# Calorimetric Study of the Heat and Cold Denaturation of β-Lactoglobulin†

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ABSTRACT: Temperature-induced changes of the states of β-lactoglobulin have been studied calorimetrically. In the presence of a high concentration of urea this protein shows not only heat but also cold denaturation. Its heat denaturation is approximated very closely by a two-state transition, while the cold denaturation deviates considerably from the two-state transition and this deviation increases as the temperature decreases. The heat effect of cold denaturation is opposite in sign to that of heat denaturation and is noticeably larger in magnitude. This difference in magnitude is caused by the temperature-dependent negative heat effect of additional binding of urea to the polypeptide chain of the protein upon its unfolding, which decreases the positive enthalpy of heat denaturation and increases the negative enthalpy of cold denaturation. The binding of urea considerably increases the partial heat capacity of the protein, especially in the denatured state. However, when corrected for the heat capacity effect of urea binding, the partial heat capacity of the denatured protein is close in magnitude to that expected for the unfolded polypeptide chain in aqueous solution without urea but only for temperatures below 10 °C. At higher temperatures, the heat capacity of the denatured protein is lower than that expected for the unfolded polypeptide chain. It appears that at temperatures above 10 °C not all the surface of the β-lactoglobulin polypeptide chain is exposed to the solvent, even in the presence of 6 M urea; i.e., the denatured protein is not completely unfolded and unfolds only at temperatures lower than 10 °C. The heat capacity decrease of denatured β-lactoglobulin upon heating above 10 °C in urea solution proceeds in a sigmoidal fashion, suggesting that compaction of the protein, associated with desolvation of some urea bound to protein, is to some extent a cooperative process.

Reversible disruption of the native protein structure upon cooling, known as cold denaturation, appears to be a very general property of all globular proteins with an extended hydrophobic core, but for most proteins it is expected at too low a temperature for its experimental observation (Privalov, 1990). One can observe cold denaturation either by supercooling the aqueous protein solution, as we did in the case of myoglobin and apomyoglobin (Privalov et al., 1986; Griko et al., 1988), or by adding urea or guanidinium chloride (GdmCl) to the protein solution, which shifts the temperature of cold denaturation above the freezing point of solution (Pace & Tanford, 1968; Nojima et al., 1978; Griko & Privalov, 1986; Griko et al., 1988 a,b; Chen & Schellman, 1989).

The main characteristic of cold denaturation, distinguishing it qualitatively from heat denaturation, is that it proceeds with a negative heat effect, i.e., with negative enthalpy and entropy change. However it appeared that both these thermodynamic parameters are represented by the same temperature-dependent functions for both heat and cold denaturation, as determined by the denaturation heat capacity increment (Privalov, 1990). This means that the final state in cold and heat denaturation is the same macroscopic state; i.e., the denatured state is universal.

In this paper we consider an example, β-lactoglobulin, which on first sight at least represents a somewhat different case. This is one of the first proteins in which the cold denaturation was observed in the presence of urea (Jacobsen & Christiensen, 1948; Schellman, 1958; Pace & Tanford, 1968). Temperature dependence of the apparent denaturation enthalpy of this protein showed a clear kink at 10 °C, which was explained by assuming that the denaturation of β-lactoglobulin at temperatures below 10 °C was not a simple two-state transition (Pace & Tanford, 1968). The temperature-induced change of the order of denaturation reaction was surprising and deserved detailed investigation by direct calorimetric methods.

## EXPERIMENTAL PROCEDURES

β-Lactoglobulin A was prepared from the milk of individual cows according to Armstrong et al. (1967). The homogeneity of the preparation was checked by electrophoresis in the native and denatured conditions according to Laemmli (1970).

The concentration of protein was determined by its extinction coefficient. In acidic solution in the presence of urea, $E_{278} = 9.6$ (Byler et al., 1983).

All reagents used were of analytical grade. Urea was purified and checked according to Hodman et al. (1962).

Most of the measurements were done in 0.1 KCl/HCl buffer solutions at pH 2.0 with different concentrations of $NaH_2PO_4$ and urea.

The calorimetric measurements were performed using a DASM-4 scanning microcalorimeter in a broad temperature range, including subzero temperatures achieved by supercooling the solution (Privalov et al., 1986). Most experiments were done at a scan rate of 1.0 K/min and protein concentrations of 0.8–3.0 mg/mL. In some cases a lower scan rate, 0.5 K/min, was used.

The partial specific heat capacity of β-lactoglobulin was calculated as a function of temperature as described by Privalov and Potekhin (1986), measuring solution density by a digital densimeter DNA-60 (Anton Paar).

The circular dichroism spectra, CD, in the range of 183–320 nm were measured with a Jasco-41-A spectropolarimeter in thermostated cells.

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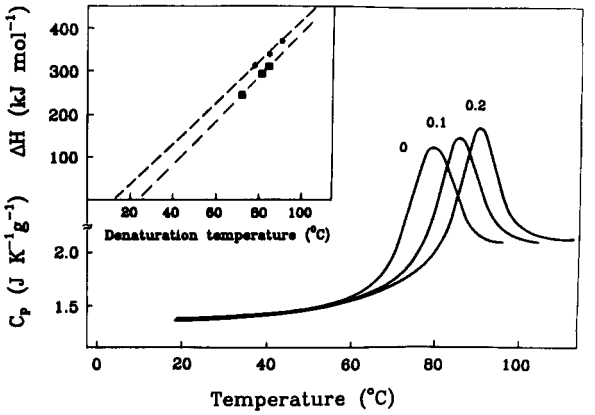


FIGURE 1: Temperature dependence of the β-lactoglobulin partial heat capacity in solutions with different concentration of phosphate (molarity of phosphate is indicated on the curves) and the dependence of denaturation enthalpy on the temperature of denaturation for the solutions without urea (●) and in the presence of 2 M urea (■).

## RESULTS AND DISCUSSION

*Heat Denaturation of β-Lactoglobulin in Aqueous Solutions with Various Concentrations of Phosphate.* It is known that β-lactoglobulin forms dimers in solutions with pH >2. To exclude complications connected with the dissociation of dimers upon denaturation, all experiments were carried out at pH 2.0, where the protein is in monomeric form (Pace & Tanford, 1968; Kella & Kinsella, 1988).

A remarkable feature of β-lactoglobulin is that its thermal denaturation in acidic solutions does not depend significantly on the pH and ionic strength of the solution but is sensitive to the presence of phosphate ions (Arakawa & Timasheff, 1984; Kella & Kinsella, 1988). Figure 1 represents the temperature dependence of the partial heat capacity of β-lactoglobulin in solutions with different concentrations of $NaH_2PO_4$. With the increase of phosphate concentration from 0 to 0.2 M, the denaturation heat absorption peak shifts to higher temperatures and increases in size. Denaturation also results in a significant heat capacity increase which amounts to 0.31 ± 0.04 $J \cdot K^{-1} \cdot g^{-1}$ or, calculated per mole of protein, to 5.70 ± 0.70 $kJ \cdot K^{-1} \cdot mol^{-1}$ (using a molecular mass of 18 000). The partial heat capacity of the native β-lactoglobulin at 25 °C is 1.37 ± 0.07 $J \cdot K^{-1} \cdot g^{-1}$ or 25.4 ± 1.3 $kJ \cdot K^{-1} \cdot mol^{-1}$. Therefore, the partial specific heat capacity of denatured protein at this temperature is 1.68 ± 0.09 $J \cdot K^{-1} \cdot g^{-1}$.

The values of the calorimetric enthalpies determined from the area under the calorimetrically observed heat absorption peaks, $\Delta H^{cal}$, are given in Table I. In the same table are given the van't Hoff enthalpies of the observed process, $\Delta H^{vH}$, calculated from the sharpness of the heat absorption peak by the van't Hoff equation, assuming that denaturation is a two-state transition. The close correspondence of these two enthalpies shows that the heat denaturation of β-lactoglobulin can be regarded as a two-state transition, i.e., that the concentration of states intermediate between the native and denatured is negligibly small.

The dependence of the denaturation enthalpy on the denaturation temperature at different concentrations of phosphate is shown in Figure 1. The slope of this function equals 4.90 ± 0.40 $kJ \cdot K^{-1} \cdot mol^{-1}$ or 0.27 ± 0.02 $J \cdot K^{-1} \cdot g^{-1}$. This is very close to the calorimetrically determined denaturation heat capacity increment, $\Delta C_p$. Since

$$\frac{d\Delta H_G}{dT} = \left(\frac{\partial \Delta H}{\partial T}\right)_\omega + \left(\frac{\partial \Delta H}{\partial \omega}\right)_T \frac{d\omega}{dT} \quad (1)$$

Table I: Thermodynamic Characteristics of β-Lactoglobulin Heat Denaturation in Various Buffer Solutions

| urea concn (M) | $T_d$ (°C) | $\Delta H^{cal}$ (kJ/mol) | $\Delta H^{vh}$ (kJ/mol) | $R^a$ | $\Delta C_p$ ($kJ \cdot K^{-1} \cdot mol^{-1}$) |
|---|---|---|---|---|---|
| 0.1 M KCl/HCl, pH 2.0 | | | | | |
| 0 | 78.0 | 312 ± 15 | 319 ± 15 | 0.98 | 5.58 ± 0.7 |
| 2.0 | 70.8 | 243 ± 12 | 248 ± 12 | 0.96 | 5.82 ± 0.7 |
| 4.4 | 56.0 | 120 ± 20 | 155 ± 20 | 0.92 | 7.89 ± 0.8 |
| 0.1 M Sodium Phosphate, pH 2.0 | | | | | |
| 0 | 85.0 | 340 ± 15 | 313 ± 15 | 1.09 | 5.99 ± 0.7 |
| 2.0 | 81.3 | 296 ± 14 | 278 ± 14 | 1.07 | 6.14 ± 0.7 |
| 4.4 | 68.6 | 204 ± 20 | 230 ± 14 | 0.91 | 6.86 ± 0.7 |
| 0.2 M Sodium Phosphate, pH 2.0 | | | | | |
| 0 | 91.0 | 371 ± 14 | 328 ± 15 | 1.00 | 5.66 ± 0.7 |
| 2.0 | 84.0 | 313 ± 14 | 285 ± 15 | 1.10 | 6.29 ± 0.7 |
| 4.4 | 76.0 | 248 ± 15 | 265 ± 15 | 0.96 | 6.64 ± 0.7 |

$^a$ $R = \Delta H^{cal}/\Delta H^{vh}$.

and

$$\left(\frac{d\Delta H}{dT}\right)_\omega = \Delta C_p \quad (2)$$

where ω is the concentration of phosphate, the correspondence of these two temperature derivatives means that the enthalpy of phosphate interaction with protein is small.

*Cold Denaturation of β-Lactoglobulin in Urea Solutions.* A linear extrapolation of the enthalpy function to lower temperatures shows that the temperature $T_H$, at which the denaturation enthalpy becomes zero, is about 18 °C (Figure 1). If $T_G$ is the temperature at which the Gibbs energy difference of the native and denatured states is zero and the protein denatures upon heating, one should expect that this parabolic function should also be zero at the temperature $T'_G$, at which the cold denaturation of the protein should take place (Privalov, 1990):

$$T'_G = \frac{(T_G)^2}{3T_G - 2T_H} \quad (3)$$

It follows from the values of $T_G$ (see Table I) that the cold denaturation of β-lactoglobulin in an aqueous solution should occur at temperatures below −20 °C, i.e., below the freezing point of the solution and, therefore, cannot be observed practically.

According to eq 3 the temperature of cold denaturation can be raised by raising $T_H$. This can be done by adding urea to the solution. The enthalpy of urea solvation by a protein is negative, its magnitude depends upon the urea concentration and the temperature, and consequently, it considerably increases the partial heat capacity of protein (Makhatadze & Privalov, 1992). Since urea solvates the groups of the protein which are exposed upon denaturation, the overall enthalpy of protein denaturation in the presence of urea is smaller than in its absence and it is a steeper function of temperature (Figure 1). Correspondingly $T_H$ is higher in urea solutions and therefore $T'_G$ should be higher.

Figure 2 shows the heat effects recorded upon calorimetric scanning of urea solutions of β-lactoglobulin over a wide temperature range. The pronounced exothermic peak observed upon cooling results from cold denaturation. Upon subsequent heating of the same sample, an endothermic peak appears which corresponds to renaturation of the cold-denatured protein. Cold denaturation and renaturation are completely reversible processes, but in calorimetric recordings, which are scanned at a considerable heating rate (1.0 K/min), they are shifted in the temperature scale because of the slow kinetics of these processes at low temperatures. Because of this tem-

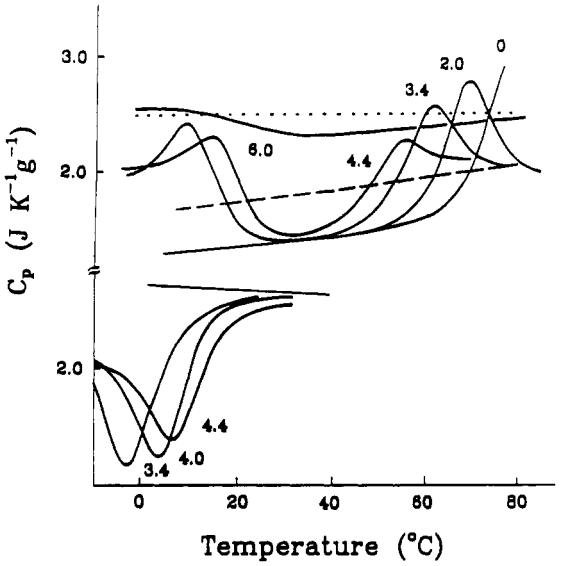


FIGURE 2: Temperature dependence of the partial heat capacity of β-lactoglobulin in urea solutions with phosphate buffer, pH 2.0, in cooling and heating experiments. The concentration of urea is indicated on the curves. The dotted line represents the heat capacity of apocytochrome *c* in 6 M urea solution.

Table II: Thermodynamic Characteristics of β-Lactoglobulin Cold Denaturation and Renaturation in Solutions with Various Urea Concentrations

| urea concn (M) | $T_d$ (°C) | $\Delta H^{cal}$ (kJ/mol) | $\Delta H^{vh}$ (kJ/mol) | $R^a$ | $\Delta C_p$ (kJ·K$^{-1}$·mol$^{-1}$) |
|---|---|---|---|---|---|
| | | Cold Denaturation | | | |
| 3.4 | −3.0 | −271 ± 30 | 154 ± 30 | 1.76 | 11.0 ± 1.2 |
| 4.0 | 4.0 | −260 ± 26 | 171 ± 30 | 1.52 | 12.0 ± 1.2 |
| 4.4 | 6.3 | −220 ± 24 | 169 ± 30 | 1.30 | 11.9 ± 1.2 |
| | | Renaturation | | | |
| 3.4 | 9.0 | 212 ± 31 | | | 11.6 ± 1.5 |
| 4.0 | 13.0 | 208 ± 31 | | | 11.9 ± 1.5 |
| 4.4 | 16.0 | 180 ± 30 | | | 13.1 ± 1.5 |

$^a$ $R = \Delta H^{cal}/\Delta H^{vh}$.

perature shift, the values of these two heat effects are different: the heat effect of renaturation is smaller since it proceeds at a temperature closer to $T_H$ where the enthalpy of denaturation becomes zero (Table II). It should be noted that, notwithstanding the considerable hysteresis, the deviation of the cold denaturation enthalpy from the equilibrium value at the temperature of the peak maximum is rather small, as the calorimetric enthalpy values are on exactly the same line as the van't Hoff enthalpies found by Pace and Tanford (1968) in the equilibrium study above 10 °C and the colorimetric enthalpies of heat denaturation (Figure 3). However, at temperatures below 10 °C a clear deviation of the van't Hoff enthalpy from the calorimetric enthalpy is observed (Table II). This deviation, which is responsible for the kink on the apparent enthalpy function found by Pace and Tanford (1968), means that at temperatures below 10 °C denaturation of β-lactoglobulin does not represent a two-state transition.

It is remarkable that the negative enthalpies of cold denaturation are considerably larger than the positive enthalpies of heat denaturation in the same solvent and the latter are smaller than the enthalpies of heat denaturation in the absence of urea (see Tables I and II). Plotted against the denaturation temperature, the enthalpies of denaturation in the absence and in the presence of urea are represented by two different and almost linear functions which have different slopes (Figure 3).

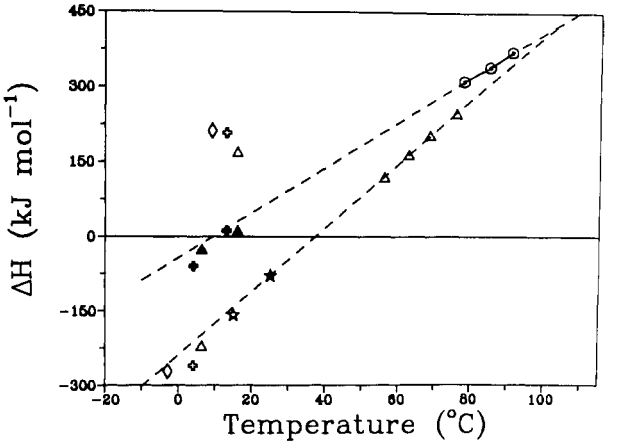


FIGURE 3: Temperature dependence of the enthalpy of β-lactoglobulin denaturation and renaturation (upper branch at low temperatures) for solutions containing various concentration of phosphate and urea. Symbols: (○) without urea (◇) 3.4 M urea (+) 4.0 M urea, and (△) 4.4 urea; (☆) results obtained by Pace and Tanford (1968). The filled symbols below 35 °C indicate the enthalpies corrected for the urea solvation effect.

It should be noted also that there is a very large increase of the heat capacity associated with cold denaturation which is much larger than that found for heat denaturation (Figure 2). This surprising heat capacity increase is inconsistent with the calorimetrically determined linear dependence of denaturation enthalpy on temperature. The heat capacity increment upon heat denaturation of β-lactoglobulin in the presence of 4.4 M urea is 0.39 ± 0.04 J·K$^{-1}$·g$^{-1}$ and it is in good agreement with that estimated from the enthalpy dependence on temperature, $d\Delta H/dT$, which is 0.36 ± 0.04 J·K$^{-1}$·g$^{-1}$ (Table I). The heat capacity increment upon cold denaturation in the presence of 4.4 M urea is, according to direct calorimetric measurements, 0.67 ± 0.07 J·K$^{-1}$·g$^{-1}$ (Figure 2).

We know that urea solvation increases the partial heat capacity of proteins and their denaturation heat capacity increment (Makhatadze & Privalov, 1992). This is why the heat capacity increment of β-lactoglobulin heat denaturation in the presence of urea is larger than in the absence of urea. But the heat capacity increment caused by the presence of urea does not depend noticeably on temperature. Therefore it is far from clear why it should be twice as large for cold denaturation as for heat denaturation.

Some light is shed on this problem by considering the temperature dependence of the partial heat capacity of β-lactoglobulin in 6 M urea solution, in which this protein is in the denatured state and does not show either heat or cold denaturation (Figure 2). The partial heat capacity of the protein in this solution is significantly higher than it is in the aqueous solution without urea, as it should be. It is surprising, however, that it does not decrease at temperatures decreasing below 35 °C but increases in a sigmoidal way. This is very unusual behavior for the heat capacity of a denatured protein. As seen from the figure, the partial specific heat capacity of apocytochrome *c* in the same solution of 6 M urea, in which this protein is in the completely unfolded state (Privalov et al., 1989), is an almost linear function of temperature with very small slope. It coincides with the heat capacity function for β-lactoglobulin only at temperatures below 10 °C. At all higher temperatures it is considerably higher than the partial heat capacity of denatured β-lactoglobulin.

To clarify the situation we need first of all to exclude from consideration the trivial heat effects of protein interaction with urea, which are known (Makhatadze & Privalov, 1992).

*Contribution of Urea Solvation in the Enthalpy of β-Lactoglobulin Denaturation.* Calorimetrically measured, the overall enthalpy of protein denaturation at temperature $T$ in a solution which contains urea in concentration $\omega$ consists of several components: enthalpy of conformational transition of protein, $\Delta H^{con}(T)_{pr}$, ionization of protein, $\Delta H^{ion}(T)_{pr}$, ionization of buffer, $\Delta H^{ion}(T)_{buf}$, and preferential solvation of urea, $\Delta H^{sol}(T,\omega)$:

$$\Delta H^{cal}(T,\omega) = \Delta H^{con}(T)_{pr} + \Delta H^{ion}(T)_{pr} + \Delta H^{ion}(T)_{buf} + \Delta H^{sol}(T,\omega) \quad (4)$$

The enthalpies of ionization of protein and buffer are usually small and opposite in sign. Therefore, they almost completely compensate each other (Privalov, 1979).

The enthalpy of preferential solvation of urea by protein is negative and large and increases in magnitude as the temperature decreases, which results in a significant heat capacity increment of protein solvation dependent on the urea concentration, $\Delta C_p^{sol}(\omega)$ (Makhatadze & Privalov, 1992).

The enthalpy of conformational transition for a protein from the native to unfolded state is strongly temperature dependent: it is positive and large at high temperatures, it decreases as temperature decreases, and it can even change its sign at low temperatures (Privalov & Gill, 1989; Privalov, 1990). Therefore, while upon heat denaturation of a protein the enthalpy of conformational transition and the enthalpy of urea solvation have different signs, upon cold denaturation they have the same sign. This explains why the calorimetrically observed heat effect of protein heat denaturation in the presence of urea is smaller in magnitude than the heat effect of cold denaturation.

The enthalpy of conformational transition of a protein can be calculated for any temperature $T$ from the calorimetrically measured overall denaturation enthalpy, if we know the enthalpy of urea solvation by protein upon unfolding at the considered temperature and urea concentration. The enthalpy of urea solvation can be determined by comparing the enthalpies of protein denaturation with and without urea at some temperature $T'$ and then correcting them for the temperature effect:

$$\delta\Delta H(T)^{sol} = \Delta H(T') - \Delta H(T')^{urea} + (T' - T)\delta\Delta C_p^{sol} \quad (5)$$

where

$$\delta\Delta C_p^{sol} = [(C_p^D + \delta C_p^D) - (C_p^N + \delta C_p^N)] - (C_p^D - C_p^N) = \delta C_p^D - \delta C_p^N \quad (6)$$

In 4.4 M urea solution without phosphate, β-lactoglobulin denatures at 56.0 °C with an enthalpy of 120 ± 8 kJ·mol$^{-1}$ (Table I). Its denaturation enthalpy in solution without urea at the same temperature can be found by extrapolation of the enthalpy function (see Figure 3) and is 200 ± 15 kJ·mol$^{-1}$. Therefore the enthalpy of urea solvation at 56 °C in 4.4 M solution is −80 ± 23 kJ·mol$^{-1}$.

The heat capacity increment upon protein denaturation in 4.4 M urea is 7.9 ± 0.8, and without urea, 5.6 ± 0.6 kJ·K$^{-1}$·mol$^{-1}$. Their difference amounts to 2.3 kJ·K$^{-1}$·mol$^{-1}$. Thus, for the temperature correction of the solvation enthalpy in the 50 K temperature range we get −115 kJ·mol$^{-1}$; i.e., at 6 °C the solvation enthalpy should be −195 kJ·mol$^{-1}$. Since the enthalpy of cold denaturation at this temperature is −220 kJ·mol$^{-1}$, for the conformational transition of the protein at 6 °C we obtain −25 kJ·mol$^{-1}$, which is exactly on the extrapolation line of the enthalpy of protein denaturation without urea (Figure 3).

Table III: Partial Specific Heat Capacity of Unfolded Polypeptide Chain of β-Lactoglobulin Calculated for Different Urea Solutions and Temperatures

| urea concn (M) | $C_p$ (J·K$^{-1}$·g$^{-1}$) | | | | |
|---|---|---|---|---|---|
| | 5 °C | 25 °C | 50 °C | 75 °C | 100 °C |
| 6.0[a] | 2.56 ± 0.13 | 2.39 ± 0.12 | 2.37 ± 0.12 | 2.45 ± 0.12 | 2.50 ± 0.13 |
| 0 | 2.07 ± 0.10 | 2.19 ± 0.11 | 2.30 ± 0.12 | 2.34 ± 0.12 | 2.38 ± 0.12 |
| 2.0 | 2.32 ± 0.12 | 2.37 ± 0.12 | 2.42 ± 0.12 | 2.46 ± 0.12 | 2.50 ± 0.13 |
| 4.4 | 2.48 ± 0.12 | 2.52 ± 0.12 | 2.55 ± 0.12 | 2.58 ± 0.13 | 2.58 ± 0.13 |
| 6.0 | 2.53 ± 0.13 | 2.56 ± 0.13 | 2.58 ± 0.12 | 2.59 ± 0.13 | 2.59 ± 0.13 |

[a] Measured experimentally.

On the other hand, the enthalpy of urea solvation by the unfolded polypeptide chain has been measured calorimetrically and it was found that it is universal for all polypeptide chains and depends only on the concentration of urea and the temperature (Makhatadze & Privalov, 1992). In 4.4 M urea solution at 6 °C it is equal to 36.5 J·g$^{-1}$. For the native globular protein it is usually half that value. Therefore, assuming that denatured β-lactoglobulin is completely unfolded, we find that the solvation enthalpy change upon its denaturation at 10 °C should be on the order of −18 J·g$^{-1}$ or −320 kJ·mol$^{-1}$. This value is 1.7 times larger than the previous one found for the solvation enthalpy calculated from direct experimental results obtained on β-lactoglobulin. The discrepancy between these two values means that either β-lactoglobulin in the denatured state is not completely unfolded or its native state is not quite compact. To resolve these two possibilities we have to analyze the partial heat capacities of β-lactoglobulin in the native and denatured states.

*Partial Heat Capacity of β-Lactoglobulin.* The partial specific heat capacity of native compact globular proteins in aqueous solutions at 25 °C usually varies in the range 1.30–1.50 J·K$^{-1}$·g$^{-1}$ (Privalov et al., 1989). Therefore the partial specific heat capacity of β-lactoglobulin in solution without urea, which is 1.37 ± 0.05 J·K$^{-1}$·g$^{-1}$, is within the range expected for a compact globular proteins.

The values of the partial specific heat capacity of unfolded polypeptide chains at 25 °C are usually within the range 1.90–2.20 J·K$^{-1}$·g$^{-1}$ (Privalov et al., 1989). The partial specific heat capacity of denatured β-lactoglobulin at this temperature is, as shown above, 1.68 ± 0.09 J·K$^{-1}$·g$^{-1}$, which is significantly lower than that expected for the completely unfolded polypeptide chain. To confirm this conclusion we calculated, for the whole considered temperature range, the partial heat capacity of the unfolded polypeptide chain of β-lactoglobulin according to its sequence, using the partial heat capacity values of amino acid residues reported by Privalov and Makhatadze (1990). We found that at 25 °C the heat capacity of this unfolded chain should indeed be 2.19 ± 0.10 J·K$^{-1}$·g$^{-1}$.

Since the heat capacity increment of the unfolded polypeptide chain interaction with urea is known (Makhatadze & Privalov, 1992) we also calculated the partial heat capacity function of the unfolded polypeptide chain of β-lactoglobulin in 2.0, 4.4, and 6.0 M urea solutions for the whole considered temperature range. These functions are given in Table III and presented by the dashed lines in Figure 4. As can be seen, in all cases they are higher than the heat capacities of denatured proteins measured calorimetrically under similar solvent conditions and coincide with them only at temperatures below 10 °C. In the presence of urea the deviation between the heat capacities calculated for the unfolded polypeptide chain and measured for denatured protein are even more pronounced than in the absence of urea. This makes almost certain that

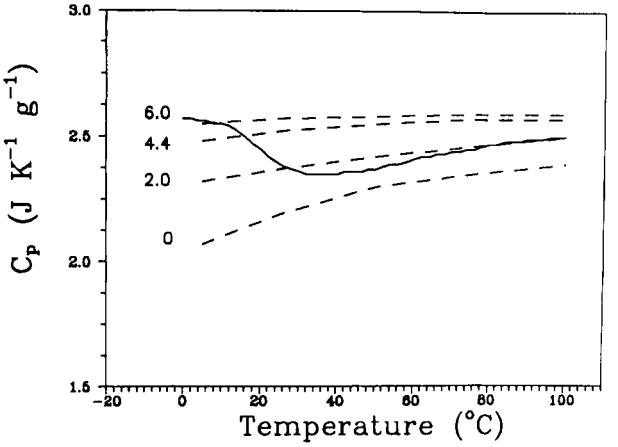


FIGURE 4: Calorimetrically determined partial specific heat capacity of denatured β-lactoglobulin in 6.0 M urea solution (solid line) and calculated partial specific heat capacity of unfolded polypeptide chain of β-lactoglobulin in aqueous solutions in the presence of 0, 2.0, 4.4, and 6.0 M urea (dashed line).

in the denatured lactoglobulin not all the groups are exposed to solvent. Judging by the observed and calculated heat capacity increment, the increase of solvent accessible surface area upon β-lactoglobulin denaturation is only half the size that it should be upon complete unfolding.

It appears that the polypeptide chain of β-lactoglobulin unfolds only upon cooling below 10 °C, perhaps as a result of extensive solvation by urea. Since the enthalpy of urea solvation is negative, its solvation should certainly increase as temperature decreases. Most intriguing is that this process does not seem to be gradual. It definitely shows some cooperativity judging by the sigmoidal shape of the heat capacity curve. However, it is far from clear whether this process can be regarded as a phase transition, particularly as a second-order phase transition, which is associated with the change of the second derivative of thermodynamic potential, i.e., the heat capacity (Karplus & Shakhnovich, 1992). It is not excluded that what we observe as the increase of heat capacity upon cooling is only the upper part of the extended heat absorption curve.

*Enthalpy of β-Lactoglobulin Denaturation.* Other evidence that denatured β-lactoglobulin is not completely unfolded at temperatures above 10 °C is presented by the enthalpy of its denaturation. As was shown for a number of globular proteins, when extrapolated to 110 °C, specific enthalpies of protein denaturation have universal values of 50 ± 5 J·$g^{-1}$ (Privalov, 1979; Privalov & Gill, 1989). The deviation from this value usually means that either the native protein is not quite compact or the denatured protein is not quite unfolded and retains some residual structure.

If we linearly extrapolate the specific enthalpy of β-lactoglobulin denaturation in the absence of urea to 110 °C, we find that it is about 27 ± 3 J·$g^{-1}$; i.e., it is only half the expected value. Since the native state, judging by its specific heat capacity, does not appear as noncompact, we can assume that the denatured state of β-lactoglobulin retains considerable residual structure stabilized by enthalpic interactions. The question is, however, what kind of structure is that: it is a domain which does not denature upon heating and can only unfold upon cooling in the presence of urea, or is it an unstructured aggregate of the polypeptide chain, a "molten globule"? One cannot solve this problem without additional information on the conformation of denatured β-lactoglobulin.

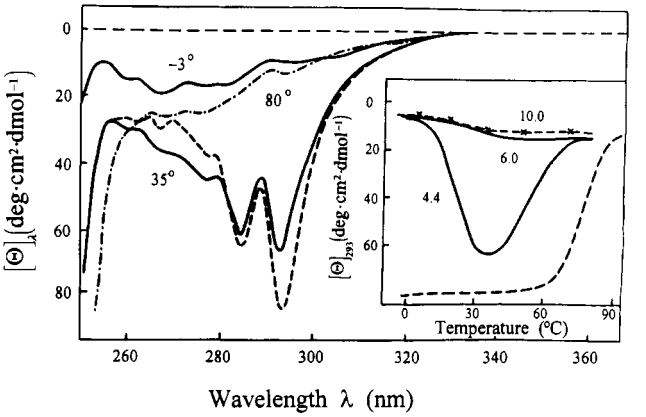


FIGURE 5: CD spectra of β-lactoglobulin in solution containing 4.4 M urea without phosphate at different temperatures and the temperature dependence of ellipticity at 293 nm in solutions with different urea concentrations. The dashed line in the inset shows the results for a solution containing 0.2 M phosphate.

*CD Studies of β-Lactoglobulin Denaturation.* Studies of β-lactoglobulin circular dichroism (CD) spectra show drastic changes in the near UV upon cooling in urea solution below 30 °C or heating above 40 °C (Figure 5). At 0 °C the ellipticity at 293 nm approaches zero. Upon consequent heating in the 6 M urea solution to 35 °C the ellipticity slightly increases. As the ellipticity in the near UV is supposed to be an indicator of the existence of tertiary structure in a protein, we can assume that β-lactoglobulin in 4 and 6 M urea solutions does not possess extended tertiary structure at 0 °C. It regains it completely at higher temperature if the urea concentration is 4 M, but in 6 M urea solution the increase in ellipticity is so small that one can hardly assume the appearance of an extended tertiary structure.

CD spectra in the far UV do not bear much structural information, as lactoglobulin is a β-structure protein with a small content of α-helix (Papiz et al. 1986).

*Temperature-Induced Change of the Order of Denaturation Reaction.* The fact that β-lactoglobulin denaturation is a two-state transition at temperatures above 10 °C and below this temperature shows clear deviation from a two-state transition is very intriguing. We encountered a similar situation before with the phosphoglycerate kinase denaturation (Griko et al., 1988). Phosphoglycerate kinase is a two-domain protein. However, its heat denaturation appears as a two-state transition; i.e., at the elevated temperatures the protein figures as a single cooperative system, which means that at these temperatures the domains are strongly interacting with each other. On the other hand, upon cooling these domains unfold almost independently. Therefore, the interaction between the domains appears to be temperature dependent, as if it caused by hydrophobic forces (Freire et al., 1991). The similarity with β-lactoglobulin denaturation leads us to the assumption that β-lactoglobulin structure might also be subdivided into the domains linked together by hydrophobic interactions. The known three-dimensional structure of β-lactoglobulin does not exclude this possibility, showing two densely packed blocks in its structure (Papiz et al., 1986).

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**Registry No.** $PO_4^{3-}$, 14265-44-2; urea, 57-13-6.