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Thermal stability of β-lactoglobulins A and B: effect of SDS, urea, cysteine and N-ethylmaleimide

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Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) were used to monitor changes in the secondary structure and thermal stability of β-lactoglobulin A and B in the presence of sodium dodecyl sulphate (SDS), N-ethylmaleimide (NEM), urea and cysteine. An increase in the thermal stabilities of both proteins was noted in the presence of 10 mM-SDS. In the presence of 50 mM-SDS, there was extensive denaturation of both variants. In general, the β-strand/β-sheet regions in the secondary structure of both variants were very susceptible to denaturation by SDS and cysteine, suggesting that these regions may be held by hydrophobic and disulphide bonds. At ambient temperature and physiological pH, a notable difference was observed in the 1636 and 1627 cm^{-1} regions of the FTIR spectra of the two β-lg variants. The results suggest possible differences in the nature of the β-sheet/β-strand distribution/content of the two proteins. Urea and NEM at a concentration of 50 mM, had little effect on the secondary structure and denaturation of both variants. New findings are presented which further indicate that although the β-lg B variant showed greater thermal stability than the A variant in all the cases studied, its denaturation temperature and secondary structure were affected to a greater extent by the protein perturbants than β-lg A.

Keywords: β-Lactoglobulin, denaturation, differential scanning calorimetry, infrared spectroscopy, FTIR.

The use of whey protein concentrates and isolates in the food industry has grown tremendously in the last decade. The excellent performance of these whey products in food formulation and product development may be attributed, primarily, to the useful functional properties of β-lacto-globulin (β-lg), the major protein in the whey fraction of bovine milk. Important properties of this protein include its ability to act as a gelling, thickening, emulsifying and foaming agent. Much effort has been expended in recent times to understand the molecular structure of this protein and its structure–function properties.

The three-dimensional structure of β-lg is now well characterized (Papiz et al. 1986; Brownlow et al. 1997; Bewley et al. 1997; Qin et al. 1998a, b, 1999). The protein is a member of the lipocalin protein superfamily, and contains 162 amino acid residues which are folded into an 8-stranded antiparallel β-barrel, lined with hydrophobic residues to create a deep hydrophobic pocket. This cavity is believed to be the main binding site for a number of hydrophobic molecules (Banaszak et al. 1994). A 3-turn α-helix is attached to the outside of the barrel. The molecule has two distinct β-sheets, an antiparallel four-stranded curved β-sheet formed by the first four β-strands (β-sheet 1), and a flatter orthogonally packed antiparallel β-sheet (β-sheet 2) formed by the remaining four stands and part of the first β-strand (Qin et al. 1998b). A ninth β-strand lies at the exterior of the β-barrel and is critically involved in dimer formation.

At physiological pH, β-lg exists as a dimer with a molecular weight of 36·2 kDa. Seven different genetic variants have been identified, the most abundant being the A and B forms (Eigel et al. 1984). The primary structures of these two variants differs in positions 64 and 118 where the aspartic acid and valine of β-lg A are replaced by glycine and alanine in β-lg B. Recent studies have shown that the seemingly minor difference in the primary structure of the

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A and B variants has significant effects on their trigonal crystal structure as well as their functional characteristics (Imafidon et al. 1991; Huang et al. 1994a, b). In a recent study, Qin et al. (1999) reported that the D64G substitution on an exposed flexible loop may be responsible for lowered solubility and enhanced oligomerization and gelation propensity of variant A, while the V118A substitution on a buried β-strand may be responsible for differences in thermal stabilities of the two variants.

In earlier studies (Boye et al. 1995, 1996, 1997), we determined the effect of pH on the thermal denaturation of β-lg AB and the two β-lg variants A and B and on the microstructure of their gels. Our results showed the two variants to behave differently when subjected to similar heat and pH treatments. We found β-lg B to have greater thermal stability than the A variant. The β-lg B variant also required more energy to denature and its denaturation was more cooperative than β-lg A. We further found that at acidic pH values, β-lg B had a higher proportion of β-sheet structure, and on heating formed larger aggregate structures than the A variant.

In the present work, we continue to investigate differences in the secondary structure, denaturation and thermal stability of β-lg A and B in the presence of several protein structure perturbing agents. The reagents used (SDS, cysteine, urea and NEM) are chosen for the following reasons; SDS is known to disrupt hydrophobic interactions while cysteine, urea and NEM are known to interfere with disulphide–sulphydryl exchange, hydrogen/hydrophobic bonding and sulphydryl oxidation, respectively. These interactions, as well as electrostatic attractions, have been shown to be primarily responsible for the functional properties exhibited by most proteins (Cheftel et al. 1985; Kinsella & Whitehead, 1989). The concentrations of the reagents selected for study are based on work reported in earlier studies (Harwalkar & Ma, 1989; Shih, 1992; Boye et al. 1996a) which show these reagents at the selected concentrations to alter protein structure (urea being the exception). Most studies on the effect of urea on protein structure have been carried out at high concentrations (2–8 M) (Harwalkar & Ma, 1989; Xiong & Kinsella, 1990). It was impossible, in this work, to study the effect of urea at these high concentrations because of interferences in the infrared region of interest. Much lower concentrations are therefore used. It should also be mentioned that while recent studies on the effect of SDS on β-lg have focused mainly on its stoichiometric binding to the central cavity of the protein, the focus of this paper is on its effect on denaturation and thermal stability and not on the specific mechanisms of its binding. The two techniques used in this study are Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). FTIR has proved to be very useful for monitoring changes in protein secondary structure during thermal treatment while DSC provides specific information on the thermal characteristics (e.g., denaturation temperature, cooperativity of unfolding) of proteins, during heat treatment.

Materials and Methods

Materials

β-Lg A (L-7880) and B (L-8005) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. Deuterium oxide (D_2O) (product 15, 188-2, minimum 99·9 at.% D) was purchased from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical grade.

Sample preparation

Solutions (100 g/l) of β-lg A and B were prepared by dispersing the proteins in 10 and 50 mM solutions of SDS, urea, cysteine and N-ethylmaleimide (NEM). For the FTIR study, the solutions were prepared in D_2O instead of H_2O because of its greater transparency in the infrared region of interest (1600–1700 cm^{-1}).

Fourier transform infrared spectroscopy

Infrared spectra of the β-lg solutions were recorded with a 8210 Nicolet FTIR spectrometer (Nicolet Instrument Corporation, Madison, WI) equipped with a deuterated triglycine sulphate detector as described previously (Boye et al. 1995). 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, USA). The samples were placed in an IR cell with a 50-μm pathlength and CaF_2 windows. The temperature of the sample was regulated by placing the cell in a thermostatted holder controlled by an Omega temperature controller (Omega Engineering, Laval, QC, Canada). 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 within ±0·5 °C. Deconvolution of the observed spectra were performed using the Nicolet FTIR Software, Omnic 3.0. Deconvolution of the infrared spectra were performed as described by Kauppinen et al. (1981). The signal to noise ratio was >20 000 : 1, and the bandwidth used for deconvolution was 13 cm^{-1} with a narrowing factor of 2·4. All FTIR experiments were done in duplicate.

Differential scanning calorimetry

Thermal properties of the β-lg variants A and B were studied by DSC using a DuPont 1090 thermal analyser (DuPont Co., Wilmington, DE, USA) equipped with a 910 DSC cell base and a high pressure cell as described previously (Ma & Harwalkar, 1988). Aliquots (10 μl) of each solution were placed in pre-weighed DSC pans, hermetically sealed and weighed accurately. A sealed empty pan was used as a reference (Harwalkar & Ma, 1990 and references therein; Boye et al. 1995, 1996b, 1997; Gao et al. 2001; Sobral & Habitante, 2001). The samples were heated from 20 °C to 120 °C at a programmed heating rate of 5 deg
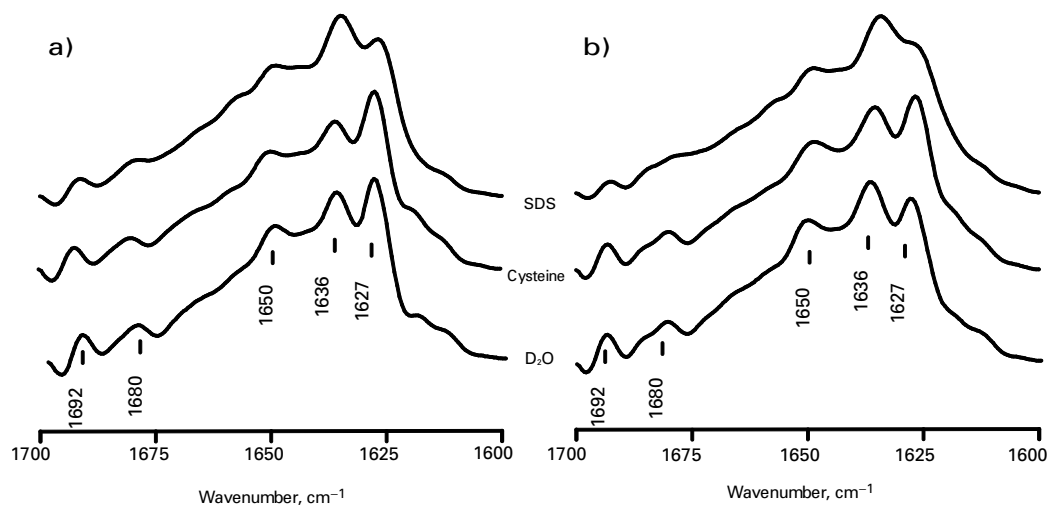
Fig. 1. Deconvolved infrared spectra of (a) β-lactoglobulin A and (b) β-lg B (100 g/l, 25 °C) in the presence of 50 mM-SDS, 50 mM-cysteine and D_2O.

C/min. Indium standards were used for temperature and energy calibrations. Onset temperature (T_s), peak temperature of denaturation (T_m), and heat of transition or enthalpy (ΔH), were computed from the thermograms by the 1090 analyser. The ΔH values were based on the protein content of the solution placed in the DSC pan. The width at half-peak height (T_w) was also measured. All DSC measurements were done in triplicate.

Statistical analysis

Statistical analysis of the DSC data was conducted using the SAS software developed by the SAS Institute Inc. (Cary, NC, USA). The general linear models (GLM) procedure was used for analysis of variance. Differences between sample means were analysed using Scheffe's test. Significance was defined at $P=0.05$.

Results

Effects of perturbants at 25 °C

The FTIR spectra of β-lg A and B in D_2O (Fig. 1), shows the spectrum of the genetic variant B to have a higher, more intense peak at 1636 cm^{-1} and a much less intense peak at 1627 cm^{-1} relative to the A variant. In earlier studies (Arrondo et al. 1988; Boye et al. 1996b, 1997; Subirade et al. 1998) the band appearing at 1636/35 cm^{-1} was attributed to β-sheet, while that at 1627/26 cm^{-1} was attributed to β-strands. No major differences were observed in the bands at 1650 cm^{-1} of the two proteins; these bands have been attributed to α-helical structure (Susi & Byler, 1988). In the presence of cysteine (50 mM), an increase in the intensity of the band at 1627 cm^{-1} relative to that at 1636 cm^{-1} was observed for both proteins. The reverse was observed in the presence of SDS (50 mM), where a sharp

decrease in the intensity of the 1627 cm^{-1} bands was observed for both proteins. The spectra of the two proteins in SDS also showed decreases in the intensities of the 1692 and 1680 cm^{-1} bands, attributed to β-type and β-sheet structures respectively (Casal et al. 1988; Susi & Byler, 1988; Boye et al. 1996a, b). A decrease in the intensities of these bands has been associated with the onset of unfolding (Boye et al. 1996a, b).

Effects of perturbants at elevated temperatures

Figure 2 shows the FTIR spectra of β-lg A and B heated in the presence of 50 mM-SDS. Heating above 35 °C resulted in the complete disappearance of the 1692 cm^{-1} band which is indicative of extensive unfolding of both proteins. The loss of this band was accompanied by a decrease in the intensities of the bands at 1680 and 1627 cm^{-1}, which is further indication of a breakdown of native secondary structure. Between 45 and 75 °C the spectra of both proteins showed two new bands at 1683 and 1629 cm^{-1}; bands at these wavelengths have been attributed to β-type structures (Krimm & Bandekar, 1986); these structures were not present in the native state. Above 75 °C there was the appearance of a new band at 1619/1618 cm^{-1} and an increase in the intensity of the band at 1683 cm^{-1}. The increase in the intensities of these two bands has been associated with aggregate formation (Clark et al. 1981; Boye et al. 1996a, b).

Samples of the proteins (100 g/l) were also dispersed in 10 mM-SDS solutions and analysed by FTIR, to determine the effect of a lower concentration of SDS on thermal stability. The spectrum of β-lg A at 25 °C (not shown) showed a decrease in the intensity of the 1627 cm^{-1} band when compared with that recorded in D_2O, but an increase when compared with the spectrum recorded in the presence of 50 mM-SDS. Similar results were obtained for β-lg B
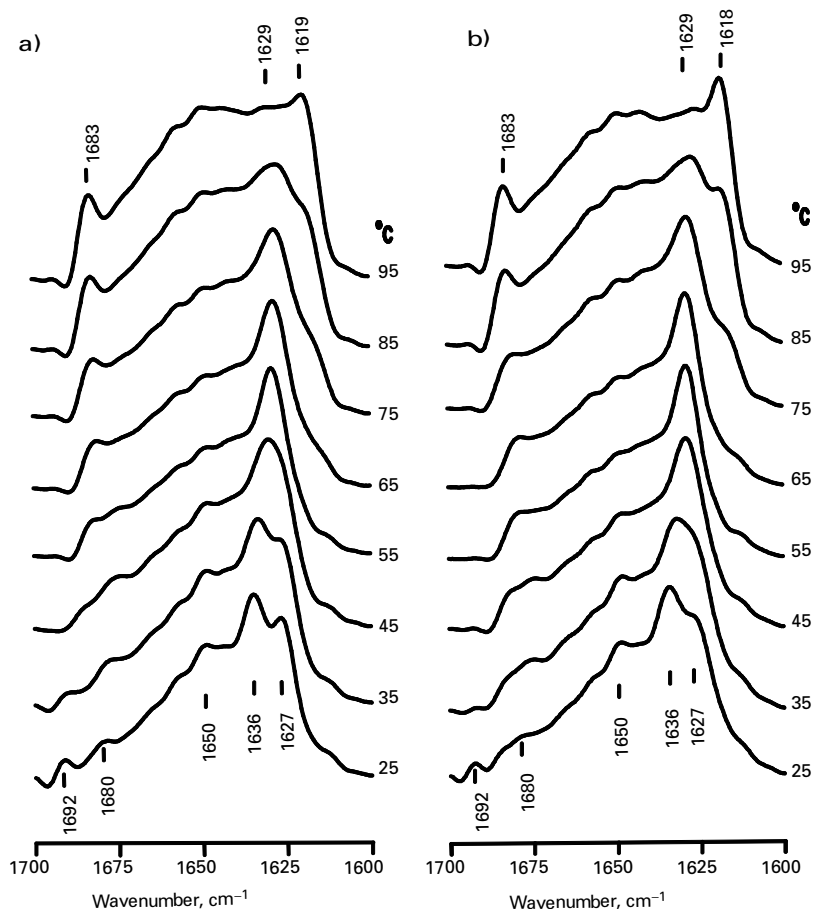
Fig. 2. Stacked plot of the deconvolved infrared spectra of (a) β-lactoglobulin A and (b) β-lg B (100 g/l) heated in the presence of 50 mM-SDS.

suggesting that both proteins were less denatured in the presence of 10 mM-SDS. Major changes in the spectra of the proteins were observed only when they were heated above 65 °C, which further confirmed that both variants were thermally more stable in the 10 mM-SDS solution than in the 50 mM-solution.

Figure 3 shows the spectra of the two proteins heated in the presence of 50 mM-cysteine. As the temperature was increased from 25 to 55 °C, an increase in the intensity of the 1636 cm^{-1} band was observed while that of the 1627 cm^{-1} band decreased. Above 65 °C, there was a notable decrease in the intensities of the bands at 1692, 1680, 1650, 1636 and 1627 cm^{-1}, indicative of de-naturation and a general breakdown in secondary struc-ture. This was followed by aggregation (increase in the intensity of the two bands at 1683/2 and 1623/1 cm^{-1}) as the proteins were heated further. The width of the aggre-gation band (1623/21 cm^{-1}) in the spectra of β-lg A was larger than in the spectra of β-lg B which may indicate differences in the type and firmness of the aggregates formed by the two proteins (Ismail et al. 1992).

The FTIR spectra of the proteins in urea (in the 1600–1700 cm^{-1} region, not shown) was, in general, very similar to that obtained in D$_2$O, suggesting that urea at a concentration of 50 mM, had very little effect on the protein secondary structure as expected. Figure 4a shows a plot of the integrated intensities of the 1690–1692 cm^{-1} band in the spectra recorded of β-lg A and B in 50 mM-urea. This plot is useful in comparing the rate of unfolding of the two proteins when heated. The plot for β-lg A showed a gradual decrease in the intensity of the band as the protein was heated from 25 to 60 °C indicative of a slow unfolding of the protein. Between 60 and 70 °C there was a sharp drop in the intensity of the band as the protein started to de-nature. The plot for β-lg B showed a much slower rate of decrease between 25 and 60 °C. The sharp drop in the in-tensity of the band (indicating denaturation), was observed only between 65 and 75 °C, which indicates a greater thermal stability of the B variant.

Figure 4b shows a comparison of the rate of unfolding in the presence of 50 mM-urea and 50 mM-SDS for β-lg A. The plot for 50 mM-SDS showed a sharp drop in the intensity of the 1692 cm^{-1} band between 30 and 50 °C which in-dicates that the protein unfolded very quickly when heated in the presence of this reagent. The decrease in the intensity of the band in the presence of urea (between 30 and 65 °C),
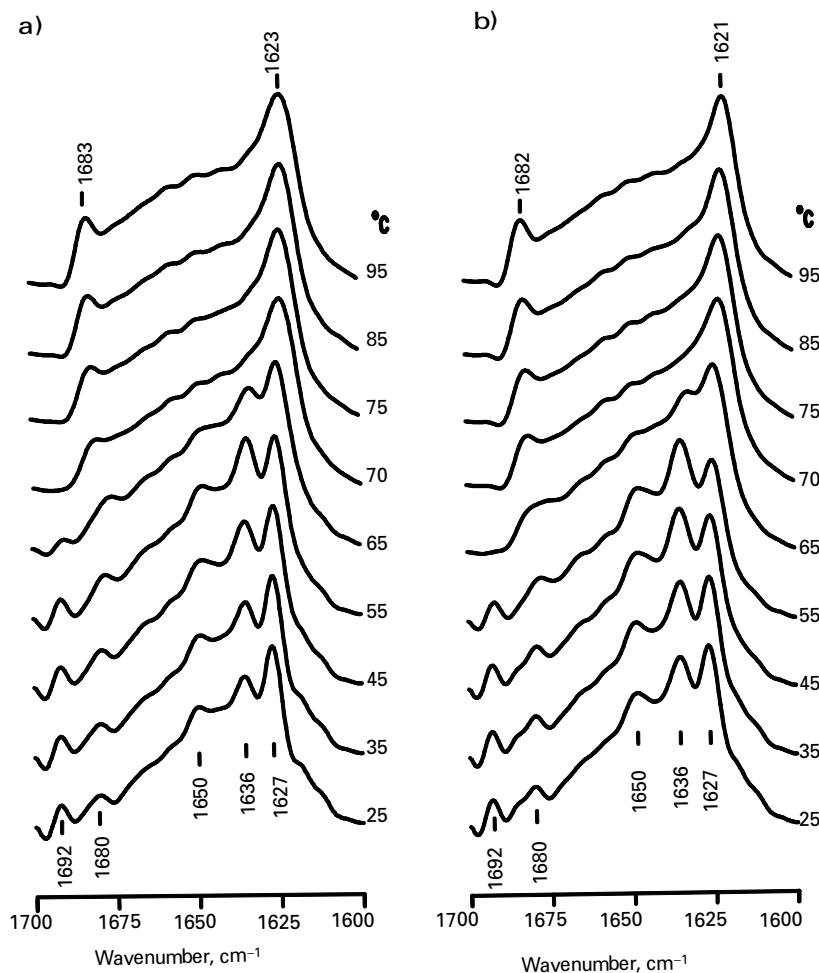
Fig. 3. Stacked plot of deconvolved infrared spectra of (a) β-lactoglobulin A and (b) β-lg B (100 g/l) heated in the presence of 50 mM-cysteine.

occurred at a much slower rate, indicating a much slower process of unfolding. Similar findings were observed for the B variant.
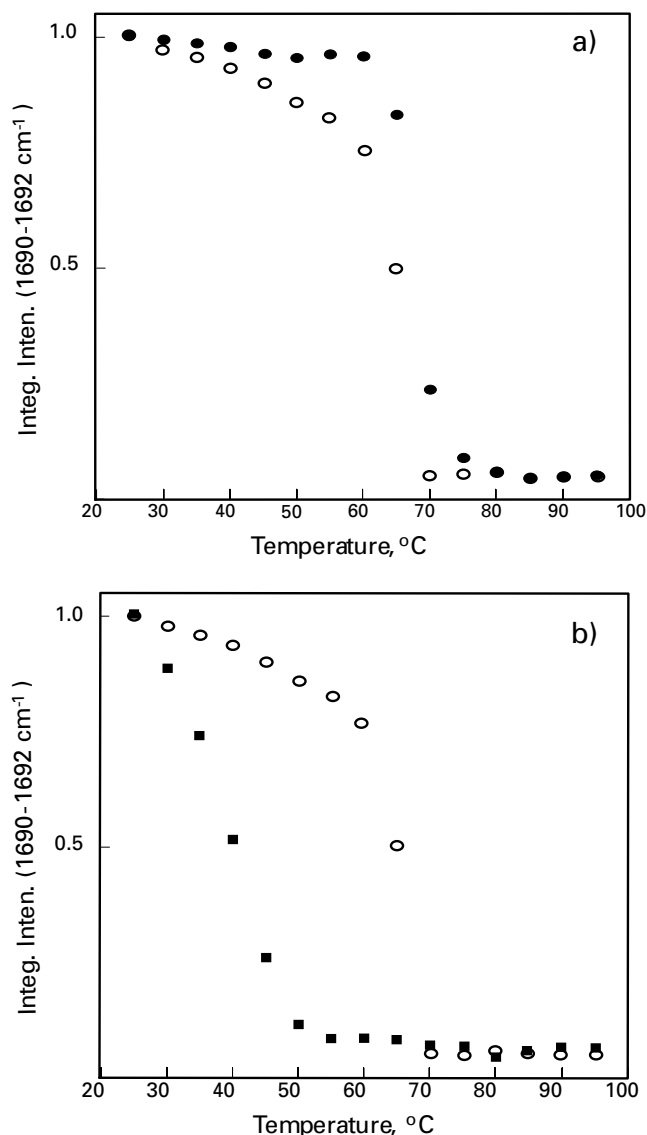
The FTIR spectra of β-lg A and B heated from 25 to 95 °C in the presence of 50 mM-NEM (spectra not shown) were also similar to those observed in D_2O, suggesting that there was very little effect of this reagent on protein secondary structure. As with the other perturbants, the presence of NEM did not inhibit aggregation of the proteins; the two bands at 1683 and 1621 cm^{-1} which are attributed to aggregate formation were observed following denaturation of both proteins. This is particularly interesting, and agrees with other research findings that suggest that aggregation of β-lg can occur in the absence of sulphydryl-disulphide interchange reactions (Manderson et al. 1998, 1999a, b).

Differential scanning calorimetric studies

The results obtained by FTIR were confirmed by DSC (Table 1). Onset (T_s) and peak (T_m) temperatures of denaturation were, in general, significantly larger ($P<0·01$) for β-lg B than for β-lg A in the presence of all four perturbants and at all concentrations studied. The only exception was in the presence of 50 mM-NEM where the increase in the T_s of β-lg B was only marginal. The results confirm those obtained by FTIR which showed the B variant to have greater thermal stability to denaturation and aggregation.

Both β-lg variants were thermally more stable in the presence of 10 mM-SDS than in H_2O but were completely denatured in the presence of 50 mM-SDS. Cysteine, at a concentration of 10 mM, had little effect on the DSC characteristics of both proteins. At higher concentrations (50 mM) there was a decrease in both T_s and T_m values, and an increase in T_w (broadening of the DSC peak), particularly for β-lg B, which is indicative of a decrease in thermal stability and in the cooperativity of unfolding of the protein. A significant decrease in ΔH (enthalpy) ($P<0·05$) was also observed. The decrease in enthalpy value was, however, greater for β-lg B than for β-lg A. ΔH values have been correlated with the content of ordered secondary structure of a protein (Kosiyama et al. 1981) and represent the total amount of energy required to denature a protein.

greater extent in the presence of 50 mM-NEM than β-lg A. ΔH values in the presence of 50 mM-NEM were significantly lower ($P<0{\cdot}05$) than those obtained in H_2O, urea and 10 mM-cysteine and SDS.

Discussion

X-ray studies to date have shown that there are hardly any differences in the secondary structures of the β-lg A and B variants (i.e, both variants contain same amount of β-strands, β-sheets, α-helix and turns) (Qin et al. 1999). The critical difference, according to these studies, exists mainly in the monomer-dimer equilibrium. Bewley et al. (1997) explain that the Ala[118]Val substitution (variant B/A) which introduces two extra methyl groups in the A variant perturbs the surrounding structure by displacing the polypeptide chain at residues 37–40 and affects the monomer-dimer equilibrium, and also the stability of the protein. The differences observed in the FTIR spectra of the two β-lg variants may possibly be a reflection of this shift in the monomer-dimer equilibrium. In our earlier work (Boye et al. 1997) we found that at pH 3 (when β-lg exists mostly in the monomeric state (Timasheff & Townend, 1961)), there was a decrease in the intensity of the 1636 cm^{-1} band. As the pH was increased to 7 (when the protein exists as a dimer), there was a dramatic increase in the intensity of this band. These findings may suggest, that the band observed at 1636 cm^{-1} in the FTIR spectra of β-lg represents the absorption of the β-lg β-sheet (in the dimeric state), while that at 1627 cm^{-1} represents the absorption of the β-sheet in the monomeric state (Qin et al. 1999 and references therein). These results could suggest that at ambient temperature and physiological pH, a notable difference exists in the monomer-dimer ratio of the two β-lg variants. The higher intensity of the 1636 cm^{-1} band in the spectra of the B variant could potentially suggest that this variant had a higher dimeric content than the A variant. β-lg was earlier reported to form dimers with dissociation constants in the micromolar range at pH ~ 7 and stability constants in the order β-lg C $\gg$ β-lg B $>$ β-lg A (Timasheff & Townend, 1961; McKenzie & Sawyer, 1972; Thresher & Hill, 1997). The results reported here would agree with these findings.

The β-strand/β-sheet regions in the secondary structure of both β-lg A and B were, in general, very susceptible to denaturation by SDS and cysteine (Fig. 1), suggesting that these regions may be held, somewhat, by hydrophobic and disulphide bonds. In the presence of SDS, the 1627 cm^{-1} band was more susceptible to denaturation while hardly any changes were observed in the 1636 cm^{-1} band. On the contrary, in the presence of cysteine, the 1636 cm^{-1} band was the more susceptible to denaturation. Cysteine is known to disrupt disulphide bonding. The decrease in the dimeric β-sheet content in the presence of cysteine, may therefore suggest that these β-sheet regions (specifically, the β-sheets involved in dimer formation) are held together

Fig. 4. (a) Plot of the integrated intensity (Integ. Inten.) of the 1690–1692 cm^{-1} band in the infrared spectra of β-lactoglobulin (100 g/l) A (○) and B (●) in 50 mM-urea as a function of temperature. (b) Plot of the integrated intensity (Integ. Inten.) of the 1690–1692 cm^{-1} band in the infrared spectra of β-lactoglobulin A (100 g/l) in 50 mM-urea (○) and 50 mM-SDS (■) as a function of temperature.

The DSC characteristics of β-lg A and B in the presence of 10 and 50 mM-urea (Table 1) were similar to those obtained in H_2O which again confirmed that urea (at these concentrations) had little effect on the thermal stabilities and cooperativity of unfolding of the proteins. T_s and T_m values in the presence of 50 mM-NEM were lower than those obtained in H_2O suggesting an effect of this reagent on thermal stability. While the statistical analysis showed the T_m value for β-lg A not to be significantly different from that observed in H_2O, that of β-lg B was significantly lower ($P<0{\cdot}05$). This suggests that β-lg B was perturbed to a

Table 1. Effect of perturbants on DSC characteristics, onset temperature (T_s), peak temperature of denaturation (T_m), width at half-peak height (T_w), enthalpy (ΔH) of β-lactoglobulin A and B

Values are means ± SD for $n = 3$

Perturbant	β-lg	T_s (°C)	T_m (°C)	T_w (°C)	ΔH (J/g)
H$_2$O	A	72·07 ± 0·23[a]	75·07 ± 0·15[a]	3·21 ± 0·26[a]	10·90 ± 0·20[a]
	B	75·37 ± 0·25[b]	78·37 ± 0·12[b]	2·96 ± 0·08[a]	13·27 ± 0·81[a]
10 mM, SDS	A	70·83 ± 0·68[c]	78·47 ± 0·75[c]	13·58 ± 1·91[b]	10·40 ± 0·66[a]
	B	74·57 ± 1·01[b]	82·73 ± 1·21[d]	10·25 ± 0·43[c]	12·27 ± 0·42[a]
10 mM, urea	A	72·40 ± 0·10[a]	75·53 ± 0·21[a]	3·08 ± 0·14[a]	12·50 ± 0·53[a]
	B	74·90 ± 0·36[b]	77·63 ± 0·29[b]	3·13 ± 0·13[a]	13·00 ± 1·18[a]
50 mM, urea	A	72·07 ± 0·29[a]	74·83 ± 0·32[a]	2·97 ± 0·06[a]	10·67 ± 1·04[a]
	B	75·07 ± 0·06[b]	77·97 ± 0·12[b]	3·08 ± 0·07[a]	12·73 ± 1·75[a]
10 mM, cysteine	A	71·43 ± 0·12[a,c]	74·90 ± 0·10[a]	3·67 ± 0·29[a]	12·40 ± 0·75[a]
	B	74·20 ± 0·46[b]	77·73 ± 0·15[b]	3·83 ± 0·38[a]	13·93 ± 1·55[a]
50 mM, cysteine	A	70·60 ± 0·26[c]	74·63 ± 0·15[a]	4·67 ± 0·29[a]	8·83 ± 1·10[b]
	B	72·50 ± 0·30[d]	76·97 ± 0·06[b,e]	5·00 ± 0·00[a,d]	9·77 ± 0·55[b]
10 mM, NEM	A	n/a	n/a	n/a	n/a
	B	n/a	n/a	n/a	n/a
50 mM, NEM	A	68·50 ± 0·11[e]	74·50 ± 0·10[a]	7·51 ± 0·24[e]	7·75 ± 0·29[b]
	B	68·79 ± 0·19[e]	75·44 ± 0·10[e]	8·79 ± 0·41[f]	9·91 ± 0·05[b]

n/a – data not available

[a,b,c,d,e] Means in a column with the same superscript letters are not significantly different

by disulphide bonds. It is known that Cys[106] and Cys[119] which form a disulphide bond in both β-lg A and B are located on β-sheet strands G and H (Creamer, 1995; Brownlow et al. 1997; Manderson et al. 1998; Qin et al. 1998a, b, 1999) which form part of β-sheet 2 (involved in dimer formation). The free sulphydryl in both variants, Cyst[121], is also located on β-strand H and, thus, also forms part of β-sheet 2. It is not surprising therefore, that introduction of cysteine, which causes a disruption of disulphide bonds and initiates disulphide–sulphydryl interchange reactions, would result in a disruption of the structure in this β-sheet region for both variants. SDS appears not to have had any effect on this β-sheet, but rather appeared to disrupt the structure in the region of the β-sheet not involved in dimer formation (β-sheet 1) (i.e. 1627 cm^{-1} band). Our results further indicated, that when heated in the presence of SDS, both β-lg A and B assumed a secondary structure prior to aggregation, which was very different from that of the native protein and the aggregated protein. This structure may represent a meta-stable state similar to the molten globule state observed in α-lactalbumin (Ptitsyn, 1987; Lala & Kaul, 1992).

At 95 °C, residual bands could still be observed in the vicinity of the 1650 cm^{-1} band in the spectra of both proteins under most of the conditions studied; this may suggest a resistance of these α-helical regions to denaturation or formation of new helical structures as the proteins are heated.

Our results also showed β-lg B to have greater thermal stability than β-lg A when heated in the presence of all four protein perturbing agents. In an earlier paper (Boye et al. 1997) we reported that while some workers (Imafidon et al. 1991; Huang et al. 1994a, b; Prabakaran & Damodaran,

1997) have come to similar conclusions, others have found the A variant to be thermally more stable under a variety of conditions (Gough & Jenness, 1962; Hillier & Lyster, 1979; Dannenberg & Kessler, 1988; Botelho et al. 2000). Work done by Qi et al. (1995) and Nielsen et al. (1996) have shown that the different protein concentrations and scanning rates used by different workers may be responsible for these discrepancies. The results reported in this study, however, seem to indicate that although the B variant was thermally more stable (i.e., had a higher denaturation temperature), its denaturation temperature and secondary structure were affected to a greater extent by the protein perturbants than β-lg A, which may be indicative of a greater frailty in the structure of the B variant. We postulate that, if energy is initially required to dissociate dimers, into monomers prior to actual unfolding of these monomers, then differences in the dimer to monomer ratio might play a role in the energies and temperatures required for denaturation and could, thus, have an influence on the stability of these proteins. Techniques which look at the final states of the denatured molecules may come to different conclusion when compared with techniques which look at the rates of unfolding of these proteins or the initial temperatures they each require to begin to unfold. Also, the source and procedures used in the preparation of the β-lg variants, as well as conditions used during analysis (e.g., pH, ionic strength, type of buffers used; Qi et al. 1995; Nielsen et al. 1996; Holt et al. 1999a, b) can have a significant impact on the physicochemical properties of the proteins. The present work was conducted using commercial unbuffered β-lg samples, and no attempt was made to control the ionic strength of the solutions used. This must be taken into consideration in any data interpretation.

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