Differences in the Processes of β-Lactoglobulin Cold and Heat Denaturations

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ABSTRACT The changes in β-lactoglobulin upon cold and heat denaturation were studied by scanning calorimetry, CD, and NMR spectroscopy. It is shown that, in the presence of urea, these processes of β-lactoglobulin denaturation below and above 308 K are accompanied by different structural and thermodynamic changes. Analysis of the NOE spectra of β-lactoglobulin shows that changes in the spin diffusion of β-lactoglobulin after disruption of the unique tertiary structure upon cold denaturation are much more substantial than those upon heat denaturation. In cold denatured β-lactoglobulin, the network of residual interactions in hydrophobic and hydrophilic regions of the molecule is more extensive than after heat denaturation. This suggests that upon cold- and heat-induced unfolding, the molecule undergoes different structural rearrangements, passing through different denaturation intermediates. From this point of view, cold denaturation can be considered to be a two stage process with a stable intermediate. A similar equilibrium intermediate can be obtained at 35°C in 6.0 M urea solution, where the molecule has no tertiary structure. Cooling or heating of the solution from this temperature leads to unfolding of the intermediate. However, these processes differ in cooperativity, showing noncommensurate sigmoidal-like changes in efficiency of spin diffusion, ellipticity at 222 nm, and partial heat capacity. The disruption with cooling is accompanied by cooperative changes in heat capacity, whereas with heating the heat capacity changes only gradually. Considering the sigmoidal shape of the heat capacity change an extended heat absorption peak, we propose that the intermediate state is stabilized by enthalpic interactions.

INTRODUCTION

The study of protein unfolding induced by decreasing temperature, i.e., cold denaturation, is expected to shed light on the mechanism and pathway of protein folding and the nature of intermediate states. The detection of intermediates during protein folding and unfolding is difficult because they are usually only transiently populated (Kuwajima, 1989; Kim and Baldwin, 1990; Hughson et al., 1989; Freire and Biltonen, 1978). However, experimental evidence indicates that partly folded states can be stable at low temperatures (Beringer and Fink, 1982, 1988; Privalov et al., 1986a; Griko et al., 1988; Barick and Baldwin, 1993). They might in fact be detectable under conditions of cold denaturation, when the stability of individual elements of secondary structure are high even though hydrophobic interactions promote cold denaturation.

Commonly observed denaturational intermediates can be characterized by the absence of unique tertiary structure and weak long-range interactions (Kuwajima, 1989; Baum et al., 1989; Dobson et al., 1990; Baldwin, 1990; Dill and Shortle, 1991; Ewbank and Creighton, 1991; Ptitsyn, 1992). Therefore, it seems that measurements of spin diffusion can provide additional information about intramolecular changes after the loss of specific ordered structure.

It has been shown that β-lactoglobulin's cold denaturation in the presence of urea is more complicated than its heat denaturation and does not exclude the presence of intermediates (Schellman, 1958; Pace et al., 1968; Casal et al., 1988; Griko et al., 1992). In this paper, we present results of a comparative study of the heat and cold denaturation of β-lactoglobulin using NMR spectroscopy, CD, and scanning calorimetry, demonstrating that these two processes differ by the relative significance of intermediate states during the thermal transition.

MATERIALS AND METHODS

β-Lactoglobulin A was obtained from bovine milk as described previously (Armstrong et al., 1967). Protein homogeneity was checked by electrophoresis using native and denaturing conditions (Laemmli, 1970).

The calorimetric measurements were carried out using a DASM-4A microcalorimeter over a broad temperature range, including subzero temperatures, which were achieved by supercooling the solutions as described (Privalov et al., 1986b). In both the heating and cooling experiments, scanning rates of 0.5–1.0 K/min were used. All measurements were made in 0.1 M KCl-HCl buffer, pH 2.0. The urea concentration range was 0–7 M. The protein concentration in the calorimetric experiments was 3–5 mg/ml. The partial specific heat capacity of β-lactoglobulin was determined and analyzed according to the procedure described earlier (Privalov and Potekhin, 1986).

The ^{1}H-NMR experiments were carried out at an operating frequency of 400 mHz on a Brucker WM-400 spectrometer interfaced to an Aspect 2000 computer system. The concentration of the protein in the NMR experiments was 3–6 mg/ml, determined spectrophotometrically using extinction coefficient $E_{278}^{1\,cm,1\%} = 9.6$ (Byler et al., 1983).

The NOE difference spectra present the spectra for which the pool of methyl resonances centered at 0.89 ppm (each odd spectrum) was saturated minus the spectra with saturation in the range outside the spectrum (each even spectrum). The decoupler power was 40 dB (the full decoupler power −0.2 W). The saturation time was 1 s, as is regularly the case for NOE experiments when the saturation in a globular protein reach is 80% in the

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1.3–0.5 ppm region of the spectrum. The delay before starting the 90 pulse was 0.1 s (Akasaka, 1983; Noggle et al., 1971). The accumulation number depended on concentration and varied between 576 and 980. The ^{1}H-NMR spectrum was obtained at 50–100 accumulations. Temperature stability in the probe-head was provided by a B-VT1000 temperature controller with an accuracy of $\Delta T = 1$ K. All spectra were obtained in D_2O. Urea used in these experiments was twice recrystallized from deuterium oxide solution (99.9%). To avoid kinetic effects of conformational changes in β-lactoglobulin, all measurements at the low temperatures were started 40 min after stabilization of temperature.

The NOE difference spectra were the result of Fourier transformation of accumulated differences between each on resonance and off resonance of frequency induction decay (FID). At the time of saturation, the NOE spectra of a globular protein practically loses specificity, becoming similar to the ^{1}H-NMR spectrum. This spectrum can be characterized then as that of spin diffusion. Because spin diffusion efficiency correlates with interproton distances and internal motion, it might be considered to be a measure of the rigidity of a molecule.

To estimate the efficiency of the spin diffusion, the integral intensity of signals in the region of absorption of aromatic protons (7.6–6.4 ppm) and methyl protons of aliphatic groups (2.7–1.3 ppm) in NOE difference and ^{1}H-NMR spectra were measured relative to the integral intensity of proton signals of the methyl pool in the 1.3–0.5 ppm region. All spectra were normalized to this last signal. Suppose that under the described conditions, the ^{1}H-NMR spectrum can be considered as the spectrum of spin diffusion with $\eta = -1$ at $\omega^2\tau^2 \gg 1$, and that deviation of the spin diffusion from 1 can be estimated for the different ranges of the spectrum by measuring the ratio of the integral intensity for a definite range in the NOE spectrum and to that of the same range in the ^{1}H-NMR spectrum. It is convenient to call this ratio G for characterization of the efficiency of spin diffusion and physical rigidity of the protein molecule. Under the experimental condition chosen, this corresponds to measuring the stationary NOE at $\omega^2\tau^2 \gg 1$, which is true for macromolecules (ω is the resonance frequency; τ is the correlation time) (Akasaka, 1983; Kalk et al., 1976; Wagner et al., 1979). Because the mobility of the aromatic groups in most compact globular proteins is more limited than that of the aliphatic groups, comparison of this ratio for both aliphatic (G_{al}) and aromatic (G_{ar}) ranges of the spectrum allows judgment of the rigidity, and interaction between different parts, of the polypeptide. Most of the aromatic residues are involved in formation of hydrophobic clusters in the protein molecule. Thus, the efficiency of spin diffusion measured in corresponding ranges of spectra could characterize their stiffness. In this paper, the ratio was determined as an integral value for signals of aromatic amino residues (7.5–6.5 ppm), which permits assessment of the stability of hydrophobic clusters in molecule and aliphatic amino acid residues (2.7–1.4 ppm).

Consideration of this ratio as a probe for estimating the compactness and intramolecular mobility of the polypeptide chain could be especially important for analyses of the conformational changes between intermediate states and unfolded state in proteins. In this study, we have used the suggested parameter (G) together with CD and calorimetric data to compare intramolecular changes in denatured states β-lactoglobulin after heat and cold denaturation, as well as in different urea solutions.

RESULTS

Previously, we showed that the most convenient condition for observation of both heat and cold denaturation of β-lactoglobulin is a buffered solution containing 4.0–4.4 M urea at pH 2.0 (Griko and Privalov, 1992). Under these conditions, the protein exists as a monomer and is completely reversible after either heat or cold denaturation (see also Pace and Tanford, 1968).

Fig. 1 represents the ^{1}H-NMR spectrum of β-lactoglobulin at pH 2.0, 4.0 M urea, and various temperatures. The spectra at 308 K show many resonances of unexchanged amide protons, as well as a number of signals in other regions of the spectrum. These serve as indicators of the unique tertiary structure of the globular protein. Decreasing the temperature from 308 K leads to a decrease in intensity of the signals characterizing the native protein structure (secondary chemical shifts; Dobson et al., 1984). These signals completely disappear by 270 K. This result implies drastic change in the conformation of β-lactoglobulin (i.e., disruption of its unique tertiary structure). The reverse occurs upon heating to 308 K. High reproducibility of the spectra after cooling and subsequent heating to 308 K indicates that the process of cold denaturation of the β-lactoglobulin is completely reversible. Further heating to 350 K leads to spectral changes similar to those observed in the cooling experiments. The disappearance of secondary chemical shifts suggests that β-lactoglobulin is denatured at temperatures above 335 K.

From the original calorimetric curve presented in Fig. 2, it follows that the cooling of the β-lactoglobulin is accompanied by an extensive release of heat, which occurs in the temperature range of disruption of β-lactoglobulin structure, as observed by NMR. In contrast, subsequent refolding to the native protein structure after cold denaturation is accompanied by heat absorption. The cold denaturation and renaturation are shifted in the temperature scale because of the slow kinetics of these processes at low temperatures. As result of this temperature shift, the values of these two heat effects are different. The negative enthalpy of cold denaturation is considerably larger than the positive enthalpy of the renaturation because it proceeds at temperatures farther from T_{inv} where the enthalpy of denaturation becomes zero (Griko and Privalov, 1992).

The second heat absorption peak above 308 K corresponds to a disruption of the native structure, as is typical of heat denaturation. The area of this peak is the calorimetric enthalpy of heat denaturation ($\Delta H_{cal} = 140$ kJ/mol) and is in good agreement with the van't Hoff enthalpy ($\Delta H_{vh} = 155$ kJ/mol) determined from the sharpness of the peak. This suggests that the heat denaturation of β-lactoglobulin in the presence of 4.0 M urea represents a two-state transition without stable intermediates, or with a population of intermediates that is extremely low. A similar analysis of the denaturation processes at low temperatures shows that the calorimetrically measured enthalpy of β-lactoglobulin cold denaturation ($\Delta H_{cal} = -263$ kJ/mol) is larger than the van't Hoff enthalpy ($\Delta H_{vh} = -171$ kJ/mol). The deviation between these two quantities exceeds 35%. This means that the disruption of β-lactoglobulin structure induced by temperature decreasing is not a two-state transition, i.e., some stable intermediate states are present during the transition between native and denatured states.

Fig. 3 presents the NOE difference spectra of the β-lactoglobulin spin diffusion as observed in 4.0 M urea at the considered temperatures. It is known that at long saturation in any region of the protein spectrum in NOE experiments, the spectrum loses specificity and becomes very similar to the NMR spectrum of β-lactoglobulin obtained

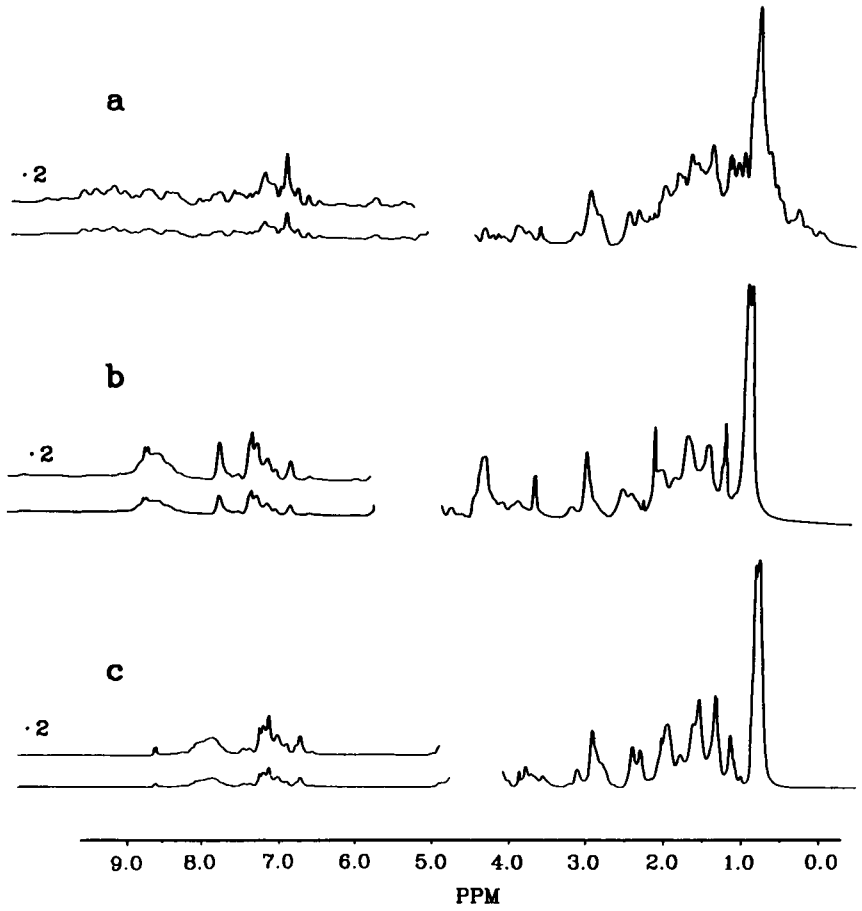

FIGURE 1 ^{1}H-NMR spectrum of β-lactoglobulin in 0.1 M KCI-HCI solution pH = 2.0 containing 4.0 M urea at different temperatures: (*a*) 308 K; (*b*) 270 K; (*c*) 353 K.

under the same conditions (Akasaka, 1983; Kutyshenko, 1991). Decreases in the integral intensity of signals in NOE difference spectrum, which are accompanied by a disappearance of the secondary chemical shifts with decreasing temperature, indicate increases in the interproton distances between groups in the protein (Akasaka, 1983; Kalk et al., 1976). Nevertheless, at 267 K the NOE resonances are still present. It is also remarkable that the spin diffusion does not disappear completely in 6.0 M urea solution, where the NMR spectrum of β-lactoglobulin at temperature of maximal stability (308 K) shows practically no secondary chemical shifts in the high-field portion of the spectrum. This suggests the absence of tertiary structure under these conditions. (Fig. 3 *c*). The near-UV CD spectrum of β-lactoglobulin also indicates the absence of asymmetric environments of aromatic amino acid side chains (Fig. 4). Thus, in 6.0 M urea, β-lactoglobulin is denatured but not completely unfolded.

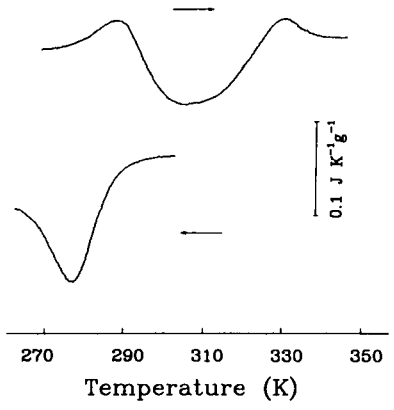

FIGURE 2 Original scanning microcalorimetric recording obtained upon cooling and consecutive heating of β-lactoglobulin in 4.0 M urea solution of 0.1 M KCl-HCl buffer, pH 2.0. The direction of temperature change is indicated by arrows.

Fig. 5 represents the temperature dependence of the parameters G_{ar} and G_{al} of β-lactoglobulin as a function of urea concentration. These data allow one to judge the changes in efficiency of spin diffusion as an order parameter (G) upon temperature-induced conformational changes (see Materials and Methods). In the absence of urea, β-lactoglobulin undergoes only heat denaturation (see Fig. 4). Increasing the temperature from 308 K elicits a monotonic decrease of the G parameter in both the aromatic and aliphatic regions of the spectrum. Their values approach one another, and at a temperatures above the transition temperature they become similar. In the presence of 4.0 M urea, the protein can be denatured upon cooling or heating (Figs. 1–4). Under these conditions, the parameter G exhibits similar behavior upon increases in temperature (Fig. 5 *b*). As before, G_{ar} and G_{al} reach a similar value at temperatures higher than that of heat denaturation. Upon decreasing the

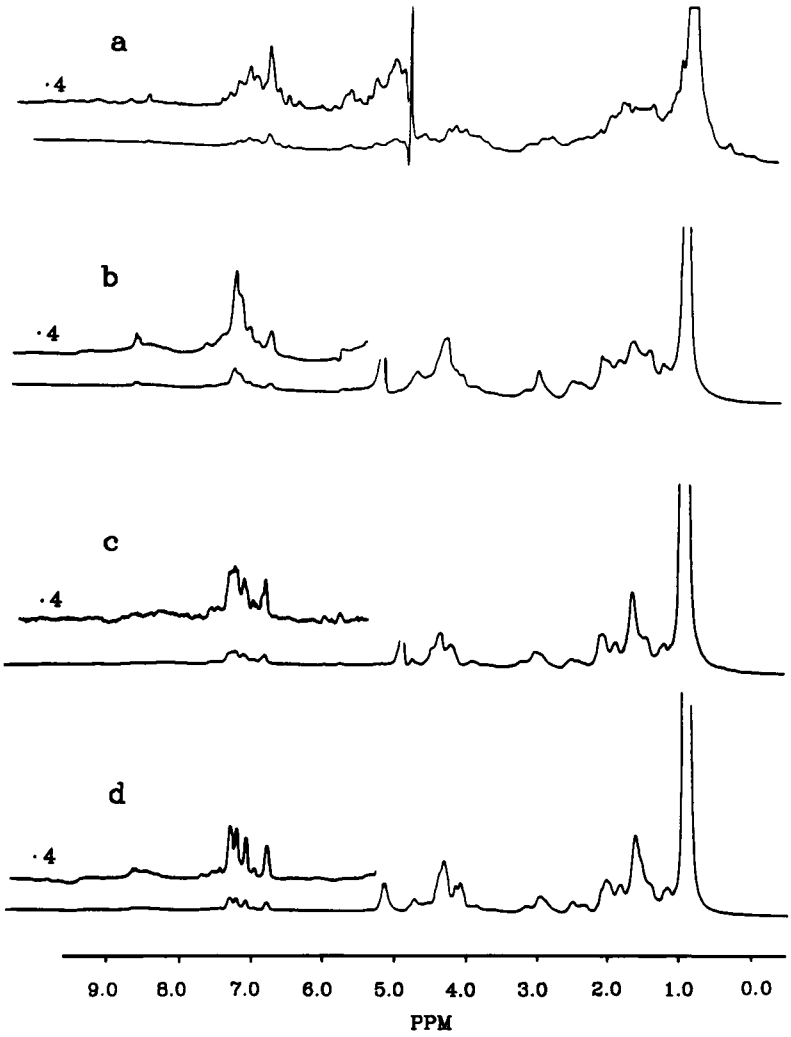

FIGURE 3 NOE spectrum of β-lactoglobulin in 4.0 M urea solutions of 0.1 M KCl-HCl buffer, pH 2.0: (*a*) at 308 K; (*b*) at 267 K, and 6.0 M urea solution: (*c*) at 308 K; (*d*) at 267 K.

temperature from 308 K, the parameters G_{al} and G_{ar} increase monotonically down to 283 K. In the temperature range of cold denaturation, both functions suddenly change their tendency to increase and remain constant down to 273 K.

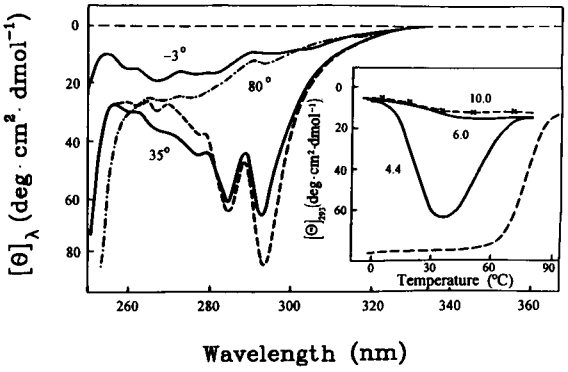

FIGURE 4 CD spectra of β-lactoglobulin in the near UV-region in a solution containing 4.0 M urea at different temperatures and the temperature dependence of the ellipticity at 293 nm, and different urea concentrations. The dashed line shows β-lactoglobulin ellipticity in the urea free solution.

Fig. 5 *c* shows the temperature dependence of the G_{al} and G_{ar} parameters of denatured β-lactoglobulin in 6.0 M urea. Note that the values of these functions are significantly lower in 6.0 M urea than in 4.0 M at 308 K, under which conditions the protein displays a unique tertiary structure. However, the behavior of these parameters resembles that observed after heat and cold denaturation for β-lactoglobulin in 4.0 M. The maximal difference between G_{al} and G_{ar} parameters in this solution is at 310 K. At higher and lower temperatures, they become similar in value to the completely unfolded β-lactoglobulin in 10.0 M urea (Fig. 5 *d*).

Fig. 6 shows the temperature dependence of the partial heat capacity of β-lactoglobulin. Remarkably, the change in heat capacity associated with cold denaturation is larger than that of heat denaturation (Fig. 6 *b*). Because the effect of urea on the heat capacity does not depend noticeably on temperature (Makhatadze and Privalov, 1992), the increase in solvent-accessible surface area upon denaturation appears to be different for heat and cold denaturation processes. Fig. 5 *c* suggests that this result also pertains to the protein denatured in 6.0 M urea solution. This heat capacity change is very unusual for denatured proteins, which usually show near linear character (Privalov et al., 1989).

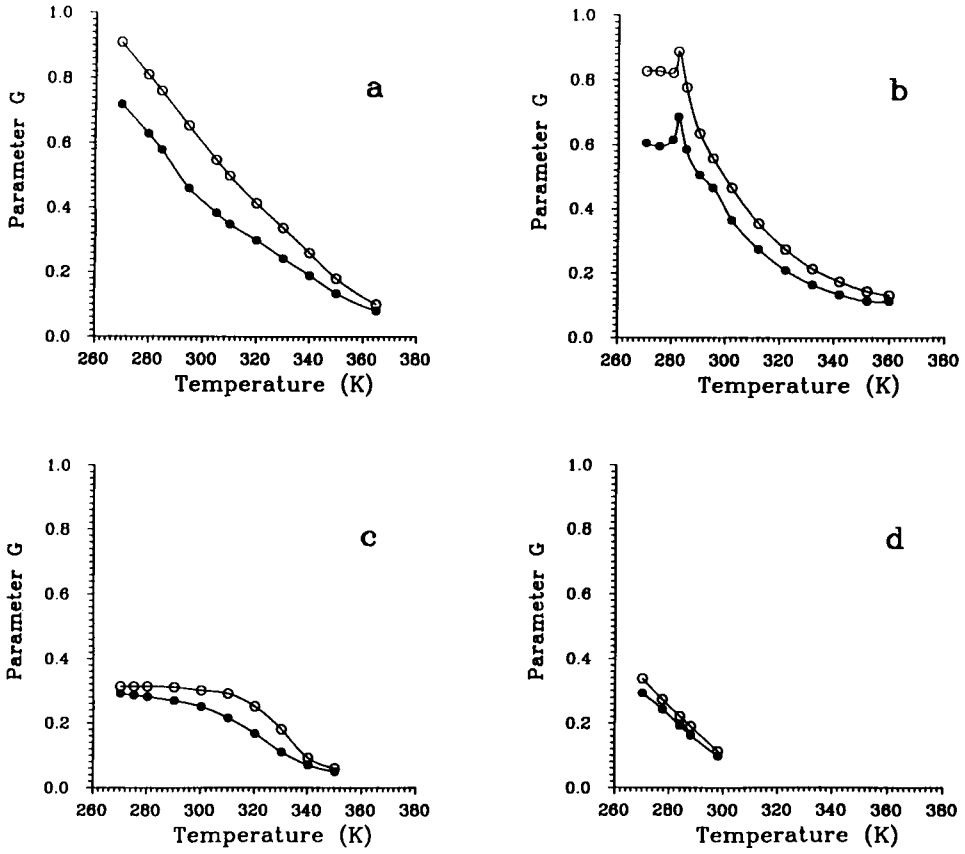

FIGURE 5 Temperature dependence of the parameter of rigidity (G) of β-lactoglobulin for aromatic (○) and aliphatic (●) protons in solutions containing 0 M urea (a), 4.0 M urea (b), 6.0 M urea (c), and 10.0 M urea (d).

DISCUSSION

The results obtained here show that the denatured states of β-lactoglobulin reached after heat and cold denaturation differ in their structural and thermodynamic characteristics. The partial specific heat capacity of the native β-lactoglobulin is 1.37 ± 0.07 J/g K at 25°C. The heat capacity does increase considerably upon heat denaturation, but it does not reach the value expected for the unfolded polypeptide chain immediately after the thermal denaturation. Instead, increasing the temperature gradually raises the heat capacity of the denatured β-lactoglobulin, so that above 110°C it becomes very close to that of the unfolded state (Fig. 6 b). Because the heat capacity change of protein unfolding is proportional to changes in nonpolar and polar surface areas, the obtained value of the heat capacity shows that in the heat-denatured protein, not all amino acid residues are exposed to the solvent. The increase in temperature leads to more complete disruption of the residual structure and hydration of hydrophobic groups.

Changes in the efficiency of spin diffusion of the denatured protein, as well as their values at specific temperatures, suggest correlated intramolecular changes upon heat and cold denaturation.

The monotonic decrease of the G-parameter, which measures the efficiency of spin diffusion (see Materials and Methods) with rising temperature, indicates an increase in different kinds of motion in the protein molecule: motion of specific side chains, sequential motion, and motion of the molecule as a whole (Akasaka, 1983; Kutyshenko and Khechinashvili, 1989). In contrast, the decrease in temperature with a concomitant decrease of dissipative forces

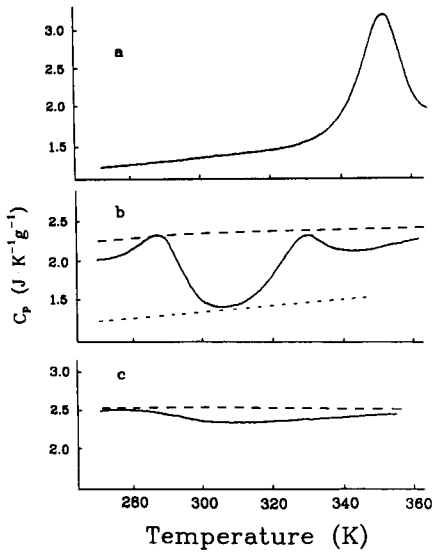

FIGURE 6 Temperature dependence of the β-lactoglobulin partial heat capacity in 0.1 M KCl-HCl solutions, pH 2.0 with different urea concentration. (a) 0 M, (b) 4.0 M, (c) 6.0 M. The dashed line shows the expected heat capacity change for the complete unfolding of β-lactoglobulin calculated from its amino acid sequence. The dotted line shows the heat capacity of native β-lactoglobulin.

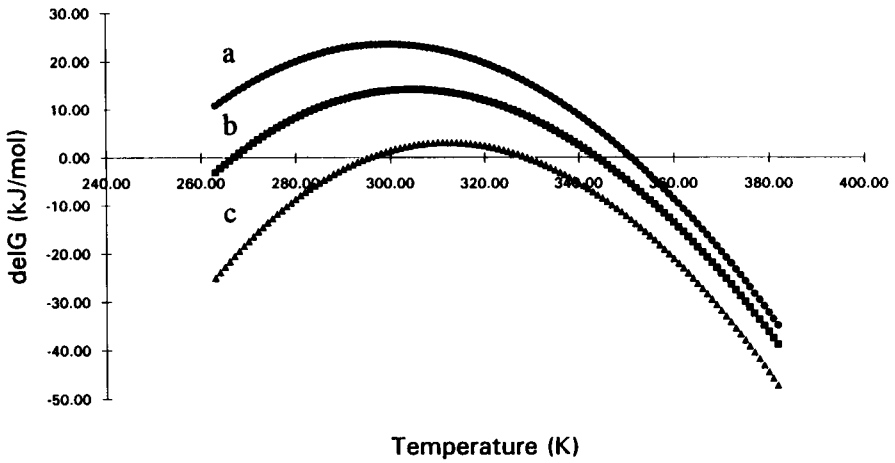

FIGURE 7 Temperature dependence of the Gibbs energy differences of the native and denatured β-lactoglobulin in 0.1 M KCI-HCI buffer, pH 2.0 with different urea concentrations. (*a*) Without urea; (*b*) 2.0 M; (*c*) 4.0 M. Values were calculated using calorimetrically obtained parameters:

(*a*)	(*b*)	(*c*)
ΔH = 312.2 kJ mol^{-1} K^{-1}	ΔH = 243.0 kJ mol^{-1} K^{-1}	ΔH = 120.8 kJ mol^{-1} K^{-1}
T_d = 351 K	T_d = 343.8 K	T_d = 329 K
ΔC_p = 5.58 kJ mol^{-1} K^{-1}	ΔC_p = 5.82 kJ mol^{-1} K^{-1}	ΔC_p = 6.9 kJ mol^{-1} K^{-1}

increases the rigidity of the regular conformation in the native protein. If spin diffusion does indeed reflect the compactness of the molecule, then it should likewise correlate with conformational entropy. Thus, the parameter G might be a good probe of the degree of unfolding. It is remarkable that the differences between G_{ar} and G_{al} for β-lactoglobulin correlate with the structural integrity of the unfolded molecule. Disruption of native β-lactoglobulin structure upon heat denaturation is accompanied by a drawing together of all motions of the constituent structural parts of the molecule (G_{al} is equal or close to G_{ar}). The conformational entropy becomes large and similar in magnitude for aromatic and aliphatic groups of the molecule. Above 110°C, where β-lactoglobulin is unfolded according to the value of the heat capacity and the ellipticity in far UV region, G_{al} is equal to G_{ar}. For β-lactoglobulin unfolded in 10 M urea, G_{ar} is close to G_{al} in the considered temperature region (Fig. 5). Thus, detectable differences between the efficiency of spin diffusion for aromatic and aliphatic amino acids after heat denaturation transition correlate with the value of the heat capacity, showing that heat-denatured β-lactoglobulin is not completely unfolded below 110°C. The difference between G_{ar} and G_{al} is maximal at temperatures where the native state is stable. The thermal stability of the protein depends on Gibbs energy, $\Delta_d G(T)$. For two-state processes, $\Delta_d G(T)$ can be calculated from experimentally obtained values of ΔC_p, T_d, and T_{inv}. using the following expression:

$$\Delta_d G(T) = \Delta_d H(T_d) - T\Delta_d S(T_d) = \Delta C_p \cdot \left[\frac{T \cdot T_{inv}}{T_d} - T_{inv.} + T \ln \frac{T_d}{T}\right]. \quad (1)$$

Here ΔC_p is the difference in heat capacity between the native and denatured states of β-lactoglobulin, T_d is the temperature of denaturation, and $T_{inv.}$, is the temperature at which the denaturation enthalpy is zero. As seen in Fig. 7, $\Delta_d G(T)$ has an extremum at room temperatures, as determined by the condition

$$\left(\frac{\partial \Delta_d G}{\partial T}\right)_{T_{max}} = -\Delta_d S(T_{max}) = 0. \quad (2)$$

It is remarkable that the T_{max} shifts toward higher temperatures with increasing concentrations of urea. The $\Delta_d G(T)$ function crosses the zero level at two different temperatures, which correspond to the temperatures of heat and cold denaturation.

At temperatures below T_{max} the decrease of protein stability proceeds with increases in G_{al} and G_{ar}. These latter functions reach their maximal values at the temperature of the cold denaturation, which can be determined from the equation (Privalov et al., 1986)

$$T'_d = \frac{T_d^2}{3T_d - 2T_{inv.}}. \quad (3)$$

As the temperature decreases to 280 K, these functions decrease sharply; below this temperature, they do not change significantly. It is interesting to note that the disruption of the native protein structure upon cold denaturation does not lead to a convergence of the G_{ar} and G_{al} parameters, as was observed in the case of heat denaturation (Fig. 5). At present, we cannot find a reasonable explanation for this phenomenon. We suggest, however, that the result is both the decrease in entropy upon disruption of the protein tertiary structure induced by cooling and the simultaneous large-scale changes in the molecular structure. This follows from the

decrease of the G parameter, which at cold denaturation changes more drastically than at heat denaturation.

It is also in interesting that the same type of changes in G_{al} and G_{ar} also take place under conditions where β-lactoglobulin has no detectable tertiary structure (Schellman, 1958; Griko and Privalov, 1992) (Fig. 5 *c*). The spin diffusion parameters appear to indicate that β-lactoglobulin in 6.0 M urea at 308 K is not completely disordered, but still has some hydrogen bonds and hydrophobic intramolecular interactions. Under these conditions, the values of G_{al} and G_{ar} are different. However, they becomes close to one another at extremely high and low temperatures, as has been observed for β-lactoglobulin above the temperature of heat denaturation in 4.0 M urea, under which conditions the protein appears to be completely unfolded. The maximal differences between G_{al} and G_{ar} in the range 300–320 K correspond to the maximal ordered topology of the molecule under these conditions. The probability of the formation of the native structure by denatured protein molecule is highest in this temperature region. This is confirmed by appearance of some secondary chemical shifts in the 6–5 and 0.8 ppm regions of the spectrum at 308 K in 6.0 M urea solution.

Taken together, these data indicate that denatured β-lactoglobulin exhibits extensive residual hydrophobic and hydrophilic interactions. Although the structure stabilized by those interactions is much less fixed than in the native protein, it nevertheless displays a flexibility and rigidity (parameter G) similar to that of the native molecule. The conformational changes at high and low temperatures accompanying the increase in heat capacity and decrease in the spin diffusion can be classified as "unfolding." This process is characterized by a large increase in entropy caused by an increase in freedom of motion of the protein in unfolded conformation (Lumry et al., 1969). In contrast, if β-lactoglobulin does not exhibit low temperature-induced unfolding, as in the absence of urea (Figs. 4, 5 *a*, and 6 *a*), its flexibility might be caused by a trivial temperature factor (Mirsky and Pouling, 1936). In this case, the monotonic increase of the efficiency of spin diffusion (increase of parameter G with temperature decrease) is determined by the temperature dependence of solvent viscosity and appears to be similar in character for the folded and unfolded polypeptide chain (Fig. 5, *a* and *d*).

It is notable that the conformational changes in β-lactoglobulin upon cold denaturation are different in cooperativity and magnitude from those induced by heat denaturation. The decrease of ellipticity at 222 nm with decreasing temperature indicates a reduction of the residual secondary structure in the denatured β-lactoglobulin (Fig. 8). Note that the ellipticity changes are of a sigmoidal character in contrast to the gradual changes accompanying a temperature increase. The heat capacity changes (Fig. 6 *c*), which indicate an increase in the number of nonpolar side chains exposed to solvent upon the unfolding, also show some cooperativity. This follows from the sigmoidal shape of the calorimetric curve at low temperature, which only gradually rises with increasing temperature. Differences in cooperativity of these processes might be caused by different cooperation between elements of residual structure in the denatured molecule. We can expect that at low temperatures distribution of the denatured conformations will be low, whereas at high temperatures it progressively shifts towards conformations with less residual structure. As a result, the transformation of the stable intermediate into the unfolded state with increasing temperature should be a more gradual process than the unfolding that accompanies a decrease in temperature.

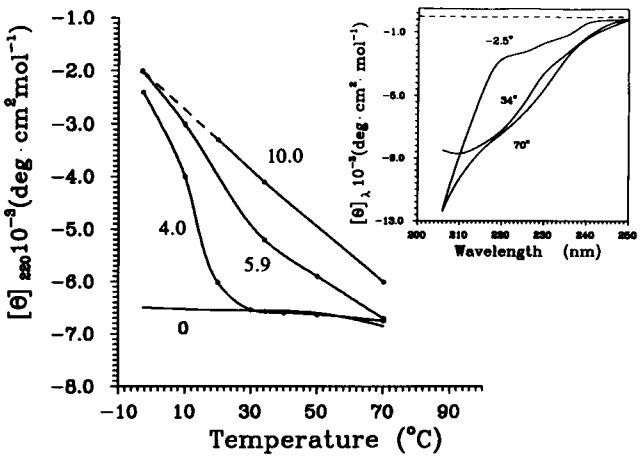

FIGURE 8 The temperature dependence of ellipticity of β-lactoglobulin at 222 nm in solutions of 0.1 M KCl-HCl buffer pH 2.0 containing different urea concentrations. The inset shows CD spectra of β-lactoglobulin in far UV-region in the solution containing 4.0 M urea at different temperatures.

If the unfolding of β-lactoglobulin is considered to be a two-stage process, involving disruption of unique tertiary structure at the first stage and disruption of persistent secondary structure at the second stage, details of denaturation should depend on whether it proceeds at low temperature (cold denaturation) or at high temperature (heat denaturation). Cold denaturation involves disruption of the intermediate simultaneously with disruption tertiary structure and accompanies by substantial changes in secondary structure, heat capacity, and efficiency of spin diffusion. As a result, the calorimetric and van't Hoff enthalpies of this transition show a marked deviation from those expected for a two-state model. In contrast, the heat denaturation of β-lactoglobulin in the same solution leads to disruption of mostly tertiary structure (Fig. 1 *c*). This process is not accompanied by substantial changes in heat capacity, ellipticity, and efficiency of spin diffusion as for unfolding, and appears to be a two-state transition. The stable intermediate, classified as heat-denatured protein, can be gradually disrupted by the further heating to 110°C, at which temperature its structural and thermodynamic parameters are no longer distinguishable from those in 10 M urea.

CONCLUSION

On the basis of the results presented here, we propose that cold denaturation of β-lactoglobulin involves an equilibrium

intermediate state, which does not appear upon heat denaturation. This intermediate is characterized by an absence of unique tertiary structure and the presence of nonspecific hydrophobic interactions, which stabilize residual secondary structure (Hughson et al., 1990; Chan et al., 1990). Because the strength of hydrophobic interactions decreases with temperature, reduction of temperature should lead to decooperation of intramolecular interactions and more complete polypeptide chain unfolding (Griko et al., 1988, 1989; Freire et al., 1992). A similar intermediate state is found in denatured β-lactoglobulin in 6.0 M urea at 308 K. Decrease of temperature leads to disruption of the intermediate, which is accompanied by a change in the heat capacity. The fact that some elements of the native-like secondary structure of proteins can persist in strongly denaturing solution was demonstrated recently by K. Wüthrich and colleagues on the 434-repressor in 7 M urea solution (Neri et al., 1992a, b).

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