Use of Differential Scanning Calorimetry and Infrared Spectroscopy in the Study of Thermal and Structural Stability of α-Lactalbumin

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Structural changes of α-lactalbumin (α-lac) in response to pH, ionic strength, sugars, and heat treatment were investigated by differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy. From DSC, two reversible transitions at 39.6 °C (A) and 64.8 °C (B) were observed when α-lac was heated. At pH 3, transition A was partially reversible (14%) while transition B was completely reversible. At pH 9, both transitions were completely irreversible. Heating α-lac at pH 3 resulted in aggregation with no observed gelation; at pH 9 a translucent gel was formed. FTIR showed that at pH 3, denaturation of α-lac resulted in the appearance of two bands at 1616 and 1685 cm^{-1} attributed to intermolecular aggregation. These bands were absent at alkaline pH. The major effects of heat treatment of α-lac were loss in the bands attributed to α-helix, 3_{10}-helix, and β-sheet and increase in the bands attributed to turns.

Keywords: *α-Lactalbumin; infrared spectroscopy; differential scanning calorimetry; denaturation; secondary structure*

INTRODUCTION

α-Lactalbumin (α-lac) is the second major globular protein in the whey of milks from various mammalian species. It is a low molecular weight (14.2 kDa) compact metalloprotein accounting for 20% of the proteins in bovine whey. α-Lac is a modifier protein of the lactose synthase complex in the mammary cell with eight cysteine residues which exist as four intramolecular disulfides. The protein contains 123 amino acid residues with a single bound calcium ion and is capable of binding zinc as well as other metals (Hiraoka et al., 1980; Bernal and Jelen, 1984; Stuart et al., 1986; Kronman, 1989). Crystallographic studies reveal a close structural homology between α-lac and lysozyme (Smith et al., 1987). As in lysozyme, the compact globular structure contains several regions of regular secondary structure including four α-helices, several regions of 3_{10}-helix, and an antiparallel β-pleated sheet separated by irregular β-turns (Acharya et al., 1989; Brew and Grobler, 1992). The existence of a hydrophobic box structure in bovine α-lac that is very similar to that of hen's egg white lysozyme has been reported (Koga and Berliner, 1985).

α-Lac is considered as the most heat-stable whey protein. Removal of the bound Ca^{2+} reduces the stability of the native tertiary structure (Hiroaka et al.,1980). The conformation around the Ca^{2+}-bound region consists of a helix−turn−helix motif with residues 76−82 forming the first helix (3_{10}-helix) and residues 85−93 forming the second α-helix (Brew and Grobler, 1992). Kuwajima et al., (1986) found that the Ca^{2+}-bound and -free forms assume essentially the same folded conformation, based on CD and proton NMR studies; they concluded that the stability of the folded state is enhanced by Ca^{2+}.

In solution, α-lac undergoes intermolecular interactions leading to varying degrees of polymerization on both sides of its zone of insolubility (∼pH 4.8; Shukla, 1973). At acidic pH values, the protein undergoes a rapid reversible association and slow aggregation (Kronman et al., 1964; Kronman and Andreotti, 1964). Between pH 6 and 8.5 there is very little association, and above pH 9.5 there is expansion without aggregation (Kronman et al., 1967).

Denaturation of the protein involves dissociation of Ca^{2+} (Mackenzie and White, 1991); this occurs regardless of whether the protein is at pH values below 4.0 or above 9.0, is heated above 50 °C, exposed to low concentrations of guanidine hydrochloride, or subjected to Ca^{2+} removal from the native form. These denaturing conditions result in a transition of the native state to a stable transient state designated as the "A" or the "molten globule" state that is different from the unfolded denatured (U) state (Ptitsyn, 1987). The molten globule state has been described as a compact intermediate protein conformation that has a secondary structure content like that of the native state but with a poorly defined tertiary structure (Lala and Kaul, 1992). α-Lac has also been shown to refold to its native conformation after exposure to denaturing conditions (Brew and Grobler, 1992); when a chelator (e.g., EGTA) is added to bind endogenous Ca^{2+}, only 2% or less of active α-lac is regenerated as compared with as much as 90−100% in the presence of 100 mm Ca^{2+}. This further suggests a Ca^{2+} dependence for refolding.

Results from X-ray scattering experiments have shown that the amino acid residues are densely packed in the interior of the molten globule structure (Timchenko et al., 1986; Damaschun et al., 1986). Hydrogen−deuterium exchange experiments using NMR show that there are specific regions of particular stability in the secondary structure which fluctuate little at room temperature, while other regions are more labile and afford only marginal protection from solvent exchange (Baum et al., 1989).

In spite of the tremendous advances made in determining the three-dimensional structure and metal-binding properties of α-lac, there is comparatively less information about its structure and functionality in

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different environments. Salts, for example, alter the native conformation of food proteins and affect their temperature of denaturation (Arntfield et al., 1990). In solution, ions exert their influence by affecting the net charge of proteins, hydration, and electrostatic interactions (Kinsella and Whitehead, 1989). Sugars and polyols stabilize proteins against heat denaturation by increasing the structure of water which indirectly strengthens hydrophobic interactions and stabilizes protein conformation (Back et al., 1979). In this study the combination of differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR) were used to investigate the thermal and structural changes in α-lac on heat treatment, in particular its refolding behavior after heat treatment. DSC has been established as a technique for studying thermal denaturation and conformational transition of proteins and is used in the study of various food systems (Arntfield et al., 1990). Infrared spectroscopy constitutes one of the oldest methods for studying the secondary structure of polypeptides and proteins. Several systematic studies of the IR spectra of α-lac in D_2O have been reported (Prestrelski et al., 1991a,b). More recently, the application of resolution enhancement and band-narrowing techniques in FTIR spectroscopy has increased its use in protein research. The amide I′ band (1700−1620 cm^{-1}) which arises mainly from backbone C═O stretching is sensitive to small variations in molecular geometry and hydrogen bonding. Different patterns of folding of the peptide backbone result in the rise of bands at discrete frequencies (Krimm and Bandekar, 1986; Prestrelski et al., 1991a) within the amide I′ band. These two techniques were used to study the unfolding and refolding behavior of α-lac (both apo- and holo-forms) at various pH values, in the presence of NaCl, sucrose, and glucose.

MATERIALS AND METHODS

Materials. α-lactalbumin (product L6010, containing 0.3 mol of Ca^{2+}/mol of α-lac) 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. Solutions of α-lac (mixture of apo- and holo-forms) were prepared by dissolving the protein in deuterium oxide to make 10% and 20% (w/v) concentration; holo-α-lac was obtained by adding $CaCl_2$ to induce Ca^{2+} binding of the apo-conformers. The final concentration of $CaCl_2$ was 0.5 M. The apo-conformer was obtained by dissolving the protein in deuterated Na_2-EDTA (ethylenediaminetetraacetic acid tetrasodium salt, pH 6.8, 0.1 M) for the DSC study. The Na_2-EDTA was used as a chelating agent for Ca^{2+} (Relkin et al., 1993). For the FTIR study, a solution of α-lac (20%, w/v) was dialyzed against 0.1 M Na_2-EDTA for 48 h, dialyzed twice against distilled water for a total of 8 h, and then lyophilized. A 10% (w/v) concentration of the lyophilized protein was prepared in D_2O and used for the FTIR study.

To study the effect of ionic strength and sugars, α-lac was dissolved in D_2O containing 0.5−1.5 M NaCl and 10% and 50% (w/v) each of glucose and sucrose. For the pH studies, the protein was dissolved in deuterated phosphate buffer at pH 3, 5, 7, and 9 (ionic strength, 0.2). In this work, pD = pH + 0.4 (Covington et al., 1968). For the sake of clarity, pH is employed in place of pD.

Differential Scanning Calorimetry (DSC). Each solution (25 μL) 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 α-lac 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. The degree of reversibility was determined from the ratio of the areas under the second and first endothermal peaks (Relkin et al., 1993). The DSC was calibrated by use of indium standards. All DSC experiments were done in triplicate.

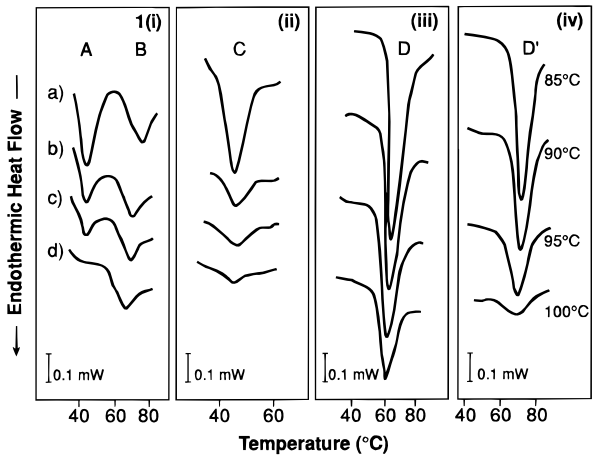

Figure 1. DSC characteristics of α-lac (20%, w/v, protein concentration) in (i) D_2O, (ii) Na_2-EDTA (0.1 M), (iii) $CaCl_2$ (0.5 M), and (iv) $CaCl_2$ (0.5 M) preheated at 85, 90, 95, and 100 °C for 10 min and cooled to room temperature before scanning in the DSC. Heating rate was 5 °C/min (a−d refer to first, second, third, and fourth heating cycles, respectively).

After scanning in the DSC, the pans were opened and visually checked for gelation. A firm gel defined a pellet that could be removed from the DSC pan: a white firm gel was classified as an opaque gel and a clear firm gel was classified as a translucent gel; a soft gel defined a pellet that was easily breakable and could not be removed from the DSC pan; a whitish coagulum that exhibited flow properties was classified as an aggregate (Clark and Lee-Tuffnel, 1986; Boye et al., 1995).

Fourier Transform Infrared Spectroscopy (FTIR). α-Lactalbumin (10%, w/v) dissolved in deuterium oxide solutions as described above was subjected to infrared spectroscopy. Infrared spectra were recorded with a 8210E Nicolet FTIR spectrometer equipped with a deuterated triglycine sulfate detector. A total of 512 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 thermostat 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 except in the case of the NaCl concentration and pH studies where the spectra were recorded at the temperatures indicated; temperatures were verified with a thermometer. 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 >20 000:1, and the bandwidth used for deconvolution was 18 cm^{-1} with a narrowing factor of 2.8 (Prestrelski et al., 1991a,b). All FTIR experiments were done in duplicate.

RESULTS AND DISCUSSION

Thermal Stability. Figure 1 shows the DSC thermograms of α-lac heated in D_2O, Na_2-EDTA (0.1 M), and $CaCl_2$ (0.5 M). Two transitions at 39.6 ± 0.2 °C (peak A) and 64.8 ± 0.5 °C (peak B) with enthalpies (calculated as the area under the peaks) of 0.95 ± 0.11 and 0.52 ± 0.01 J/g respectively, were observed with α-lac

Table 1. DSC Characteristics of Apo-α-lactalbumin and Holo-α-lactalbumin[a]

					% reversibility (%)	
treatment	T_d (A)	H (A)	T_d (B)	H (B)	A	B
D_2O (apo ± holo-α-lac)						
1st heating cycle	39.6 ± 0.2	0.95 ± 0.11	64.8 ± 0.5	0.52 ± 0.01		
2nd heating cycle	38.7 ± 0.4	0.58 ± 0.07	65.6 ± 1.1	0.53 ± 0.03	61	100
3rd heating cycle	39.2 ± 0.5	0.45 ± 0.02	65.1 ± 1.0	0.58 ± 0.04	47	100
4th heating cycle	37.4 ± 0.2	0.07 ± 0.01	63.2 ± 0.1	0.55 ± 0.04	7	100
Na_2-EDTA (apo-α-lac)						
1st heating cycle	42.5 ± 0.4	1.50 ± 0.00				
2nd heating cycle	43.2 ± 0.0	0.74 ± 0.00			49	
3rd heating cycle	44.0 ± 0.5	0.52 ± 0.05			35	
4th heating cycle	43.3 ± 0.2	0.38 ± 0.04			25	
$CaCl_2$ (holo-α-lac)						
1st heating cycle			70.1 ± 0.1	2.7 ± 0.30		
2nd heating cycle			70.5 ± 0.1	2.2 ± 0.02		81
3rd heating cycle			70.3 ± 0.1	1.8 ± 0.07		66
4th heating cycle			70.7 ± 0.2	1.2 ± 0.10		44

[a] A, B, first and second transitions observed in the DSC thermogram of α-lac; T_d, peak temperature of transitions A and B; H, enthalpy of transitions A and B calculated as area underneath the peaks; % reversibility, enthalpy calculated from each heating cycle divided by enthalpy of first heating cycle × 100.

Table 2. DSC Characteristics of α-Lactalbumin in $CaCl_2$, NaCl, Sucrose, and Glucose

				% reversibility	
treatment	T_d (A)	T_d (B)	H (B)	A	B
$CaCl_2$ (0.5 M)					
preheated at 85 °C		70.4 ± 0.2	2.15 ± 0.00	80	
preheated at 90 °C		71.0 ± 0.1	1.66 ± 0.00	61	
preheated at 95 °C		71.7 ± 0.1	0.96 ± 0.01	45	
preheated at 100 °C		72.1 ± 0.2	0.33 ± 0.02	12	
NaCl (M)					
0.0	39.6 ± 0.2	64.8 ± 0.5		61	100
0.5	48.3 ± 0.2	69.9 ± 0.1		63	89
1.0	52.6 ± 0.1	72.2 ± 0.3		63	82
1.5	53.2 ± 0.1	73.1 ± 0.2		48	76
sucrose (%, w/v)					
10	39.6 ± 0.2	65.5 ± 0.01		42	78
50	44.6 ± 0.1	69.7 ± 0.3		32	100
glucose (%, w/v)					
10	40.0 ± 0.1	67.4 ± 0.2		70	94
50	43.7 ± 0.2	68.1 ± 0.1		70	100

[a] See footnotes to Table 1.

in D_2O (Figure 1i.a). (The enthalpy values are based on the weight of the protein solutions.) Cooling of the sample followed by reheating (second heating cycle) (Figure 1i.b) resulted in the reappearance of peaks A and B indicating that these transitions were reversible. The enthalpies of transitions A and B in the second heating cycle were 0.58 ± 0.07 and 0.53 ± 0.03 J/g, respectively which indicates a percent reversibility of ~60% for transition A and 100% reversibility of transition B. After the fourth heating cycle, the enthalpy of transition A was 0.07 ± 0.01 J/g and that for B was 0.55 ± 0.04 J/g (Table 1). No transitions were observed in the fifth heating cycle. In Na_2-EDTA, only one thermal transition was observed at 42.5 ± 0.4 °C (peak C, Figure 1.ii). Like the transitions at 39.6 and 64.8 °C (peaks A and B), this transition was also reversible. The enthalpy of this transition decreased from 1.5 ± 0.001 to 0.74 ± 0.003 J/g on reheating (second cycle) and subsequently to 0.38 ± 0.04 J/g after the fourth heating cycle; the percent reversibility was 49% after the second heating cycle and 25% after the fourth cycle (Table 1). In $CaCl_2$ (0.5 M) one thermal transition was observed at 70.1 ± 0.1 °C (peak D) with an enthalpy of 2.7 ± 0.3 J/g. Cooling followed by reheating indicated reversibility of this transition whose enthalpy decreased to 2.2 ± 0.02 J/g after the second heating cycle (~80% reversibility); after the fourth heating cycle, the enthalpy value was 1.2 ± 0.1 J/g (56% reversibility). The presence of one thermal transition in α-lac treated with Na_2-EDTA (peak C) and $CaCl_2$ (peak D) suggests that the first and second transitions in the thermogram of α-lac (peaks A and B) were due to the thermal transitions of Ca^{2+}-free (apo) and Ca^{2+}-bound (holo) α-lac, respectively; the higher transition temperatures of peaks C and D in comparison to A and B reflect the stabilizing effect of the Ca^{2+} and Na^+ ions (Relkin et al., 1993).

The transitions observed in the DSC analysis demonstrate the reversible denaturation of α-lac in $CaCl_2$ when heat was applied at a temperature gradient of 5 °C/min. To confirm that this reversibility is independent of the method of heating, α-lac in $CaCl_2$ (0.5 M) was preheated at 85−100 °C for 10 min and then subjected to the DSC analysis. The results (Figure 1iv) show the transition at 70.4 °C (peak D) which confirms the thermal reversibility of α-lac regardless of the mode of heating. The enthalpy decreased from 2.15 ± 0.003 J/g (preheating at 85 °C) to 0.33 ± 0.02 (preheating at 100 °C) J/g which indicates 80% and 12% reversibility, respectively (Table 2). No transitions were observed when α-lac was preheated at 100 °C for 30 min suggesting that the protein was irreversibly denatured. Denaturation of food proteins is frequently accompanied by aggregation and gelation. Dissociation and/or unfolding of protein molecules generally increases the exposure of reactive groups, especially the hydrophobic groups of globular proteins. Protein−protein hydrophobic, electrostatic, and hydrogen-bonding interactions are favored; these cross-linking interactions, as well as

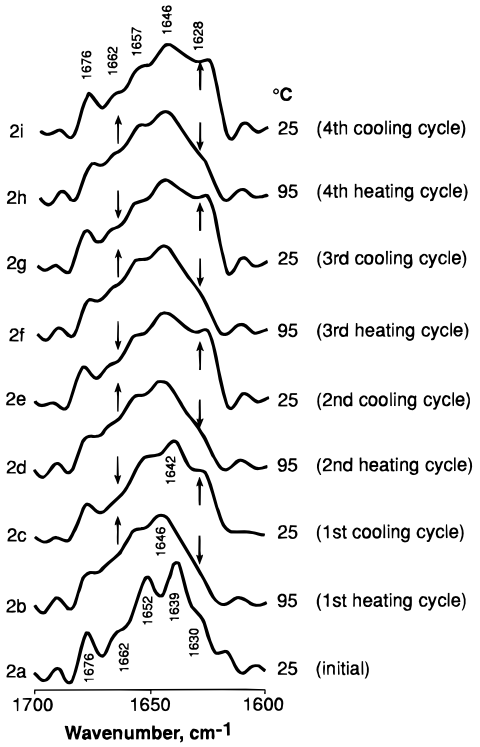

Figure 2. Deconvoluted infrared spectra of α-lac in D_2O (10%, w/v, pH 6.8) heated and cooled (four heating/cooling cycles) at the temperatures indicated. The numbers refer to the wavenumbers (cm^{-1}) of the main bands observed.

disulfide−sulfhydryl interchange reactions, are primarily responsible for the subsequent aggregation and gelation of these proteins (Clark and Lee-Tuffnell, 1986; Ziegler and Foegeding, 1990). The heated α-lac solutions were, therefore, observed for gelation in order to determine if a relationship existed between their thermal reversibility and their gelling properties. The α-lac solution in $CaCl_2$ heated at 85 °C formed an opaque curd which is indicative of aggregation with no observed gelation. α-Lac in $CaCl_2$ when heated at 95 °C gave a soft opaque gel, and at 100 °C gave a firm opaque gel both of which are indicative of aggregation with gelation. In the absence of $CaCl_2$, α-lac did not gel on heating. A soft translucent gel was formed only after the fifth heating cycle and also on heating in the oven at 100 °C for 30 min. In the presence of $CaCl_2$ and Na_2-EDTA, an opaque gel was formed after the fourth heating cycle. This suggests that the ability of α-lac to renature after heat treatment is directly related to the presence of Ca^{2+} and the extent to which it aggregates or gels on heat treatment. When no aggregation or gelation occurs, the percent reversibility increases.

In this study, transitions affecting position and intensities of the amide I′ band (1700−1600 cm^{-1}) and the amide II band (1550 cm^{-1}) were considered. The amide I′ band involves transitions relating to C═O-stretching vibrations, while the amide II involves −CONH vibrations of the peptide backbone and can be used to study rates of hydrogen−deuterium exchange and unfolding of the protein (Clark et al., 1981). Figure 2 shows the FTIR spectra of α-lac (20%, w/v) in D_2O heated from 25 to 90 °C, cooled to 25 °C, and subjected to four heating/cooling cycles. Spectra were collected during both the heating and cooling cycles. The spectrum at 25 °C of the first heating cycle gave four main bands at 1676, 1662, 1652, and 1639 cm^{-1} and a shoulder at 1630 cm^{-1}. The assignment of these bands

Table 3. Peak Positions of the Amide I′ Regions of α-Lactalbumin

peak position (cm^{-1})	assignment	peak position (cm^{-1})	assignment
∼1613	side chain	1644 ± 3	unordered
1618 ± 3	β-sheet	1654 ± 3	α-helix
1624 ± 4	β-sheet	1663 ± 4	turns
1632 ± 2	β-sheet	1669 ± 2	turns
1638 ± 2	β-sheet	1675 ± 4	β-sheet
∼1639	3_{10}-helix	∼1684	β-sheet

(Table 3) are as follows: 1676 cm^{-1} attributed to β-sheet (Casal et al., 1988; Susi and Byler, 1988) and turns (Krimm and Bandekar, 1986); 1662 and 1652 cm^{-1} bands attributed to turns and α-helices, respectively (Susi and Byler, 1988); 1639 cm^{-1} previously assigned to antiparallel β-sheet structure and 1630 cm^{-1} attributed to extended antiparallel β-sheet (Susi et al., 1985). Holloway and Mantsch (1989) have recently attributed bands at or near 1639 cm^{-1} to the 3_{10}-helices of α-lac. The resolution enhancement factor (*K*) used in this study did not permit us to observe the 1645 cm^{-1} band which was reported in α-lac by Prestrelski et al., (1991a).

Heating (α-lac in D_2O, pH 6.8) to 95 °C (first cycle, Figure 2b) resulted in a loss of the 1630 and 1639 cm^{-1} bands and a split of the 1652 cm^{-1} band accompanied by the appearance of two new bands at 1657 (α-helix) and 1646 (random coil) cm^{-1}. The difference spectra (not shown) further showed a slight increase in the band at 1662 cm^{-1} (turns). Cooling of this heated solution (first cooling cycle, Figure 2c) gave a band at 1628 cm^{-1} (β-sheet) accompanied by a shift of the band at 1646 to 1642 cm^{-1} and a decrease in the intensity of the 1662 and 1657 cm^{-1} bands suggesting that on cooling, turns and α-helical structures were transformed to β-sheet. Reheating (second heating cycle) gave a spectra (Figure 2d) similar to that obtained from the first heating cycle (Figure 2b). When the sample was recooled, the spectrum obtained (Figure 2e) was similar to that from the first cooling cycle (Figure 2c). The third heating cycle (Figure 2f) showed a decrease in the intensity of the bands at 1628 and 1676 cm^{-1} and an increase in the intensity of the bands at 1662 and 1657 cm^{-1}. Cooling (third cycle, Figure 2g) again showed a decrease in the intensity of the 1662 and 1657 cm^{-1} bands and an increase in the intensity of the bands at 1676 and 1628 cm^{-1}. The transitions in the fourth heating and cooling cycles (Figure 2h,i) were similar to that in the third cycle. The data suggest that α-lac has a high degree of thermal stability and that the unfolding observed on heating is reversible (Relkin et al., 1993). The antiparallel β-sheet at 1628 cm^{-1} is highly mobile and is transformed to turns and unordered structure at high temperatures. Upon cooling these turns realign to form extended β-sheet with antiparallel orientation. This supports the results obtained from the DSC which showed reversibility of both transitions (A and B) on reheating.

Prestrelski et al., (1991a) reported that removal of bound Ca^{2+} from α-lac resulted in the transition from a well-defined coordination complex to an essentially orderless structure. To determine the contributions of apo-α-lac (Ca^{2+}-free) and holo-α-lac (Ca^{2+}-bound) to the transitions observed in the FTIR and DSC, the FTIR spectra of α-lac dialyzed in EDTA (0.1 M) were recorded. Figure 3 shows the spectrum of α-lac (dialyzed); four bands at 1659, 1650, 1640, and 1624 cm^{-1} were observed at 25 °C. The spectrum was very different from that

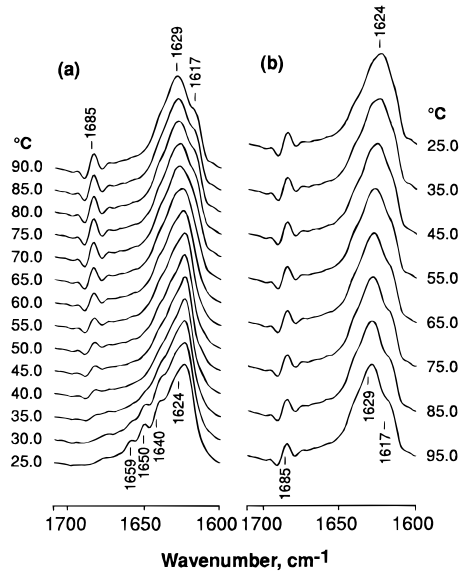

Figure 3. Stacked plot of deconvoluted infrared spectra of α-lac (10%, w/v) dialyzed in EDTA (0.1 M): (a) heating cycle and (b) cooling cycle.

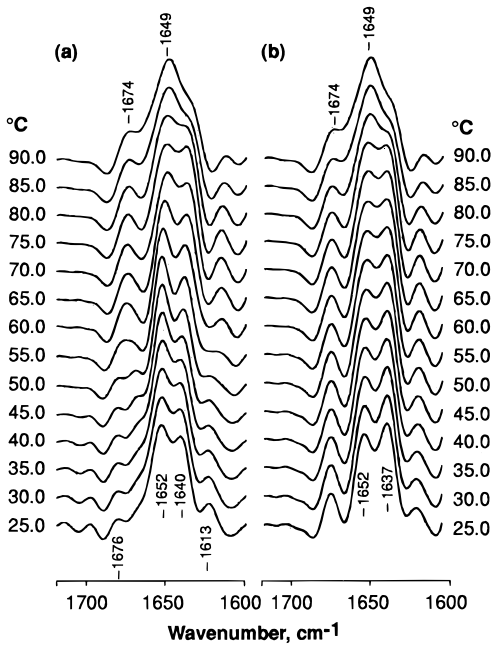

Figure 4. Stacked plot of deconvoluted infrared spectra of α-lac (10%, w/v) heated through two cycles in $CaCl_2$ (0.5 M) as a function of temperature: (a) first heating cycle and (b) second heating cycle.

obtained for α-lac in D_2O (containing 0.3 mol of Ca^{2+}/mol of α-lac) (Figure 2a), suggesting that removal of the Ca^{2+} caused marked changes in the secondary structure of the protein. The spectrum also shows a reduction in α-helix and 3_{10}-helix content and a relative increase in β-sheet content (1624 cm^{-1} band). The conformation of α-lac around the Ca^{2+}-bound region consists of a helix−turn−helix motif (Brew and Grobler, 1992); removal of Ca^{2+} may therefore have destabilized the helical structures in this region causing them to unfold or to form other secondary structures. Heating to 35 °C resulted in a decrease in the intensities of the bands at 1659, 1650, and 1640 cm^{-1} indicative of the loss of secondary structure (denaturation). This temperature is very close to the first transition (peak A, 39.6 °C) observed in the DSC of α-lac in D_2O, suggesting that the transition represents the denaturation of apo-α-lactalbumin. At 40 °C the band at 1685 cm^{-1} started to increase in intensity. This was accompanied by a slight increase in the band at 1617 cm^{-1} as the temperature was increased to 90 °C. An increase in the intensity of these two bands has been associated with intermolecular hydrogen-bonded antiparallel β-sheet structures resulting from reassociation of unfolded peptide segments which leads to formation of gel and aggregate structures (Clark et al., 1981); this suggests that the protein had started to aggregate. Figure 3b shows the spectra of the cooling cycle (95−25 °C). The spectrum of α-lac cooled back to 25 °C after heating was very similar to the one at 95 °C which further suggests that the thermal transitions that occurred during the heat treatment were irreversible.

The spectrum in $CaCl_2$ (Figure 4a) shows four main bands at 1613, 1640, 1652, and 1676 cm^{-1}. These bands are similar to the ones observed for α-lac in D_2O (Figure 2a). [The band at 1613 cm^{-1} has been attributed to side chain absorbances (Table 3; Chirgadze et al., 1975).] Heating to 50 °C gave a band at 1665 cm^{-1} (attributed to turns) which indicates unfolding of the protein. The band at 1640 cm^{-1}, attributed to 3_{10}-helix, decreased consistently with increasing temperature, suggesting an unfolding of the helical structure probably in the Ca^{2+}-binding elbow. The α-helix band (1652 cm^{-1}), however, was affected only slightly with increasing temperature. Above 50 °C, the 1640 cm^{-1} band increased in intensity along with the bands at 1674 and 1613 cm^{-1}. These bands again decreased in intensity above 75 °C, suggesting a rearrangement of the protein secondary structure (Casal et al., 1988). The increase in the band at 1649 cm^{-1} can be attributed to an increase in random coil structures. Figure 4b shows the spectra of α-lac in $CaCl_2$ during the second heating cycle. The transitions were similar to that observed during the first heating cycle. The spectrum recorded at 25 °C of the second heating cycle was different from that at 95 °C of the first heating cycle but similar to that at 25 °C of the first heating cycle (Figure 4a) suggesting that the thermal transitions are reversible.

The above observations have considerable transitions in the amide I′ band. In addition to the structural data obtained in this region, information on transitions associated with protein gelation can be obtained by examination of the amide II regions of the spectrum near 1550 cm^{-1} (Clark et al., 1981). To study the rate of hydrogen−deuterium exchange in the presence of $CaCl_2$, the integrated intensity of the band in the 1525−1555 cm^{-1} region (amide II) of the four heating cycles of α-lac in $CaCl_2$ was plotted (Figure 5). An intense band was observed at 25 °C; heating (first heating cycle) resulted in a decrease in the intensity of this band to a minimum value at 75 °C; no further decrease in intensity was observed in the second, third, and fourth heating cycles. The presence of this band in the first heating cycle indicates that hydrogen−deuterium exchange was incomplete and suggests that the protein structure was compact and inaccessible to solvent (Clark et al., 1981). Heating resulted in unfolding of the protein and enhanced hydrogen−deuterium exchange, which was completed during the first heating cycle (Prestrelski et al., 1991b). The transitions observed in the spectrum, especially in the amide I′ region (Figure 4b), after the first heating cycle can therefore be attributed to conformational changes in the protein structure and not to hydrogen−deuterium exchange. In EDTA (spectra not shown) no bands were observed in this region in all four heating cycles suggesting that

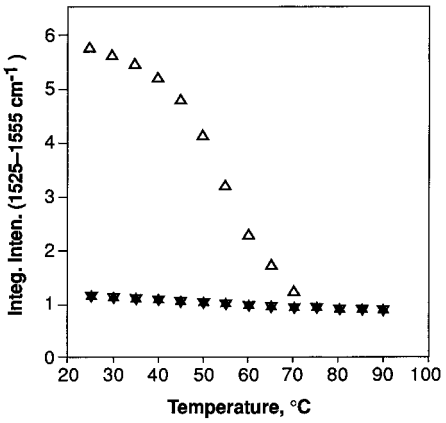

Figure 5. Integrated intensity of the 1525−1555 cm^{-1} region of the spectra of α-lac heated in the presence of $CaCl_2$ (0.5 M) as a function of temperature: (△) first heating cycle, (▲) second heating cycle, (★) third heating cycle, and (●) fourth heating cycle.

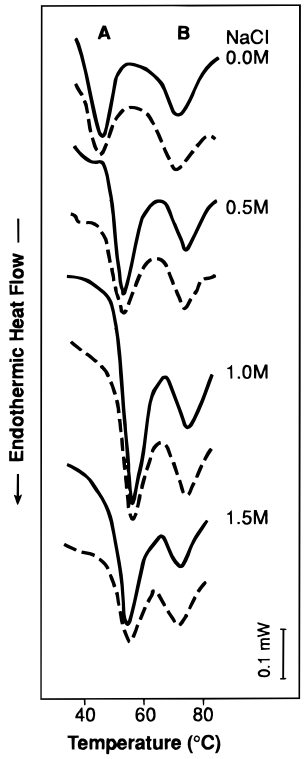

Figure 6. Effect of NaCl concentration on the DSC thermogram of α-lac (20%, w/v). Heating rate was 5 °C/min: (—) first heating cycle; (− −) second heating cycle.

hydrogen-deuterium exchange was immediate; this indicates that in the presence of EDTA the protein had a more open structure which was more accessible to solvent.

Protein−Salt Interactions. The thermograms (Figure 6) of α-lac heated in 0.5 M NaCl from 15 to 100 °C revealed two transitions at 48.3 ± 0.2 °C (peak A) and 69.9 ± 0.1 °C (peak B); this represents a shift from 39.6 °C for peak A and from 64.8 °C for peak B in the absence of NaCl. In 1.5 M NaCl, peak A shifted further to 53.2 ± 0.1 °C and peak B to 73.1 ± 0.2 °C, which suggests that NaCl has a stabilizing effect on the thermal denaturation of both apo- (peak A) and holo (peak B) α-lac. Table 2 shows the effect of NaCl concentration on the reversibility of α-lac. The percent reversibility in 0.5 M NaCl was 63% (transition A) and 89% (transition B). In 1.5 M NaCl the percent reversibility

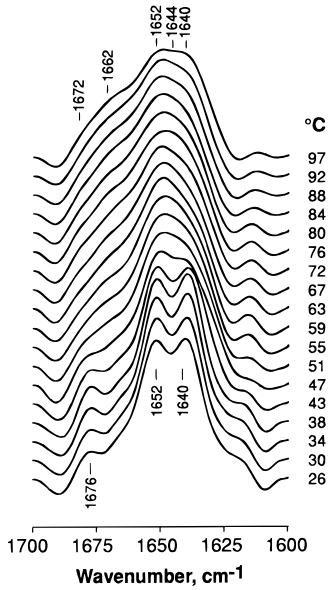

Figure 7. Stacked plot of deconvoluted infrared spectra of α-lac (10%, w/v, pH unadjusted) as a function of temperature in 0.5 M NaCl.

decreased to 48% (transition A) and 76% (transition B) suggesting that the protein was more irreversibly denatured at higher concentrations of NaCl. It is possible that the high salt concentration promoted protein−protein aggregation after denaturation, thereby preventing protein renaturation on cooling (Morrissey et al., 1987).

Figure 7 shows the FTIR spectra of α-lac heated from 26 to 97 °C in the presence of 0.5 M NaCl. Above 43 °C there was a marked decrease in the intensity of the 1676 and 1640 cm^{-1} bands in the spectra of α-lac dissolved in 0.5 M NaCl, suggesting a loss of both β-sheet and 3_{10}-helical structure. Above 47 °C, an increase in intensity of the bands at 1672 and 1662 cm^{-1} was observed, which further suggests an increase in turns and unordered structures; this could be due to partial unfolding as the hydrogen bonds in the secondary structures were broken (Ismail et al., 1992). The band at 1640 cm^{-1} increased in intensity above 72 °C which suggests a reformation of ordered structure: either 3_{10}-helix or β-sheet. In the spectra of α-lac in 1.5 M NaCl (Figure 7b), two bands at 1684 and 1618 cm^{-1} associated with the formation of gel and aggregate structures (Clark et al., 1981) were observed above 55 °C. The 1684 and 1618 cm^{-1} bands were absent in the spectrum of α-lac in NaCl concentrations lower than 1.5M, which suggests that α-lac only formed aggregates at high NaCl concentrations. When the solution of α-lac in 0.5 M NaCl was cooled from 97 to 20 °C (spectra not shown), the bands at 1672, 1652, and 1640 cm^{-1} decreased in intensity while the β-sheet bands at 1682 and 1624 cm^{-1} (Table 3) associated with β-sheet (Susi and Byler, 1988) increased in intensity. This suggests that the denaturation of α-lac in the presence of 0.5 M NaCl was reversible; however, when cooled the protein could have assumed a conformation different from that of the native protein. The presence of both the apo- and holo- forms of α-lac in the sample used confounds further discussion of the changes observed. In the spectra of α-lac in 1.5 M NaCl (not shown), two bands at 1684 and 1618 cm^{-1} associated with the formation of gel and aggregate structures (Clark et al., 1981) were observed above 55 °C. The 1684 and 1618 cm^{-1} bands were absent in the spectrum of α-lac in NaCl concentrations lower than

Table 4. Effect of pH on the DSC Characteristics of α-Lactalbumin[a]

treatment	T_d (A)	T_w(A)	H (A)	T_d (B)	T_w(B)	H (B)	% reversibility A	% reversibility B
pH								
3	39.0 ± 0.1	7.8	0.64 ± 0.01	67.5 ± 0.1	13.3	0.40 ± 0.02	14	100
5	39.3 ± 0.2	7.8	0.75 ± 0.03	67.1 ± 0.2	11.4	0.42 ± 0.02	38	87
7	43.2 ± 0.1	7.5	1.00 ± 0.01	66.0 ± 0.1	9.4	0.56 ± 0.01	38	38
9	44.4 ± 0.1	7.3	1.14 ± 0.02	65.3 ± 0.1	6.0	0.57 ± 0.01		

[a] See footnote for Table 1. T_w, width of the peaks observed in the DSC at half-height.

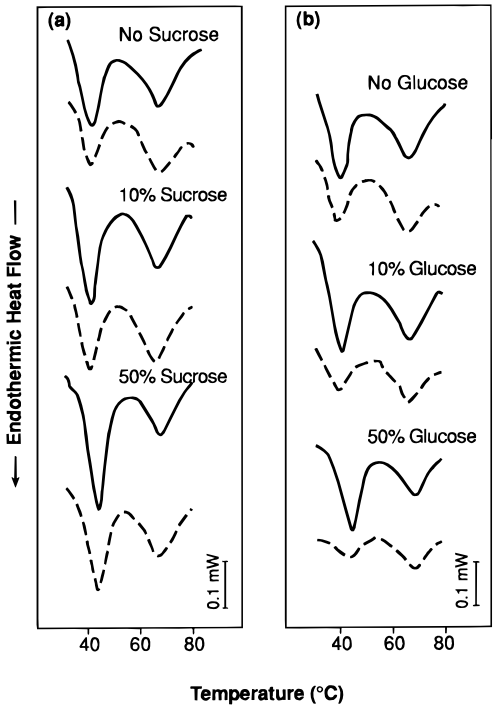

Figure 8. DSC characteristics of α-lac (20%, w/v, protein concentration, pH unadjusted) in the absence and presence of (a) sucrose and (b) glucose (10% and 50%, w/v) in D_2O. Heating rate was 5 °C/min: (—) first heating cycle; (– –) second heating cycle.

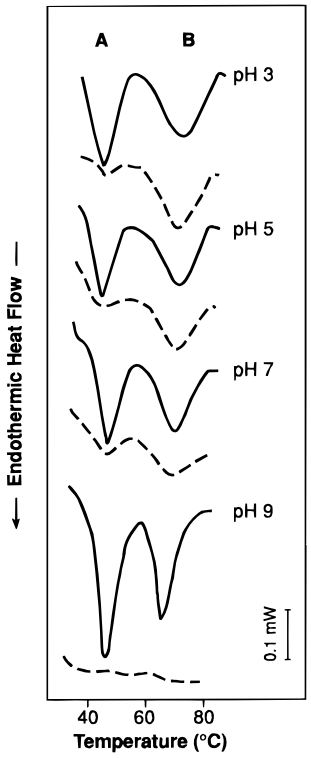

Figure 9. Effect of pH on the DSC characteristics of α-lactalbumin (20%, w/v, in deuterated phosphate buffer, ionic strength, 0.2). Heating rate was 5 °C/min: (—) first heating cycle; (– –) second heating cycle.

1.5M, which suggests that α-lac only formed aggregates at high NaCl concentrations.

Protein−Sugar Interactions. The thermograms of α-lac heated with sucrose and glucose (10% and 50% solutions, w/v, in D_2O) are shown in Figure 8. Two reversible transitions at 40.0 ± 0.1 °C (peak A) and 67.4 ± 0.2 °C (peak B) were observed in the 10% (w/v) sucrose solution (Figure 8a). These transitions represent a shift from 39.6 °C (peak A) and 64.8 °C (peak B) in the absence of sucrose (Table 2). In the presence of 50% (w/v) sucrose, the transitions shifted to 43.7 ± 0.2 °C (peak A) and 68.1 ± 0.1 °C (peak B), which suggests that sucrose had a stabilizing effect on the denaturation temperature of both holo- and apo-α-lac at high concentrations. When the samples were reheated, transition A was 70% reversible in both solutions while transition B was 94% reversible in the 10% (w/v) sucrose solution and 100% reversible in the 50% solution. The thermograms of α-lac in 10% and 50% (w/v) glucose solution and in the absence of glucose are shown in Figure 8b. Two transitions at 39.6 ± 0.2 °C (peak A) and 65.5 ± 0.1 °C (peak B) were observed in the 10% (w/v) glucose solution. These transitions shifted to 44.6 ± 0.1 °C (peak A) and 69.7 ± 0.3 °C (peak B) in the presence of 50% (w/v) glucose which indicates that glucose had a greater stabilizing effect on the denaturation of α-lac than sucrose; Harwalkar and Ma (1992) reported similar results for β-lactoglobulin. When the samples were reheated, transition A was 42% reversible while transition B was 78% reversible in the 10% (w/v) glucose solution. In the 50% (w/v) solution, transition A was 32% reversible while transition B was 100% reversible. The FTIR spectra (not shown) of α-lac heated in 50% (w/v) glucose and sucrose (heating and cooling cycles) were similar to those observed for α-lac heated in 0.5 M NaCl (Figure 7a); this indicates that in the presence of these sugars there was no aggregation (absence of 1684 and 1618 cm^{-1} bands) and denaturation on heating was reversible.

Effect of pH. Figure 9 shows the thermograms of α-lac heated in deuterated phosphate buffer at different pH values. Two thermal transitions were observed at all the pH values studied. At pH 3, the first transition (peak A) occurred at 39.0 ± 0.1 °C and the second transition (peak B) at 67.5 ± 0.1 °C (Table 4) which indicates an increase in thermal stability in the apo-form but a decrease in the stability of the holo-form. With increasing pH, transition A shifted to 44.4 ± 0.1 °C at pH 9 while transition B shifted to 65.3 ± 0.1 °C. At all the pH values studied (except pH 9), the width of the peak at half-height (T_w) was smaller for transition A than transition B (Table 4). With increasing pH, however, the T_w for transition B decreased from 13.3 °C at pH 3 to 6 °C at pH 9. The width of the peak at half-height (T_w) is a measure of the sharpness of an

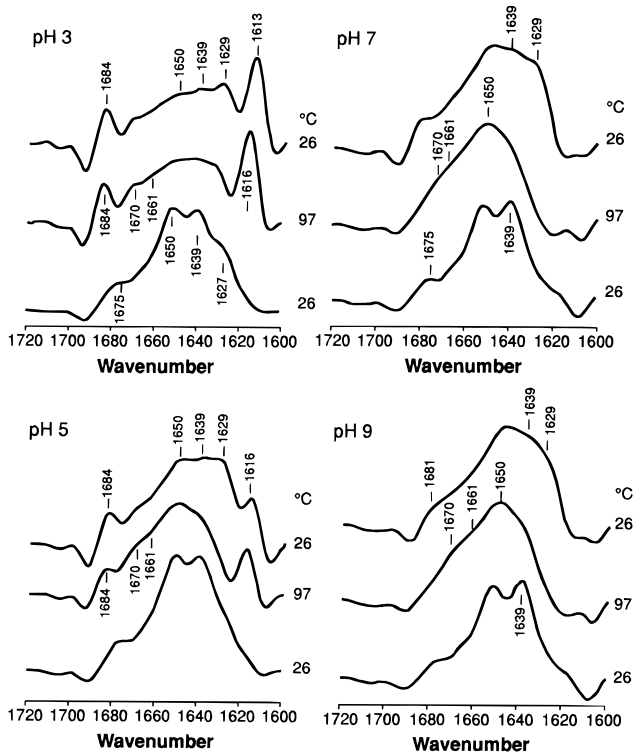

Figure 10. Stacked plot of deconvoluted infrared spectra of α-lactalbumin (10%, w/v, in deuterated phosphate buffer, ionic strength, 0.2) at 26, 97, and cooled to 26 °C.

endothermic peak and gives an indication of the cooperative nature of the transition from native to denatured state (Wright et al., 1977); if denaturation occurs within a narrow range of temperature, the transition is considered highly cooperative. [In an earlier study (Boye et al., 1995), it was observed that pH affected the cooperativity of denaturation of whey protein concentrate and β-lactoglobulin. T_w was measured to determine if a similar effect existed for α-lac.] The data suggest that transitions occurring in peak A were generally more cooperative than in peak B; with increasing pH, the cooperativity of transition B increased. When the samples were reheated, transition A was 14% reversible and transition B was 100% reversible at pH 3. At pH 5 and 7, transition A was 38% reversible, while transition B was 87% reversible at pH 5 and 38% reversible at pH 7. At pH 9 both transitions were completely irreversible. Our data (Table 4) show a progressive decrease in enthalpy for both transitions A and B, with decreasing pH. At pH 9, the enthalpy was 1.14 and 0.57 J/g for transitions A and B respectively. These values decreased to 0.64 and 0.40 J/g at pH 3. Enthalpy values obtained from the DSC have been correlated with the content of ordered secondary structure of a protein (Koshiyama et al., 1981) and can be used to monitor the proportion of protein in an isolate that is not denatured during processing (Arntfield and Murray, 1981). The decrease in enthalpy with decreasing pH observed in this study may be attributed to denaturation of α-lac at acidic pH, since a partially unfolded protein would require less heat energy to denature completely than a native protein (Ma and Harwalkar, 1988). The decrease in enthalpy may also be due to protein aggregation, which is considered an exothermic reaction (Jackson and Brandts, 1970).

Figure 10 shows the FTIR spectra of α-lac at 26 °C, heated to 97 °C, and then cooled to 26 °C at pH 3, 5, 7, and 9. The spectra at 26 °C showed a band at 1627 cm^{-1} at pH 3 which was absent at pH 9; this band, assigned to antiparallel β-sheet structure (Table 3), suggests an increase in extended β-sheet formation. At 97 °C, the spectra showed two β-sheet bands at 1684 and 1616 cm^{-1} at pH 3 and 5 but not at pH 7 and 9; these bands, attributed to intermolecular β-sheet formation resulting from aggregation of the protein, suggest that α-lac formed aggregates at pH 3 and 5 but not at pH 7 and 9. Lala and Kaul (1992) have reported the exposure of two tryptophan residues which increase the surface hydrophobicity of α-lac in the molten globule state (acid pH). This increased hydrophobicity might be responsible for the intermolecular interactions that result in the irreversible aggregation at acid pH values (1684 and 1618 cm^{-1} bands).

At both acid and alkaline pH values, cooling the heated samples resulted in a loss of the 1670 and 1661 cm^{-1} bands (turns) and the appearance of a band at 1629 cm^{-1} band (β-sheet). At pH 7 and 9 there was a notable increase in the 1639 cm^{-1} band on cooling. At pH 5, this band increased in intensity together with the band at 1684 cm^{-1}. At pH 3, the band at 1616 cm^{-1} shifted to 1613 cm^{-1} on cooling which suggests an increase in hydrogen bonding and a more compact folding of the aggregates on cooling.

Visual observation of the α-lac gels (Clark and Lee-Tuffnell, 1986) showed that at pH 3 and 5, α-lac aggregated without forming a firm gel. At pH 7 no gel or aggregate formation was observed. At pH 9, a firm translucent gel was formed which did not revert to a solution state on cooling. Hydrophobic interactions normally result in the formation of opaque coagulum-type gels (Damodaran, 1994). The formation of a translucent gel at pH 9, coupled with the absence of a peak in the DSC thermogram on reheating of α-lac at pH 9, suggests that the protein may have undergone irreversible intermolecular disulfide bond formation when heated, in contrast to the hydrophobic interactions that resulted in aggregate formation at pH 3. The absence of the 1684 and 1616 cm^{-1} bands in the IR spectra at pH 9, associated with hydrogen-bonded aggregate (gel) structure, further suggests that the translucent gel formed at pH 9 does not result from hydrogen bonding.

CONCLUSION

The two endothermic peaks observed in α-lac, previously associated with the denaturation of the Ca^{2+}-free (apo) and -bound (holo) forms by Relkin et al. (1993), were confirmed in this study. Comparison of the DSC and FTIR data shows that the first of two transitions from the DSC is related to the breakdown of the 3_{10}-helix conformation and an increase in β-sheet structure in apo-α-lac as determined by FTIR. The second transition observed from the DSC occurred within the temperature range of 65−70 °C which was similar to the temperature associated with complete unfolding of holo-α-lac as determined by FTIR; this unfolding resulted in the loss of 3_{10}-helix structure and an increase in the formation of turns. The spectra of heated apo-α-lac showed the presence of the two bands at 1617 and 1685 cm^{-1} which indicates that denaturation of apo-α-lac resulted in the formation of β-sheet structures (aggregation), and in the case of holo-α-lac denaturation resulted in unfolding into turns (increase in 1665 cm^{-1} band). The ability of holo-α-lac to renature on cooling can be attributed to the absence of aggregation (absence of 1685 and 1617 cm^{-1} bands) on heat treatment.

The present work demonstrated that α-lac is a thermally labile protein whose secondary structure is easily altered by heating but has the ability to refold to a conformation similar to that of its native state in the presence of $CaCl_2$.

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