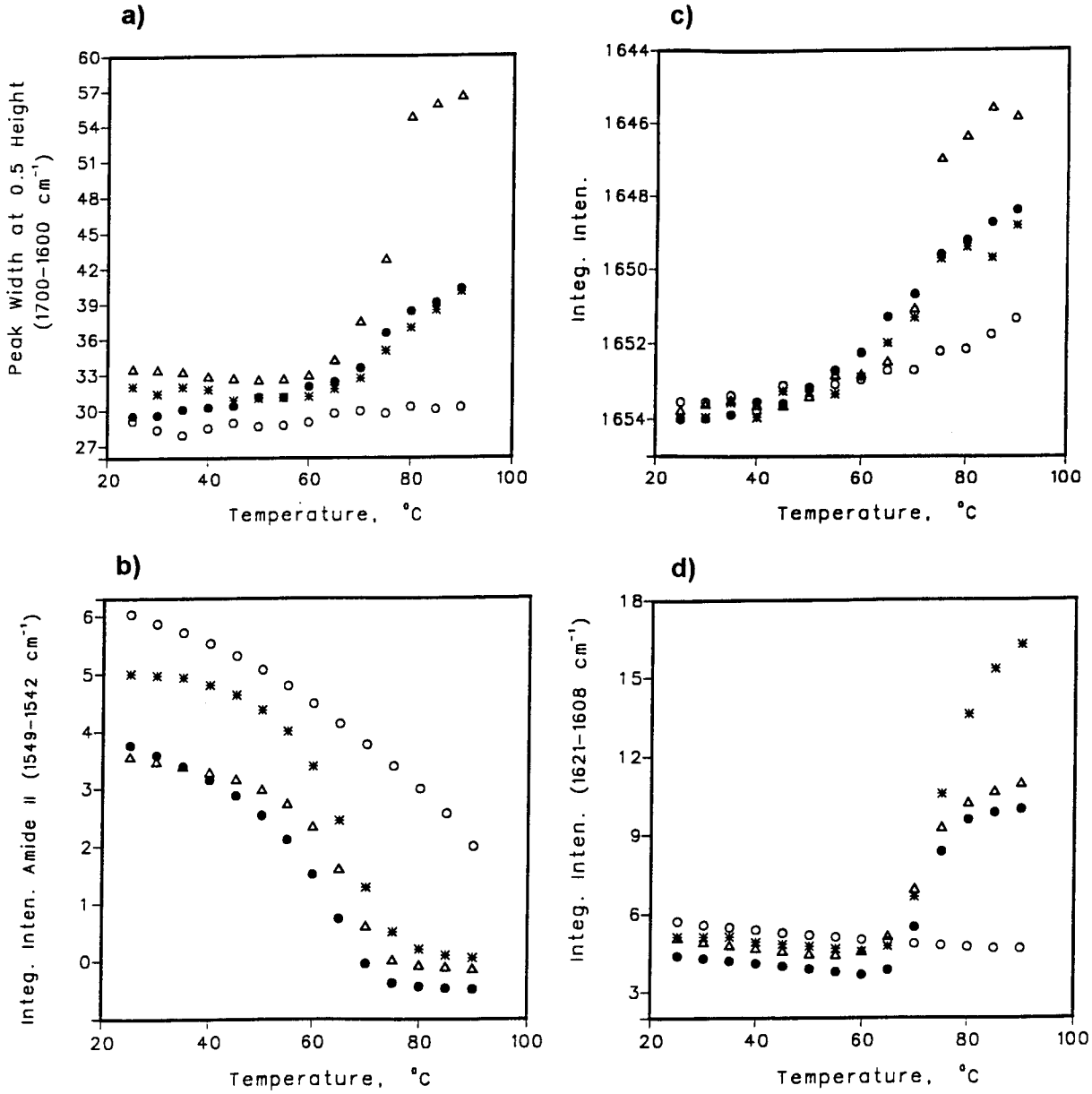

Figure 8. (a) Plot of the width at half-height of the 1700−1600 cm^{-1} region (amide I′) of BSA in the presence of cysteine (Δ), urea (●), SDS (○), and NEM (∗). Integrated intensity of the (b) 1549−1542 cm^{-1} region (amide II′) (c) 1654−1644 cm^{-1} bands, and (d) 1621−1608 cm^{-1} region, as a function of temperature.

cm^{-1} band attributed to aggregation) is shown in Figure 8d. No increase in the intensity was observed below 65 °C in the presence of the four denaturing agents. Above 65 °C, a marked increase in the intensity of the band was observed in the presence of NEM followed by cysteine and then urea. In the presence of SDS, no increase in the intensity of the band was observed at any temperature. This suggests that BSA did not aggregate when heated to 90 °C in the presence of SDS, but aggregated in the presence of cysteine, urea, and NEM; the aggregation in NEM was more intense than in cysteine and urea.

BSA was heated in the presence of the four denaturing agents at 85 and 95 °C for 10 min to check for gel formation. At 85 °C, a translucent gel was formed in the presence of both 50 mM and 2 M urea. In the presence of cysteine, an opaque gel was formed. The gel formed in NEM was opaque and easily breakable. Matsudomi et al. (1991) reported that in the presence of NEM there was a marked decrease in the hardness of BSA gels; the gels formed were transparent, fragile, and less elastic than control gels. No gel was formed when BSA was heated at 85 °C in the presence of SDS; however, at 95 °C a translucent gel was formed. The absence of the 1616 and 1684 cm^{-1} bands (attributed to aggregate formation) in the spectra of BSA heated in 50 mM SDS (Figure 7) corroborated the earlier finding which showed no gel formation on heating BSA in the presence of SDS at 85 °C for 10 min, since aggregation has been shown to be an integral step in protein gelation (Edwards et al., 1987).

The FTIR spectrum of BSA in SDS was recorded as a function of time at 86, 90, and 95 °C (Figure 9a) to check for aggregation. The spectrum (at 86 °C) showed no bands at 1684 and 1616 cm^{-1} after 2.5 h of heating, indicating an absence of aggregate formation. At 90 °C, there was a slight increase in the 1684 cm^{-1} band with time, which suggested that the protein had started to aggregate. At 95 °C a marked increase in the intensity of the 1684 cm^{-1} was observed after 20 min (Figure 9b) which increased with time, suggesting increased formation of aggregate structures.

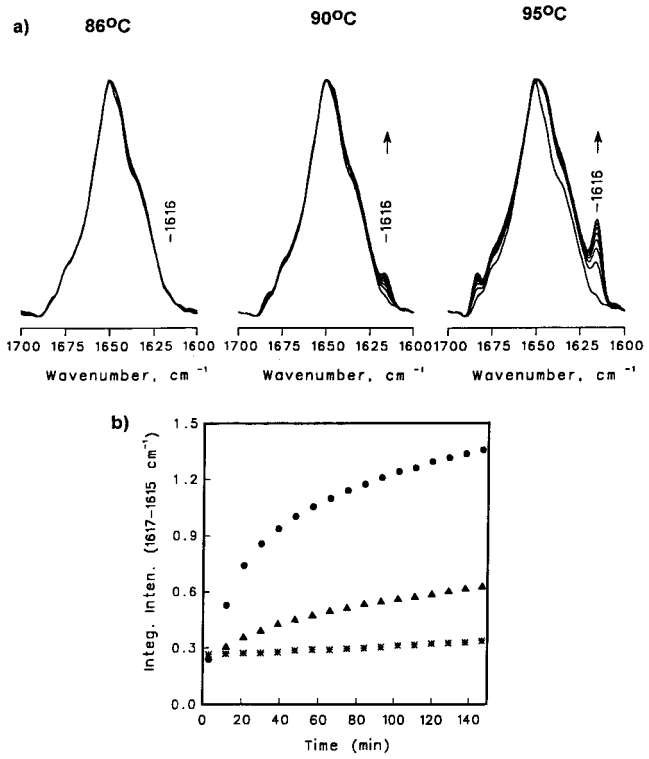

Figure 9. (a) Superimposed deconvoluted spectra of BSA in 50 mM SDS heated at 86, 90, and 95 °C for 2.5 h. (b) Plot of the integrated intensity of the 1617−1615 cm^{-1} region of the infrared spectrum of BSA heated at 86 (∗), 90 (▲), and 95 °C (●) as a function of time.

No peaks were observed in the DSC thermogram when the heated BSA sample was rescanned in the presence of all the reagents studied, which suggests that the denaturation was completely irreversible.

Conclusion. In the presence of NaCl, sucrose, glucose, cysteine, urea, and NEM and at pH values between 3 and 9, denaturation of BSA resulted in a marked reduction of α-helical structure and an increase in ordered non-native β-sheet structure and random coil structures. Clark et al. (1981) also reported the formation of ordered structure during gelation of BSA.

On the basis of the enhancement of the 1616 cm^{-1} and 1684 cm^{-1} bands (aggregation bands) in the presence of NEM and cysteine, it can be proposed that sulfhydryl oxidation (disulfide bond formation) may not be essential in the aggregation step during the formation of BSA gels but may be necessary in the formation of intermolecular cross-links between the aggregates formed, as has been suggested by other workers (Hillier et al., 1980; Xiong and Kinsella, 1990; Matsudomi et al., 1991). Hydrophobic interactions may be the most important in the initial aggregation step since no aggregation bands were observed in the presence of SDS.

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