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Studies by UV spectroscopy of thermal denaturation of β-lactoglobulin in urea and alkylurea solutions

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Abstract

The thermal denaturation of β-lactoglobulin in aqueous solutions at three different pH's and in aqueous solutions of urea, methyl-, *N,N'*-dimethyl- and ethylurea was studied by UV spectroscopy. The UV-melting curves were analyzed on the basis of two-state approximation to obtain the apparent equilibrium constant, K_{app}, and the apparent standard enthalpy of transition, ΔH^0_{app}, for protein unfolding as a function of temperature. From K_{app}, calculations of ΔG^0_{app}, as functions of temperature around transition temperature, $T_{1/2}$, in urea and alkylurea solutions and different buffer solutions have been carried out. An increase in the observed transition temperature, $T_{1/2}$, and the corresponding transition enthalpies, ΔH^0_{app}, with decreasing pH or denaturant concentration indicate increased stability of protein in these conditions. However, comparison of ΔH^0_{app} with ΔH_{cal} shows that the thermal transition of β-lactoglobulin in aqueous urea and alkylurea solutions is not a two-state process. It has also been observed that urea and all alkylureas cause a red shift in the absorbance spectrum of β-lactoglobulin which increases with increasing denaturant concentration and decreasing pH. A similar increase in red shift of β-lactoglobulin absorbance spectrum has also been observed with increasing temperature.

Keywords: β-Lactoglobulin; Urea; Alkylurea; Thermal denaturation; UV spectroscopy

1. Introduction

In previous papers, studies of the solvent denaturation of β-lactoglobulin by urea and alkylureas were reported [1,2]. Separate studies dealt with protein thermal denaturation in water and buffer solution at pH 2.0 and thermal denaturation in the aqueous urea solutions of different concentration [3,4]. The methods used were batch calorimetry, circular dichroism (CD), differential scanning calorimetry (DSC) and density measurement. The main conclusion based on the comparison of the denaturing action of urea and alkylureas, respectively, was that the differences observed reflect the presence of the hydrophobic moiety in the urea molecule. The subject of this investigation was to examine by ultraviolet (UV) spectroscopy the course of thermal denaturation, i.e., the denaturing action of heat, on β-lactoglobulin at three pH's and at different concentrations of aqueous urea, methyl-, *N,N'*-dimethyl- and ethylurea solutions. The UV spectroscopy data were analyzed on the basis of a two-state

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approximation to obtain the apparent equilibrium constant, K_{app}, for protein unfolding as a function of temperature. From K_{app}, the corresponding ΔG^0_{app}, ΔH^0_{app}, $\Delta C_P^{0,app}$ and $T\Delta S^0_{app}$ can be calculated [5–7].

2. Materials and methods

Bovine milk β-lactoglobulin, a mixture of A and B variants, was obtained from Sigma as a three-times crystallized and lyophilized powder that was used without further purification. Ultra pure urea was a product of Kemika (Zagreb, Croatia). The alkylureas used were supplied by Fluka (Buchs, Switzerland). Phosphate buffer with pH 7.4 and sodium citrate/HCl buffer with pH 2.0 were used. The stock solution of protein was prepared by dissolving it in water. Concentration in water was determined at room temperature by using $E^{1\%w}_{1\,cm} = 9.6$ at 278 nm. From the stock solution 0.2% solutions of β-lactoglobulin were prepared by adding solid alkylurea on a precise analytical balance.

The UV spectra of β-lactoglobulin at three different pH's and at different concentrations of aqueous urea and alkylurea solution were recorded on a Cary 1 UV-visible spectrophotometer from Varian by using matched 1-cm path length thermostated quartz cuvettes. The electrothermal temperature controller provides thermal programmability for the multicell unit so that the absorbance measurements can be performed directly as a function of temperature. Equilibrium thermal unfolding of β-lactoglobulin was monitored at 293 nm. The sample cuvettes were filled with the protein solution whereas the reference cuvettes were filled with the pure solvent. Each cuvette was sealed with teflon stopper to avoid evaporation during the experiment.

Absorbance at 293 nm was recorded as a function of temperature over a temperature range of 20–100°C. The heating rate in the experiments was 0.5°C/min and the hold time was 1 min.

The UV spectra of protein in different aqueous urea and alkylurea solutions in the range from 190 to 300 nm were recorded from 20 to 100°C at 20°C intervals.

To obtain the UV difference spectra of β-lactoglobulin the spectrum in 8 *M* urea at 20°C was subtracted from its spectrum with the same concentration at given conditions, because it is knovn that β-lactoglobulin in 8 *M* urea at 20°C exists in random coil conformations [7].

3. Results and discussion

β-Lactoglobulin consists of 164 aminoacid residues where there are four tyrosines (Tyr), two phenylalanines (Phe) and two tryptophans (Trp) [8]. In Fig. 1 the normalized UV spectra of β-lactoglobulin in water (pH 6.5) and given urea and alkylurea solutions are presented in the range from 190 to 300 nm. It can be seen that the absorbance changes due to the unfolding process are large in the range between 200 and 250 nm. At wavelength (λ) around 280 nm where Tyr, Phe

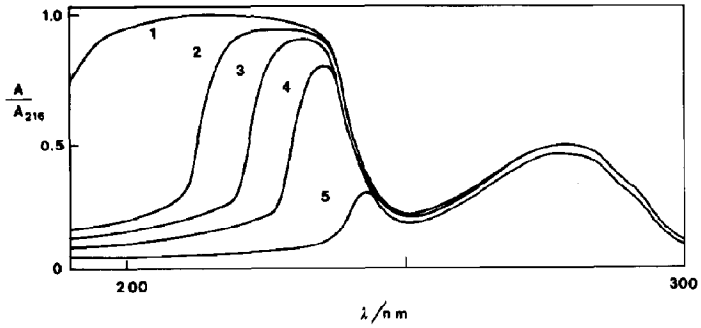

Fig. 1. Normalized UV-spectra of β-lactoglobulin in 2 *M* alkylurea solutions and in water (pH 6.5). 1 – water, 2 – urea, 3 – methylurea, 4 – *N,N'*-dimethylurea; 5 – ethylurea solution.

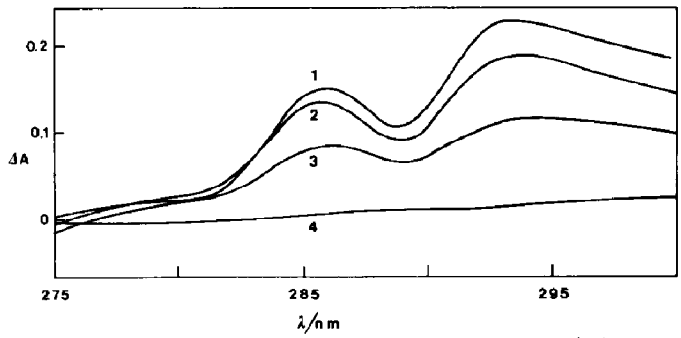

Fig. 2. UV difference spectra of β-lactoglobulin at different urea concentrations at 20°C (reference was β-lactoglobulin with the same concentration in 8 *M* urea at 20°C). 1 – water (pH 6.5), 2 – 2 *M* urea, 3 – 4 *M* urea, and 4 – 6 *M* urea.

and Trp substantially contribute to the UV spectra of proteins the changes of absorbance caused by the unfolding process are rather small. Therefore in this λ range the unfolding of β-lactoglobulin should be followed by means of difference spectra in which the spectrum of β-lactoglobulin in 8 *M* urea where it exists in random coil conformation is subtracted from the protein spectrum measured at given conditions, Fig. 2 [9,10]. It has been known that peaks in the difference spectra of proteins in the 292 to 294 nm region are assigned to Trp, in the 285 to 288 nm region to Tyr plus a small contribution by Trp, Fig. 2, and below 270 nm to Phe, [11]. The difference spectral changes in this region reflect largely the effects of the denaturant on the environment of the Trp and Tyr in protein. The unfolding of most proteins caused by a given denaturant is usually accompanied by a relatively large decrease in absorbance [9]. This is also seen in our spectra.

The stability of β-lactoglobulin was investigated quantitatively by determining its conformational stability in different aqueous urea and alkylurea solutions. This was ascertained by following equilibrium thermal unfolding of β-lactoglobulin in aqueous urea, methyl-, *N,N'*-dimethyl- and ethylurea solutions employing UV spectroscopy at 293 nm which reflects by and large the exposure of tryptophan residues (Trp) from the protein interior upon unfolding [12]. Figure 3 presents a typical sigmoidal unfolding curve of β-lactoglobulin in 2 *M* aqueous methylurea solution given in terms of protein absorbance at 293 nm as a function of temperature. From this curve the apparent or van't Hoff enthalpy of unfolding at the temperature of the half-transition and the fractions of the unfolding

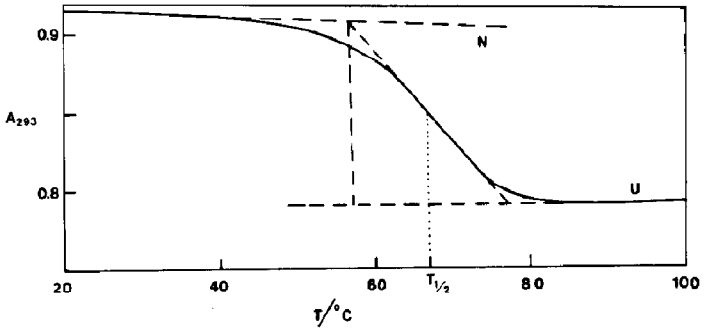

Fig. 3. A typical equilibrium unfolding curve of β-lactoglobulin (0.2%) in 2 *M* methylurea solution. ΔH^0_{app} was calculated from the slope of the curve at $T_{1/2}$. For details, see text.

protein in the temperature interval of transition were determined. The data were analyzed by assuming a two-state transition to obtain apparent equilibrium constants, K_{app}, for protein unfolding. The fraction of unfolded protein, f_U, is given by

$$f_U = \frac{A(T) - A_N(T)}{A_U(T) - A_N(T)} \quad (1)$$

where A is the absorbance; N and U refer to the native and denatured state, respectively. The relation also applies

$$K_{app} = \frac{[U]}{[N]} = \frac{f_U}{1 - f_U} \quad (2)$$

The apparent standard thermodynamic parameters, Gibbs free energy, ΔG^0_{app}, enthalpy, ΔH^0_{app}, and entropy, ΔS^0_{app}, of unfolding were determined by using the standard equations [7].

$$\Delta G^0_{app} = -RT \ln K_{app} \quad (3)$$

$$\Delta H^0_{app} = -R\left[\frac{\partial \ln K_{app}}{\partial(1/T)}\right]_P \quad (4)$$

$$\Delta S^0_{app} = \frac{\left(\Delta H^0_{app} - \Delta G^0_{app}\right)}{T} \quad (5)$$

where R is the universal gas constant.

ΔH^0_{app} was obtained directly from the slope of sigmoidal curve, see Fig. 3 [13], by using equation

$$\Delta H^0_{app} = 4RT^2_{1/2}\left(\frac{\partial f_U}{\partial T}\right)_{T=T_{1/2}} \quad (6)$$

Table 1 contains the enthalpies, ΔH^0_{app}, of protein unfolding determined from the slope of sigmoidal curves and the corresponding temperature of the half-transition, $T_{1/2}$, [13].

The van't Hoff plots based on the equilibrium thermal unfolding of β-lactoglobulin at all pH values are slightly curved meaning that the unfolding process is accompanied only by minor changes in the transition enthalpy, Fig. 4. Such behaviour has been attributed to the changes in the heat capacity of the non-polar groups that are exposed to the polar water exterior from the non-polar interior upon unfolding [14]. The values of ΔH^0_{app} and $T_{1/2}$ increase with decreasing

Table 1

The apparent standard enthalpy change, ΔH^0_{app}, of unfolding of β-lactoglobulin and the temperature of the half-transition, $T_{1/2}$, at three different pH values and at different concentrations of aqueous urea and alkylureas. The values obtained by DSC are given in parentheses. $R = \Delta H^0_{app}/\Delta H_{cal}$

Solvent	ΔH^0_{app} [a] (kJ/mol)	(ΔH_{cal})	R	$T_{1/2}$ [b] (°C)	(T_d)
pH					
2.0	262	(414)	0.644	81.4	(82.3)
6.5 (water)	232	(412)	0.564	80.1	(80.0)
7.4	167			65.9	
Urea					
2 *M*	181	(260)	0.753	69.4	(72.0)
3 *M*	129			60.0	
4 *M*	105			55.9	
Methylurea					
2 *M*	179			66.5	
3 *M*	154			56.1	
4 *M*	132			45.9	
N,N'-Dimethyl urea					
2 *M*	178	(308)	0.659	58.8	(64.0)
3 *M*	152			47.7	
4 *M*	133			35.4	
5 *M*	115			24.3	
Ethylurea					
2 *M*	161	(372)	0.565	54.8	(66.5)
3 *M*	138			41.1	
4 *M*	118			30.0	

[a] Relative error is estimated to be about 10%.

[b] Relative error is estimated to be about 1%.

pH. They agree satisfactorily with the values in the literature obtained by the same method [15]. In urea solutions ΔH^0_{app} and $T_{1/2}$ decreased with increasing urea concentration and in 6 *M* and 8 *M* urea no transition was observed. The same results have been obtained when using DSC method [4]. In methylurea solutions of β-lactoglobulin the values of ΔH^0_{app} and $T_{1/2}$ decrease with increasing denaturant concentration. In *N,N'*-dimethylurea and ethylurea solutions the behaviour of β-lactoglobulin was similar. If the values of $T_{1/2}$ and ΔH^0_{app} of β-lactoglobulin in different aqueous alkylurea solutions are compared it can be seen that the denaturing action of alkylureas increases as follows: methylurea < *N,N'*-dimethylurea < ethylurea. As already mentioned, the interactions of β-lactoglobulin with

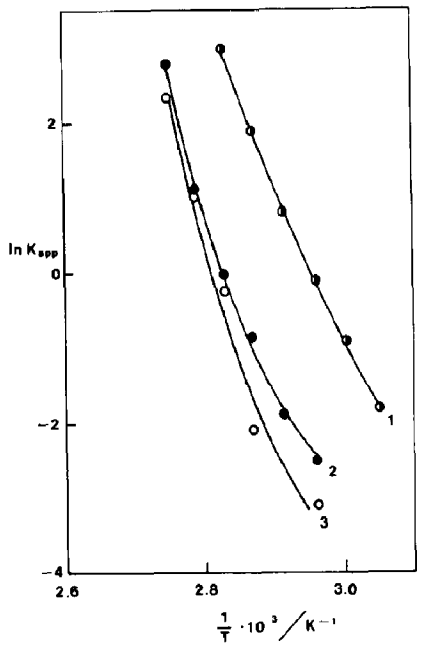

Fig. 4. Van't Hoff plot for thermal denaturation of β-lactoglobulin at different pH's. 1 – pH 7.4, 2 – pH 6.5 (water), and 3 – pH 2.0.

alkylureas were studied also by CD [1]. An analysis of the observed CD spectra at 25°C revealed differences in conformational changes produced by urea and alkylureas, respectively. Based on those results, one can conclude that only in concentrated urea solutions at 25°C the denatured form is a random coil, whereas in methylurea a transition to random coil has not been observed. Therefore methylurea is considered to be a weaker denaturant. *N,N'*-dimethylurea and ethylurea cause large $\beta \rightarrow \alpha$ conformational changes and are thus strong denaturants, although they do not turn the protein to random coils [7].

The apparent standard enthalpies of denaturation, ΔH^0_{app}, were compared with calorimetric ones, ΔH_{cal}, obtained by using DSC for β-lactoglobulin at two different pH's and in the presence of urea [3,4] and alkylureas (S. Lapanje and N. Poklar, 1991, unpublished data). These values presented in Table 1. are larger than those obtained by UV spectroscopy. It is well known that calorimetric measurements provide a direct model-independent determination of the transition enthalpy. For this reason a comparison of the model-dependent van't Hoff transition enthalpy and model-independent calorimetric enthalpy ΔH_{cal} provides insight into the nature of the transition [13]. The ratio of the van't Hoff and calorimetric enthalpies is smaller than 1 in all cases given in Table 1. The disagreement between the two enthalpies does indicate that the thermal denaturation of β-lactoglobulin cannot be regarded as a two-state transition, i.e., that the concentration of states intermediate between native and denatured is not negligibly small [7,13]. This disagreement is not surprising since it is known that β-lactoglobulin in aqueous solutions undergoes association and aggregation. At pH 2 and room temperature it is predominantly in the monomeric form, whereas around pH 7 it is in the dimeric form. Increasing the temperature shifts the equilibrium towards the monomeric form. Around 55°C the dimers are dissociated. Above 80°C unfolded species that associate appear, and at still higher temperatures aggregation that involves the sulfhydryl and disulfide groups sets in [3]. Temperature, pH and denaturant concentration thus determine the initial and the final state of protein molecules. As a consequence, majority of final states that correspond to given T, pH and denaturant concentration are not random coils; the protein molecules are not completely unfolded. However, in 8 M urea solutions at 25°C most globular proteins are known to be randomly coiled [7]. The question now is if the final state in termal denaturation in aqueous ureas and alkylureas at lower concentrations is close to random coils. Since the process is a combination of thermal and solvent denaturation, a positive answer seems reasonable. However, one has to admit that IR studies at pH 7 indicate that β-lactoglobulin during thermal denaturation up to 90°C does not unfold to a random coil before aggregating and polymerizing towards the end of the process [16].

Let us now consider the values of ΔG^0_{app} obtained from K_{app}. Around $T_{1/2}$, ΔG^0_{app} as a function of temperature shows at different concentrations of urea and alkylureas a non-linear behaviour, Fig. 5, similar to the one obtained at different pH's. The values of $\Delta G^0_{app}(T)$ as a func-

tion of temperature can be fitted to the polynomial

$$\Delta G^0_{app}(T) = \Delta G^0_{app}(T_{1/2}) + A(T - T_{1/2}) + B(T - T_{1/2})^2 \quad (7)$$

in which $\Delta G^0_{app}(T_{1/2})$ at the half-transition is zero. The A and B values for β-lactoglobulin at different concentration of urea and alkylureas and at different pH's are presented in Table 2. The stability curve (eq. 7) has some general features: ΔH^0 can be calculated by using the Gibbs–Helmholtz equation in the form $[\delta(G^0/T)/\delta(1/T)]_P = \Delta H^0$, the curvature is given by $(\delta^2 \Delta G^0/\delta T^2)_P = -\Delta C^0_P/T$, where ΔC^0_P is the difference in standard molar heat capacity of the denatured and native protein, and the slope of the curve is given by $(\delta \Delta G^0/\delta T)_P = -\Delta S^0$ [17]. The second derivative of eq. (7) was used for estimating $\Delta C^{0,app}_P$ at the temperature of half-transition. The values of $\Delta C^{0,app}_P$ are given in Table 2.

Examination of Table 2 reveals that the $\Delta C^{0,app}_P$ values are of expected sign and order of magnitude [3]. They are positive and decrease with increasing denaturant concentration. The error involved is estimated to be around 30% since $\Delta C^{0,app}_P$ is a second derivative of eq. (7). In this respect calorimetry (DSC) is superior, the error being a few percent only. Similarly as in the case of ΔH we could also in the case of ΔC_P apply the ratio $\Delta C^{0,app}_P/\Delta C^{cal}_P$ as a test for two-state behavior for which it is 1. However, the calorimetric ΔC^{cal}_P data are available only for water and a few urea solutions [3]. ΔG^0_{app} is another variable that can be used to test for two-state behavior. Considering the ΔC_P and ΔH test the ratio $\Delta G^0_{app}/\Delta G_{cal}$ is expected to be less than 1. ΔG^0_{app} is obtainable from eq. (7) but data on ΔG_{cal} are scarce. Similarly as in the case of ΔC^{cal}_P they are available only for β-lactoglobulin in aqueous solutions at different pH [3] and a few urea solutions. Using this existing data for aqueous solutions of β-lactoglobulin the ratio

Table 2

Values of coefficients for the Gibbs free energy change upon unfolding of β-lactoglobulin at different pH values and at different aqueous urea and alkylurea solutions in eq. (7), where ΔG^0_{app} is in J mol^{-1}. The heat capacity change, $\Delta C^{0,app}_P$, at temperature of the half transition in kJ mol^{-1} K^{-1}

Solvent	$-A$ (J mol^{-1} K^{-1})	$-B$ (J mol^{-1} K^{-2})	$\Delta C^{0,app}_P$ (kJ mol^{-1} K^{-1})
pH			
2.0	880±90	7.7±0.8	5.4±1.6
6.5	630±60	8.5±0.9	6.0±1.8
7.4	520±50	9.1±0.9	6.2±1.9
Urea			
2 *M*	530±50	8.3±0.8	5.7±1.7
3 *M*	350±40	8.3±0.8	5.5±1.7
Methylurea			
2 *M*	570±60	7.9±0.8	5.4±1.6
3 *M*	520±50	6.9±0.7	4.5±1.3
4 *M*	500±50	5.8±0.6	3.7±1.1
N,N'-Di-methylurea			
2 *M*	560±60	7.2±0.7	4.8±1.5
3 *M*	510±50	6.8±0.7	4.2±1.3
4 *M*	480±50	6.5±0.7	4.0±1.2
5 *M*	420±40	3.4±0.3	–
Ethylurea			
2 *M*	520±50	6.3±0.6	4.1±1.2
3 *M*	480±50	6.2±0.6	3.9±1.2
4 *M*	430±40	6.3±0.6	3.8±1.1

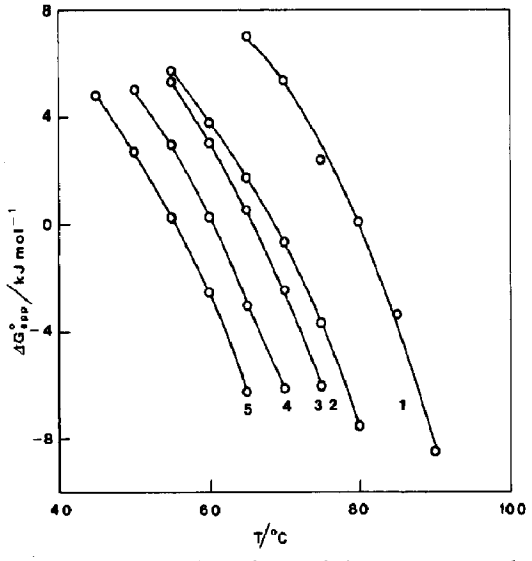

Fig. 5. Temperature dependence of the apparent standard Gibbs free energy change, ΔG^0_{app}, for the termal unfolding of β-lactoglobulin in different 2 *M* alkylurea solutions and in water. 1 – water, 2 – urea, 3 – methylurea, 4 – *N,N'*-dimethylurea, and 5 – ethylurea solution.

$\Delta G^0_{app}/\Delta G_{cal}$ is about 0.7. Therefore the transition cannot be considered a two-state one which is in accord with conclusions based on enthalpy behaviour.

From the thermodynamic point of view the reversibility of thermal denaturation in the presence of ureas is of considerable importance since it allows thermodynamic analysis of denaturation. In previous studies, where the protein concentration was between 1 and 2%, the observed 50 to 60% reversibility of thermal denaturation at pH 2.0 was very likely due to aggregation of unfolded protein molecules [3]. The results obtained in this study, where a protein concentration is only 0.2%, indicate that the reversibility of denaturation is about 90%. In other words the initial-to-final state transition observed for the β-lactoglobulin may be considered in the first approximation as a reversible one.

Recently, a DSC study of thermal and cold denaturation of β-lactoglobulin was reported [18]. Aqueous solutions at pH 2.0 (0.1 *M* KCl/HCl) were investigated. ΔH_{cal} at $T_{1/2}$ (78°C) was found to be 312 ± 15 and ΔH^0_{app} 319 ± 15 kJ mol^{-1} so that R is 1.02. ΔC_P^{cal} was 5.58 ± 0.7 kJ mol^{-1}K^{-1}. On the basis of these data one can conclude that the transition is a two-state one. The ΔH_{cal} at pH 2.0 from our previous study is 414 ± 40 kJ mol^{-1} at $T_d = 82.3$°C [3]; ΔH^0_{app} was not determined. To obtain the corresponding R value ΔH^0_{app} has to be known at the same temperature. Therefore the ΔH^0_{app} value determined in the present work of 262 kJ mol^{-1} at $T_{1/2} = 81.4$°C was adjusted to $T_{1/2} = 82.3$°C using the $\Delta C_P^{0,app}$ value presented in Table 2. This procedure was repeated for all the transitions studied. The obtained R values between 0.56 and 0.75 indicate that none of these transitions can be considered a two-state one. The low R values we report may be due to high ΔH_{cal} values determined in our previous study [3,4]. The protein concentration used in this work was in the range between 1 and 2% where a substantial association of protein molecules in native form can take place giving a positive contribution to ΔH_{cal} and thus leading to lower R values. Such difficulty can be overcome by using a more sensitive DSC instrument in which much lower ΔH_{cal} values can be determined and therefore much lower concentrations of proteins can be used. When more calorimetric data on β-lactoglobulin at low concentrations become available perhaps new conclusions will be arrived at. The situation is different with ΔH^0_{app}. In the first case the same calorimetric data have been used to calculate ΔH_{cal} and ΔH^0_{app} [18]. Thus the two quantities have nearly identical values. In our work ΔH^0_{app} has been obtained by absorbance measurement. The process of protein unfolding was monitored by following the absorbance at 293 nm where absorbance is by and large due to two Trp residues out of 164 aminoacid residues present in the molecule. In other words, the absorbance measurements at this wavelength reflect increasing exposure and solvation of Trp, that is they reflect only local conformational changes which may not accurately monitor the global denaturation event. To obtain apparent thermodynamic quantities of unfolding from these measurements one has to assume, therefore, that the other effects on the measured absorbance are negligibly small.

Changes in solvent composition of aqueous solutions of chromophoric materials generally cause small shifts in the absorption spectra peaks, $A(\lambda)_{max}$, of the chromophores. Such shifts have been attributed to changes of solvation energies of the ground and excited states, respectively. Although a detailed interpretation of the reasons behind these changes is complex, it is fairly certain that the forces responsible must be operative over short distances compared to the rather large dimensions of protein molecules [19]. Our UV spectra show that urea and all alkylureas shift both maxima (around 230 nm and 280 nm) with increasing denaturant concentration to longer wavelengths (red shift). In Fig. 2 the UV difference spectra of β-lactoglobulin in aqueous solutions are shown. It is seen that there is no difference in the positions of Trp (around 294 nm) and Tyr (around 287 nm) between 6 *M* and 8 *M* urea at 20°C indicating that β-lactoglobulin is already completely denatured in 6 *M* urea. At 294 nm a negligible red shift in difference spectra peaks, $\Delta A(\lambda)_{max}$, is observed and the intensity of peaks decrease with increasing concentration of urea. In methylurea UV difference spectra $\Delta A(\lambda)_{max}$

of β-lactoglobulin show a slight red shift and decreasing intensity in the position of Tyr (around 287 nm) and of Trp (around 294 nm) between 3 *M* and 4 *M* denaturant solutions. In *N,N'*-dimethylurea and ethylurea UV difference spectra $\Delta A(\lambda)_{max}$ show again a negligible red shift and decreasing intensity in the position of Tyr and Trp with increasing denaturant concentration.

It has been known that important factors determining the position of a peak and therefore the direction of any shift in $A(\lambda)_{max}$ are the refractive index, the dielectric constant and the density of the solvent as well as specific solvent–solute interactions, e.g., hydrogen bonding [11,20,21]. The excited state of the solute is usually more polarizable than the ground state, dispersion forces between the solvent and both states are therefore stronger for the excited state and stabilize it preferentially. This leads to the well-known "polarization red shift" which increases with the refractive index (polarizability) of the solvent [11,20,21]. The refractive index of urea and alkylurea solutions increases with increasing denaturant concentration therefore the observed red shift in $A(\lambda)_{max}$ in all urea and alkylurea solutions can be attributed to the increasied solvent polarizability. Similarly, the observed red shift in $A(\lambda)_{max}$ caused by decreasing pH of protein solution can be explained in terms of difference in the strength of hydrogen bonding to the chromophore excited and ground state which increases with decreasing pH [21].

With increasing temperature from 20 to 100°C UV spectra of β-lactoglobulin in all aqueous urea and alkylurea solutions as well as at different pH's display a red shift in $A(\lambda)_{max}$. Such behaviour was observed even in 8 *M* urea. UV difference spectra of β-lactoglobulin in 8 *M* urea at different temperature are given in Fig. 6. An increase in temperature decreases the factors determining the position of a peak in $A(\lambda)_{max}$ (refractive index, density, the dielectric constant) and therefore reduces the extent of any temperature shift. Decreasing the refractive index and density acts against the polarization red shift by weakening the solute–solvent interactions and leads to a blue shift. Measurements with Tyr and Trp models show, however, that heating them in aqueous solutions produces a distinct red shift in $A(\lambda)_{max}$ although the accompanying decrease of the solvent refractive index, dielectric constant and density should lead to a blue shift. The Tyr and Trp in dioxane, where no hydrogen bonding can occur, show blue shift in $A(\lambda)_{max}$ with increasing temperature while in water and ethanol again a red shift is observed [21]. In aqueous solution the red shift in $A(\lambda)_{max}$ of Tyr and Trp with increasing temperature is attributed to hydrogen bonds, where Tyr acts as a hydrogen donor to water [21]. The observed minor red sifts in UV spectra $A(\lambda)_{max}$ of β-lactoglobulin in aqueous urea and alkylurea solutions might be in line with such explanation.

Interpretation of the observed slight red shift in $\Delta A(\lambda)_{max}$ is more complex because of the varying contributions of spectral broadening and change in absorption in different solvents [11].

Summing up, it may be claimed that UV spec-

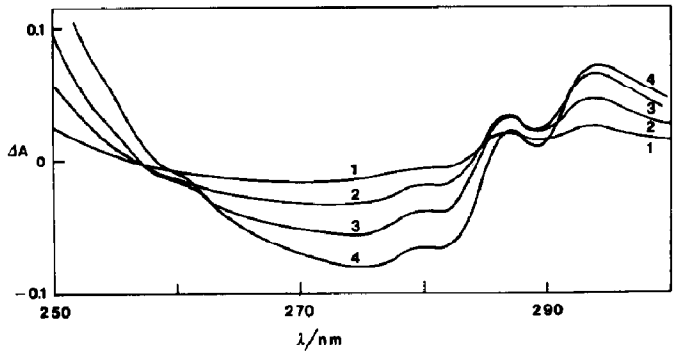

Fig. 6. UV difference spectra of β-lactoglobulin in 8 *M* urea at different temperature (reference was β-lactoglobulin in 8 *M* urea at 20°C). 1 – 40°C, 2 – 60°C, 3 – 80°C, and 4 – 100°C.

troscopy has proved useful for studying the thermal denaturation of β-lactoglobulin in the presence of urea and alkylureas. The method is simple and relatively accurate, however, one should not forget that it can give only apparent, i.e., nonthermodynamic quantities of conformational transition. A detailed analysis of the utility of apparent quantities has been given elsewhere [7].

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