Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis

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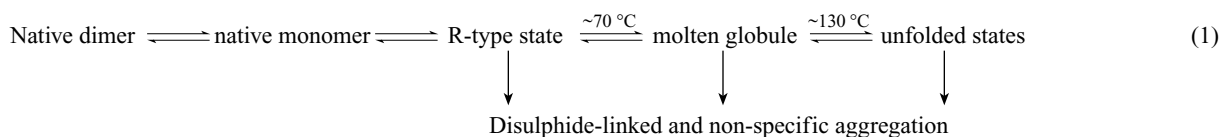*Hannah Research Institute, Ayr KA6 5HL, U.K., †BioSS, Hannah Research Institute, Ayr KA6 5HL, U.K., ‡CLRC Daresbury Laboratory, Warrington WA4 4AD, U.K. and §Centre for Molecular Recognition, University of Edinburgh, Edinburgh EH9 3JR, U.K.

Previous CD measurements of changes in the conformation of β-lactoglobulin at neutral pH as a function of temperature indicated the formation of a molten globule state above approx. 70 °C. New CD measurements are reported at temperatures up to 80 °C with an instrument on the Daresbury synchrotron radiation source which gives spectra of good signal-to-noise ratio down to 170 nm. IR spectra were recorded up to 94.8 °C with a ZnSe circle cell and a single simplified model of the substructure of the amide I′ band was used to give the fractional contents of β-sheet structure unambiguously and independently of the CD spectroscopy. The results of both techniques, however, were in agreement in showing a progressive loss of β-sheet structure with increasing temperature, beginning below the denaturation temperature. Nevertheless, the CD spectroscopy showed a fairly abrupt loss of virtually all the helical conformation at approx. 65 °C. Comparison of the present results with other studies on the molten globule formed at acid pH in the lipocalin family suggests that above 65 °C a partly unfolded state is formed, possibly by destabilization of the intermolecular β-strand I and the loss of the main helix, but it is not a classical molten globule transition.

INTRODUCTION

The thermal denaturation of β-lactoglobulin at neutral and alkaline pH values shows a pronounced dependence on protein concentration [1–5] and proceeds by a multi-step mechanism [5,6] which can be summarized as follows:

$$\text{Native dimer} \rightleftharpoons \text{native monomer} \rightleftharpoons \text{R-type state} \underset{\sim 70\,°C}{\rightleftharpoons} \text{molten globule} \underset{\sim 130\,°C}{\rightleftharpoons} \text{unfolded states} \qquad (1)$$

$$\downarrow \qquad\qquad\qquad \downarrow \qquad\qquad\qquad \downarrow$$

Disulphide-linked and non-specific aggregation

In this model, concentration dependence is introduced through the dissociation of the dimer, coupled with a conformational transition to an R-type state. At physiological concentrations this occurs at 40–55 °C. The R-state differs from the native conformation in the environment of a few side chains [7] and the accessibility of a free thiol group to intermolecular reactions but there is no major adjustment of the protein secondary structure, according to solution CD spectroscopy [8–11], even above the first denaturation transition at approx. 70 °C. The existence of a second denaturational transition at approx. 130 °C was demonstrated by de Wit and Klarenbeek [2] and Paulsson et al. [3], albeit only at the high protein concentrations dictated by the sensitivity of their instruments. From this, together with solution conformational studies, it was suggested [5,6] that between approx. 65 and 130 °C the protein could be described as being in a molten globule [12] state. Subsequently, preliminary one-dimensional ¹H-NMR spectroscopy measurements showed that while there was some decrease in chemical shift dispersion at 60

and 80 °C, there was still considerably more than would be present in a random coil (N. M. Wahlgren, X. L. Qi, C. Holt and T. Drakenberg, unpublished work). Other studies suggest a compact conformation up to 90 °C [13].

The crucial question is whether the regular secondary structure is preserved or even increased on heating, as indicated by the two studies [10,11] where a quantitative interpretation of CD spectra was reported. In a similar study but at acid pH, where the protein is considered to be more stable, a decrease in β-sheet was found on heating [14], which brings into doubt the interpretation of the spectral changes at neutral and alkaline pH values. To obtain CD spectra of better quality, a new instrument on the Daresbury Synchrotron Radiation Source was used. This new instrument can give spectra of good signal-to-noise ratio for aqueous solutions down to 170 nm, which is necessary for all-β proteins to distinguish with confidence between β-sheet and non-regular conformations [15,16].

Fourier transform IR (FTIR) spectroscopy is one of the most commonly used spectroscopic techniques for determining the secondary structure of proteins in solution [17–19] and was used as an independent technique to confirm or refute the CD

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measurements. An earlier study of the resolution-enhanced amide I′ band shape of β-lactoglobulin by Casal et al. [20] and a recent study by Boye et al. [21] showed an apparent loss of substructure as the temperature increased, but the interpretation was qualitative. The objectives were to make a quantitative analysis of the amide I′ band, to compare the results with independent CD measurements and to use both sets of results as a means of testing the hypothesis that β-lactoglobulin exhibits a molten globule state at elevated temperatures.

MATERIALS AND METHODS

Materials

The A variant of β-lactoglobulin was prepared from the milk of a homozygous cow and was purified as described previously [5]. It was at least 95 % pure β-lactoglobulin and contained no denatured or aggregated material as measured by gel filtration on a Sephadex G75 column. The sample of mixed A + B variants of β-lactoglobulin was obtained from Sigma Chemical Co. and was used without further purification in the IR measurements. However, a check was made with the pure A variant to demonstrate that there was no measurable difference by IR spectroscopy in the behaviours of the two samples. The ^2H_2O was Sigma 99.9 atom % grade and other chemicals used in this study were all of analytical grade.

CD spectroscopy

CD spectra were measured from 170 to 260 nm with the CD instrument on station 3.1 of the Daresbury Laboratory Synchrotron Radiation Source. Samples were dissolved in 10 mM phosphate buffer, pH 6.75, at a concentration of 10 mg/ml, and placed in a 10 μm path length Spectrosil cuvette. Spectra recorded in buffer containing an additional 60 mM NaCl were virtually identical apart from a lower signal-to-noise ratio below 180 nm.

The system was calibrated with a 1 mg/ml solution of (+)-10-camphorsulphonic acid, which has a known ellipticity of 33.5 millidegrees at its CD maximum of 290.5 nm. Spectra were analysed for secondary structure content by using the program Selcon [22]. The original set of basis proteins was replaced with a set collected with synchrotron radiation in the wavelength range 170–260 nm.

FTIR spectroscopy

Samples used for FTIR measurements were prepared as 20–100 mg/ml β-lactoglobulin in ^2H_2O containing 30 mM NaCl, and the p^{2}H (direct reading from the pH meter, without correction) was adjusted to 6.7 by small additions of NaO^{2}H or ^{2}HCl.

Samples (5 ml) were placed in a thermostatically controlled ZnSe circle cell with a nominal optical path length of 25 μm at 2000 cm^{-1}. The temperature was varied over the range 26.7–94.8 °C at approx. 5 °C intervals and spectra at 4 cm^{-1} resolution were recorded with a Mattson Galaxy 7000 spectrometer equipped with a linearized mercury/cadmium-telluride (MCT) detector. To decrease further the water vapour lines a wet-air spectrum was subtracted from the ^2H_2O or protein solution spectra to produce an optimally smooth baseline in the region 1750–1850 cm^{-1}.

Analysis of amide I′ band substructure

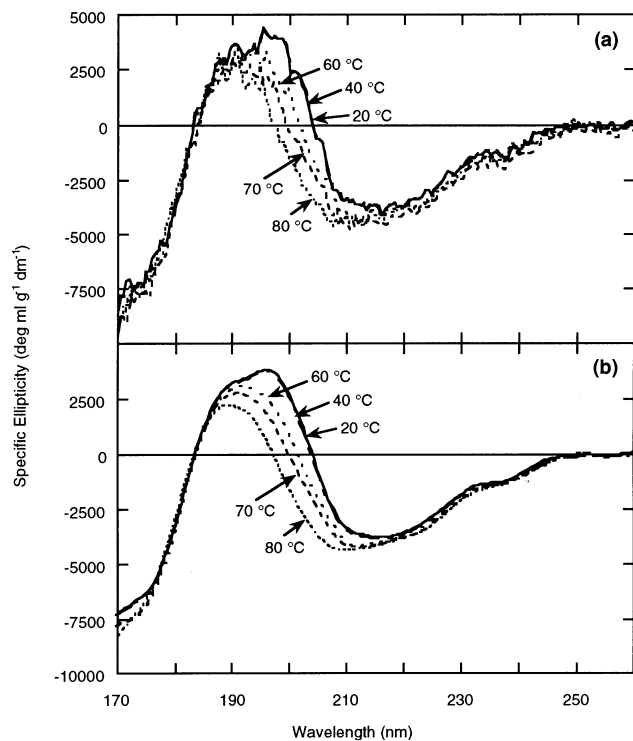The strategy used for the determination of substructure was to estimate a lower bound, n, for the number of components from the second derivative by using the shoulder resolution criterion. Estimates of the true number of components were then obtained by three statistical methods [23–28] and the positions of the peaks sought in the second derivative of the spectrum. Finally a robust, quantitative determination of the substructure was obtained by curve fitting. Second-derivative spectra recorded at the different protein concentrations (20, 50, 75 and 100 mg/ml) and temperatures were carefully examined and only those peaks detected at a given temperature by the shoulder resolution criterion in all the concentrations studied were regarded as subcomponents of the amide I′ band.

For the curve fitting a baseline-subtracted, truncated absorbance spectrum was used. The baseline was drawn from 1750 cm^{-1} to a minimum at approx. 1500 cm^{-1}; these values go well beyond the region required for quantification (approx. 1600–1700 cm^{-1}) to minimize artifacts due to the positioning of the baseline. A single simple model of substructure was used that comprises peaks in the amide I′ region whose initial maxima were at 1635, 1660 and 1680 cm^{-1}. In addition, for temperatures at or above 70 °C a fourth peak at 1614 cm^{-1} was introduced. Below the amide I′ region, peaks were placed at 1571 and 1513 cm^{-1} to fit the main and subsidiary peaks respectively in this region, and if necessary to fit the asymmetry of the 1570 cm^{-1} peak another small peak was introduced at approx. 1550 cm^{-1}. The model was then fitted to the spectrum by using standard non-linear fitting algorithms. Provided that the area fractions of the three (or four above 70 °C) peaks in the amide I′ region were found to be consistently predicted to within ± 3 %, the model was accepted provisionally and a fit was attempted of the spectrum recorded at the next highest temperature, and so on until the highest temperature spectrum had been fitted. The exercise was then reversed in descending order of temperature to verify that the parameters were not pathway-dependent to more than ± 3 %. Simpler models with fewer peaks in the central part of the truncated spectrum were readily seen to be inadequate and no objective evidence was found consistently in the second-derivative spectra for more complicated models. Moreover, if the peak at 1615 cm^{-1}, needed at 70 °C or above, was used at lower temperatures, it resulted in strong correlations between the fitted parameters at the lower temperatures, and path-dependent results.

RESULTS AND DISCUSSION

CD spectroscopy

The CD spectra up to 80 °C are shown in Figure 1; the results of applying the program Selcon [22] to the calculation of secondary structures are shown in Table 1. Essentially the same trends (± 3.3 %) with increasing temperature were found by using the basis set of 16 protein spectra of Toumadje et al. [15]. A comparison of the spectra shown in Figure 1 with those given by Griffin et al. [10] in their Figure 6 reveals a close similarity above 200 nm (their lower wavelength limit) but the quantitative analysis is quite different. Griffin et al. [10], using the CONTIN analysis procedure [29], reported virtually no change in secondary structure with increasing temperature but the results at room temperature are at variance with the X-ray crystal structure (S. Brownlow, J. H. Morais Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polikarpov, A. C. T. North and L. Sawyer, unpublished work) as shown in Table 1. Our spectra, in contrast, give good agreement with the X-ray crystal structure. When the results are compared with the findings of Griffin et al. [10], the present results give a very different picture of the changes induced by heating. The turn fraction remained constant but β-sheet and α-helix decreased and random structure increased as

Figure 1 CD spectra of β-lactoglobulin at pH 6.7 as a function of temperature before (a) and after (b) smoothing with a cubic spline
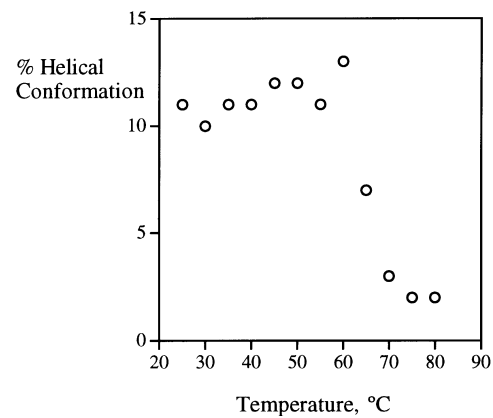

Figure 2 Variation with temperature in the calculated helical fraction of β-lactoglobulin from CD spectroscopy of a 10 mg/ml solution
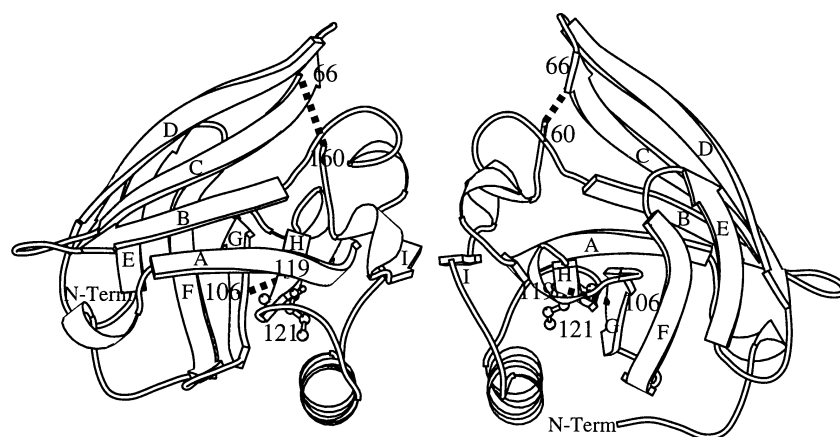
with little dependence on temperature but neither was in agreement with the X-ray crystal structure at room temperature; neither predicted the same trends with temperature as were found from the IR spectroscopy. Clearly, for satisfactory quantification of the CD spectrum of an essentially all-β protein such as β-lactoglobulin, the spectrum must be measured to well below 200 nm [15,16].

The present results therefore indicate an almost complete destruction of α-helix between 60 and 70 °C (Figure 2). The X-ray crystal structures of the lattice X (S. Brownlow, J. H. Morais Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polikarpov, A. C. T. North and L. Sawyer, unpublished work) and Y [30] forms, determined at 1.8 and 2.8 Å resolution (1 Å = 0.1 nm) respectively, show a three-turn helix to be the main source of this secondary structure although in the lattice X structure it is also clear that there are four short helical conformations in surface loops between β-strands (Figure 3). Disruption of the three-turn helix could play a significant part in helping to expose the free thiol, Cys-121, that normally lies completely buried in a cleft between the helix and β-strand H. Above approx. 65 °C the free thiol group becomes exposed and able to form intermolecular disulphide bridges [8]. The results also indicate that there is a decrease in β-sheet between 40 and 60 °C where, under the conditions of the CD experiment, the dimer is expected to have dissociated into monomers [31]. Destabilizing β-strand I, which forms an intermolecular β-sheet with its dyad-related partner in the dimer should decrease the fraction of β-sheet by nearly 4 %.

IR spectroscopy

Both the spectra and their second derivatives were very similar in appearance to spectra published by Casal et al. [20] and Boye et al. [21] by transmission measurements. Moreover, the qualitative appearance suggests a loss of regular secondary structure on heating. The correlated nature of the changes in the spectrum is demonstrated in Figure 4, where contours of constant linear correlation coefficient between absorbance values at the coordinate frequencies for a complete set of measurements as a function of temperature reveal off-diagonal peaks. These peaks are due to broadening on both wings of the amide I′ peak as a result of heating.

The statistical methods of predicting the true number of peaks usually gave no more than one additional peak over those found from the second derivative. Nevertheless, in our hands it proved impossible to do curve fitting with more than four components

Table 1 Comparison of the percentage of secondary structures of β-lactoglobulin from CD spectroscopy with those found by X-ray crystallography, and the effect of heating to 80 °C

The X-ray results are for the lattice X form (S. Brownlow, J. H. Morais Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polikarpov, A. C. T. North and L. Sawyer, unpublished work); columns headed I are results from this work using 170–260 nm; II, results from this work using 200–260 nm; III, results from [10].

	Helix			Sheet			Turn			Random		
X-ray	6.8			51.2			9.9			32.1		
T (°C)	I	II	III	I	II	III	I	II	III	I	II	III
20–25	10	28	16.6	50	22	45.2	8	17	12.3	35	33	25.8
40	11	25	–	51	25	–	7	16	–	31	34	–
60	13	25	20.3	41	27	38.5	10	14	16.0	36	34	25.1
70	3	25	23.3	43	28	45.3	7	14	11.8	47	33	19.6
80	2	21	18.2	42	23	47.8	6	18	11.6	50	37	22.3

the temperature increased, suggesting a direct conversion of regular into irregular structure.

Results were also analysed by the CONTIN program with both the full wavelength range and spectra truncated at 200 nm. With the whole spectrum a very good agreement was found with the Selcon analysis at temperatures of 70 °C or above. Below that temperature, the CONTIN analysis continued to agree with the Selcon program for helix and β-sheet but the turn conformation was too large at 20 ± 1 %, mainly at the expense of the fraction of the 'other' conformation (i.e. identified here as non-regular conformation). When the truncated spectra were analysed, the Selcon and the CONTIN methods yielded very similar results

Figure 3   Representation of the X-ray crystal structure of the lattice X form of β-lactoglobulin showing the dimer interface and the positions of the cysteine residue and two cystine bridges
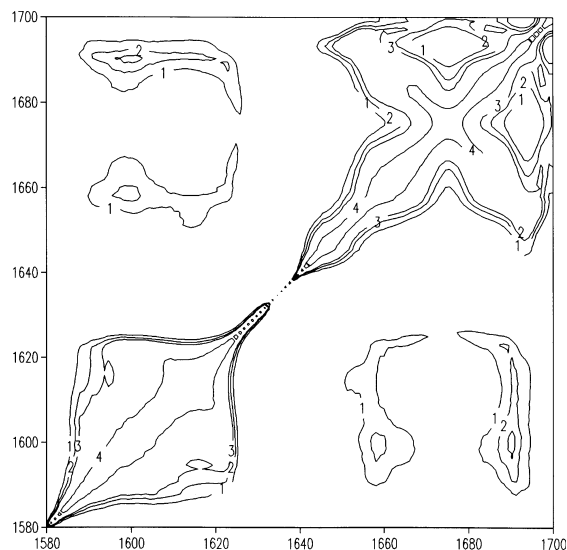
Figure 4   Contour plot of correlation coefficients relating the changes in absorbance at wavenumbers in the amide I′ region when β-lactoglobulin was heated from 26.7 to 94.8 °C

Contours 1–4 are for correlation coefficients of 0.985, 0.990, 0.993 and 0.999 respectively.

in the amide I′ region without placing additional constraints on the fitting parameters or requiring the result to agree with X-ray crystallography or CD spectroscopy. An additional component in the amide I′ region, although justified in theory, introduced strong correlations between fitting parameters. Because the object was to derive an independent estimate of secondary structure, only information recoverable from the IR spectrum could be used.
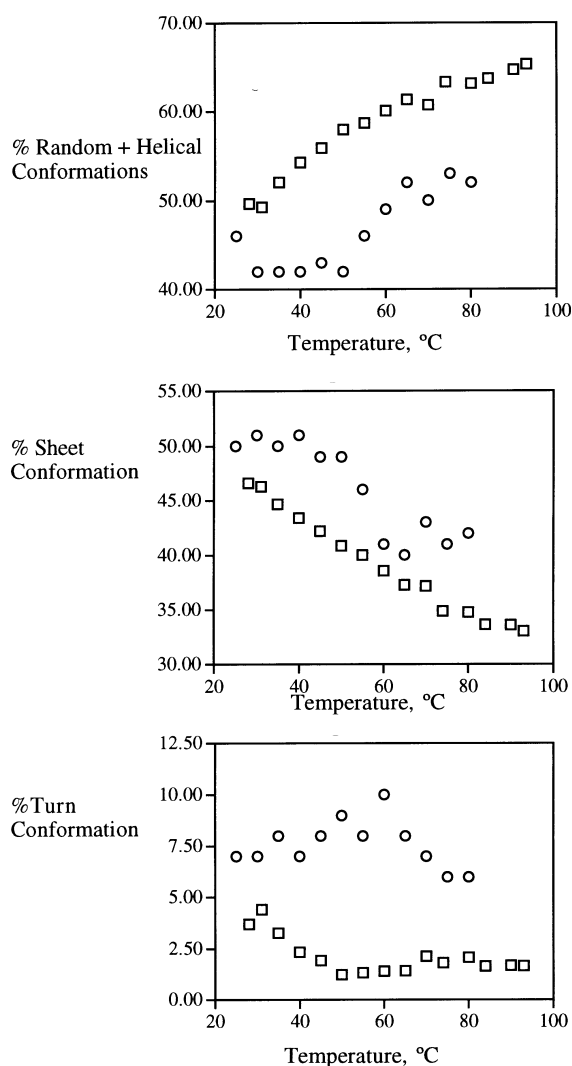
The question of the number of bands to be expected in the amide I′ region has been the subject of many theoretical and experimental studies (reviewed in [17–19]). Byler and Susi [32] identified 11 different bands by deconvolution of the spectra of 16 globular proteins. Some individual proteins, including β-lactoglobulin at p^{2}H 7, were fitted by up to nine subcomponents identified from the second derivative or from Fourier self-

deconvolved spectra. Typically, components in the range 1624–1637 cm^{-1} were assigned to the β-sheet conformation, bands at 1654 ± 2.6 and 1645 ± 1.5 cm^{-1} were identified with the α-helix and random conformation respectively, and frequencies 1663–1694 cm^{-1} to turns and bends. Complicating the latter is the possibility of a significant contribution from the normally weaker high-frequency band of the β-sheet at approx. 1675 ± 2.6 cm^{-1}. In addition, the appearance during denaturation of some proteins of a band at 1615 cm^{-1} has been explained as due to the strong hydrogen-bonding in intermolecular β-sheet formation. Although this was easily detected and quantified, it amounted to no more than 1–2 % of the total secondary structure and could not compensate for the general decrease in intramolecular β-sheet on heating.

Nevertheless, physically meaningful results can be derived from the curve fitting. The high-wavenumber band can be identified with the turn conformation (plus some possible contribution from the high-frequency β-sheet band); the middle band can be identified with the random plus α-helical conformations and the low-wavenumber band and the high-temperature band at approx. 1614 cm^{-1} can be identified with the intramolecular and intermolecular β-sheet conformations respectively. At room temperature, the percentages can be compared with the X-ray crystal structure (in parentheses) giving for β-sheet 46.6 % (51.2 %), random plus helical 49.7 % (38.9 %) and turns 3.7 % (9.9 %). A comparison of the IR and CD results in Figure 5 demonstrates that the central band in the fitting of the IR spectrum gives an overestimate of helix plus random conformations by an average of 9.7 %, and the two side bands underestimate the turns and sheet conformations by 5.3 % and 5.0 % respectively. Such agreement can be considered quite satisfactory given the accepted accuracy of IR determinations of secondary structures to be in the order of ± 5 % at best.

The molten globule state

The classical transition from a native to a molten globule state proceeds by a co-operative mechanism to produce a state that is compact and has a largely native secondary structure but in which residue side chains experience a more symmetrical environment [12,33]. The concept of a thermodynamically stable state on the folding pathway has proved attractive in explaining denaturation processes such as those induced by pH adjustment [34–36], disulphide-reducing agents [36,37], heat treatment

Figure 5   Comparison of the proportions of calculated secondary structures in β-lactoglobulin from CD ($\bigcirc$) and FTIR ($\square$) spectroscopy as a function of temperature

expose and destabilize the elements of secondary structure nearest to the homodimer interface (Figure 3).

A recent report [40] describes the partial structure of the so-called molten globule state of β-lactoglobulin-B formed at pH 2, as revealed by NMR spectroscopy. Assignments were built up from the Phe[105]-Cys-Met[107] sequence of β-strand G (Figure 3), which is linked by a disulphide bridge to Cys[119] on β-strand H and forms a particularly well defined part of the lattice X crystal structure with low mean residue temperature factors (S. Brownlow, J. H. Morais Cabral, R. Cooper, D. R. Flower, S. J. Yewdall, I. Polikarpov, A. C. T. North and L. Sawyer, unpublished work). Other assignments were made in β-strands A, E and F, all comprising part of the calyx-like fold that defines the hydrophobic pocket in the lipocalins (Figure 3). Altogether, the evidence implies that the molten globule state at pH 2 is really one in which there is a region of local disorder and increased flexibility but a resistant core of β-sheet forming part of the eight-strand calyx-like fold. Such a description is consistent with the partly unfolded state described here above 65 °C.

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[5,6,34], denaturants [33,38], dilution [35] and salt [34–36]. A molten globule state has recently been shown to be possible in all-β proteins [39] and can exist after the dissociation of homodimers [35,36]. In proteins containing both cystine and cysteine it has been observed that thiol–disulphide interchange reactions can occur in the molten globule state, resulting sometimes in almost randomized interchain bridges [37]. The proposal that β-lactoglobulin forms a molten globule state at 65–70 °C is therefore neither unreasonable nor surprising and is useful in explaining aspects of the intermolecular aggregation observed at these temperatures [8]. It is clear, however, that if the term molten globule is to be used to describe the state of β-lactoglobulin at these temperatures, it must be recognized that according to the CD and IR spectroscopy, a considerable fraction of the native regular secondary structure has been lost by 70 °C: virtually all the α-helix and up to a fifth of the β-sheet. The suggestion is that the state of β-lactoglobulin above 65 °C is not that of a classical molten globule, at least at low concentrations where dissociation of the dimer occurs below the denaturation temperature [5], to

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