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# Thermodynamic denaturation of $\beta$ -lactoglobulin in the presence of cetylpyridinium chloride

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## Abstract

In this work, we determined the stability parameters of bovine  $\beta$ -lactoglobulin, variant A, (BLG-A), in relation to their transition curves induced by cetylpyridinium chloride (CPC) as a cationic surfactant. The experiments took place over the temperature range of 298 K to 358 K. For each transition curve at any specific temperature, the conventional method of analysis, which assumes a linear concentration dependence of the pre- and post-transition base lines, gave the most realistic values for  $\Delta G_D(\text{H}_2\text{O})$ . Results show that the minimum value of  $\Delta G_D(\text{H}_2\text{O})$  occurs at  $T = 328$  K. Using the Gibbs–Helmholtz equation, the values of enthalpy,  $\Delta H_D$ , and entropy,  $\Delta S_D$ , of denaturation have been calculated considering temperature dependence of  $\Delta G_D$  at any specified concentration of CPC. The values of  $12.05 \text{ kJ} \cdot \text{mol}^{-1}$ ,  $18.54 \text{ kJ} \cdot \text{mol}^{-1}$ , and  $18.32 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ , were obtained for  $\Delta G_D(\text{H}_2\text{O})$ ,  $\Delta H_D(\text{H}_2\text{O})$ , and  $\Delta S_D(\text{H}_2\text{O})$ , respectively. The results show that the enthalpy term dominates the entropy term.

## Keywords

$\beta$ -Lactoglobulin

Cetylpyridinium chloride

Denaturation

UV–Vis

Protein stability

## 1. Introduction

The protein  $\beta$ -lactoglobulin (BLG) is the major protein of whey ruminant milk [1] and it is a member of the family so-called lipocalins. Its ease of purification and importance in the food industry explain in part the huge amount of literature pertaining to it. BLG is a small globular protein with well known structure [2], [3]. It exists at neutral pH as a dimer. Each monomer is constituted by 162 amino acids with molar mass of about 18,400 Da. This small globular protein has a three-dimensional structure consisting of one  $\alpha$ -helix and nine anti-parallel  $\beta$ -strands, with eight  $\beta$ -sheets folded into a cone-shaped barrel forming a hydrophobic pocket [4].

In order for the protein to become biologically active, it must fold and adopt one of an enormous number of possible conformations. This conformation, which we will refer to as the native state,

exists in solution as a very compact, highly ordered structure. The parameters that determine the native state stability in aqueous solutions are the amino acid sequence of the protein as well as the variable conditions of pH, temperature, and the concentrations of salts and ligands [5], [6]. The unfolding of proteins by the classical chemical denaturants, such as urea and guanidine hydrochloride (GdnHCl), has long been considered to arise because of the favourable interactions of these reagents with the protein segments [7], [8]. These denaturants can influence not only the protein unfolding, but also the ensemble of the native structure [9], [10], [11], [12], [13].

Interaction of surfactants with globular proteins was the subject of many research studies in recent years [14], [15], [16], [17], [18]. One of the important aspects of these studies is the denaturizing power of some ionic surfactants. In a few cases, the sigmoidal denaturation curves were obtained due to interaction of ionic surfactants with proteins [14], [15]. In these cases, the analysis of transition curves by using conventional method of analysis was possible, and thermodynamic parameters of protein stability were estimated.

Recently, the interaction of BLG with some ionic and non-ionic surfactants was studied by Taheri-Kafrani *et al.* [17], [18]. They showed that BLG has conformational changes during its interaction with dodecyltrimethyl ammonium bromide (DTAB), sodium *n*-dodecyl sulphate (SDS) and Triton X-100 while retinol binding properties of BLG do not show significant changes in the presence of these surfactants. However, the variation of structural index parameters of BLG *versus* these surfactants concentration did not show sigmoidal feature, so that no cooperative unfolding transitions have been detected.

Here, we report a study of interaction between BLG and cetylpyridinium chloride (CPC) as a denaturant cationic surfactant using UV–Vis spectrophotometry technique at various temperatures. The sigmoidal transition curves were obtained for BLG due to its interaction with CPC. This enables us to evaluate the thermodynamic stability of BLG at each temperature. Furthermore,  $\Delta H_D$  and  $\Delta S_D$  of protein denaturation were estimated using known Gibbs–Helmholtz equation. These valuable data provide useful information about conformational stability of BLG and forces which contribute to its stability.

## 2. Experimental

### 2.1. Materials

The  $\beta$ -lactoglobulin genetic variant A and CPC were purchased from Sigma Chemical Co., and used without further purification. All salts used for buffer preparation were analytical grade and dissolved in double distilled water. All of the solutions were used freshly after preparation.

### 2.2. Methods

The concentration of BLG was determined experimentally by using the value of  $17,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$  for the molar absorption coefficient ( $\epsilon$ ) at 279 nm [19]. For optical measurements, all solutions were prepared in 50 mM phosphate buffer at pH, 8.0. The CPC denaturation curves were determined by measuring the maximum absorption of the solutions containing BLG using a Jasco model V-730 spectrophotometer and 1-cm cuvettes thermostated to maintain the temperature at  $\pm 0.1$  K. In typical experiments,  $2.0 \text{ cm}^3$  of BLG solution were placed in the cuvette. The absorbance spectra were recorded between 200 nm and 400 nm after each addition of CPC stock solutions (1 mM), and the observed absorbance was corrected for dilution.

The CPC stock solution was added to both reference and sample cells. Fluorescence spectroscopy was used to study the conformational changes of proteins containing tryptophan residues during the denaturation, because the intrinsic fluorescence of indol chromophores in tryptophan (Trp) residues is particularly sensitive to their microenvironments. The intrinsic fluorescence of aromatic amino acids in proteins has long been used as a means of monitoring unfolding/refolding transitions induced by chemical denaturants, pH changes, temperature and pressure [20]. During fluorescence measurements, the samples were put in quartz cuvettes of 1-cm optical path. In typical experiments 2.0 cm<sup>3</sup> of BLG solution were placed into the cuvette and emission spectra were recorded after each addition of CPC stock solutions. The excitation was performed at 295 nm and the emitted fluorescence was recorded between 300 nm and 400 nm. The band slits for excitation and emission were set at 5 nm. To avoid of inner filtering effect, absorbances of the BLG solutions in 279 nm should not exceed 0.1. The final CPC concentration was about 166  $\mu$ M and there is no inner filter effect due to the presence of CPC.

### 3. Results and discussion

Relative emission intensity ( $F/F_0$ ) at 330 nm in fluorescence spectra of BLG solution at various mole ratio of [CPC]/[BLG] is shown in figure 1, where  $F$  and  $F_0$  are the emission intensities in the presence and absence of CPC, respectively. The fluorescence properties of tryptophan residues apparently are sensitive to perturbations of protein structure. Interaction of CPC with BLG changes the environment of tryptophan residue and causes to fluorescence quenching. The sigmoid feature of this curve can be related to the characteristic of a two-state mechanism. However, this also represents the denaturation power of CPC that induces a cooperative unfolding in tertiary structure of BLG. The CPC denaturation curves for BLG are shown in figure 2. In all cases, we measured the difference absorption ( $\Delta\epsilon$ ) at  $\lambda_{\max}$ . The Gibbs free energy of denaturation,  $\Delta G_D$ , was calculated as a function of CPC concentration by assuming a two-state mechanism [21], [22] and using the following equations [23]:

$$F_D = (y_N - y_{\text{obs}})/(y_N - y_D), \quad (1)$$

$$\Delta G_D = -RT \ln K = -RT \ln(F_D/(1 - F_D)) = -RT \ln[(y_N - y_{\text{obs}})/(y_{\text{obs}} - y_D)], \quad (2)$$

where  $K$  is the equilibrium constant,  $R$  is the gas constant,  $y_{\text{obs}}$  is the observed optical property,  $y_N$  and  $y_D$  are the optical properties of the native and denatured protein molecules, respectively. The latter two have been extrapolated from the pre- and post-transition baselines to the considered denaturant concentration. These pre- and post-transition baselines were determined by a linear fit of the corresponding data before and after the transition, according to the following equations:

$$y_N = a_N + b_N[\text{CPC}], \quad (3)$$

$$y_D = a_D + b_D[\text{CPC}]. \quad (4)$$

The estimated values of  $a_N$ ,  $b_N$ ,  $a_D$  and  $b_D$  are given in table 1.

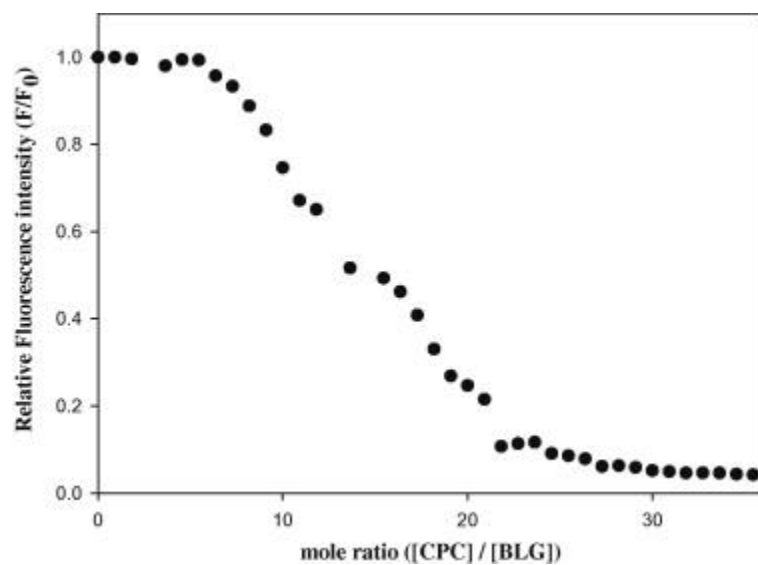


FIGURE 1. Plot of the variation of relative fluorescence intensity of BLG solution at 330 nm *versus* mole fraction ratio of [CPC]/[BLG]. The excitation wavelength was 295 nm and band slits were 5 nm for both excitation and emission wavelengths.

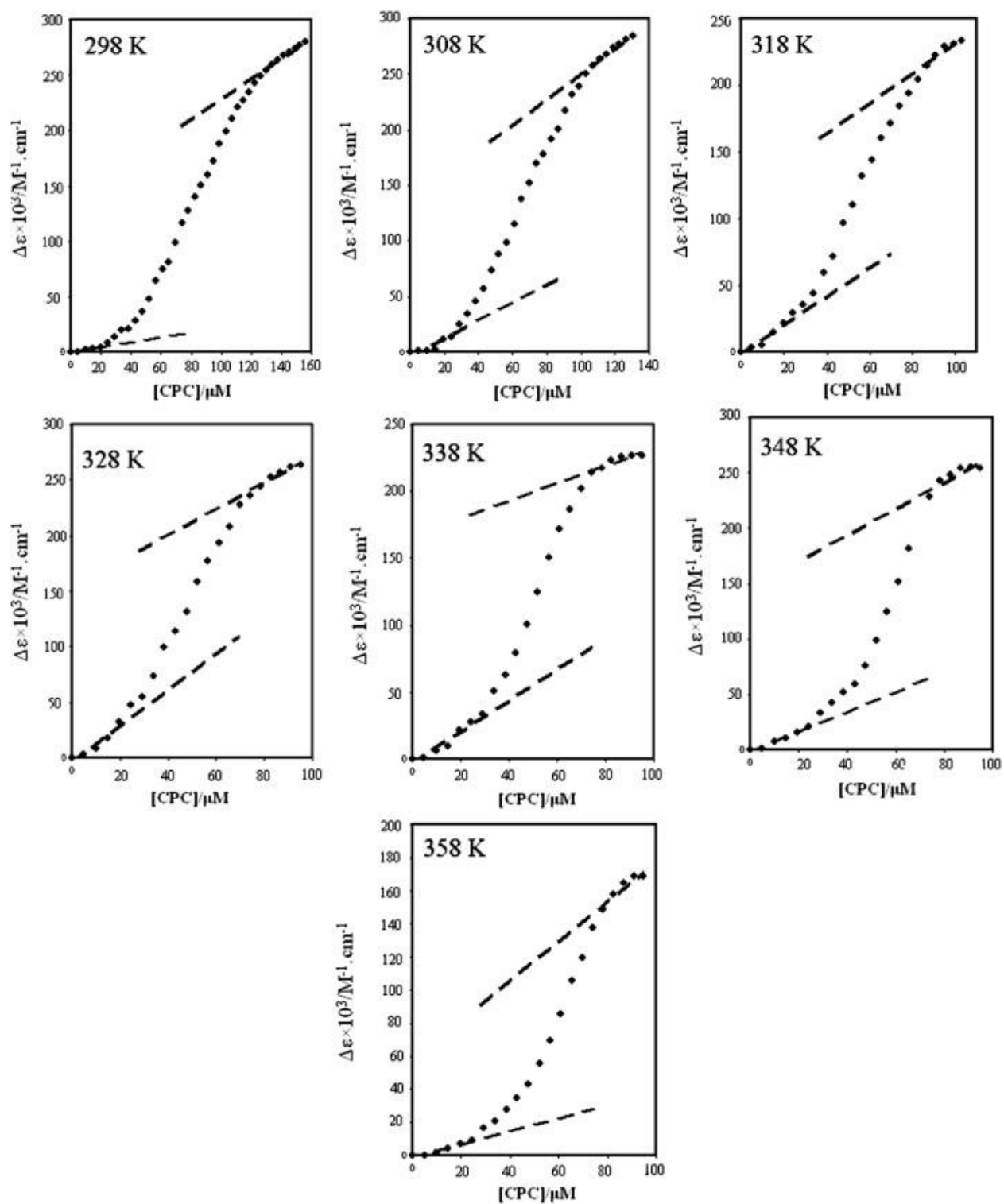


FIGURE 2. Plot of  $\Delta\epsilon$  against concentration of CPC for the denaturation curve of  $\beta$ -lactoglobulin in 50 mM phosphate buffer, pH, 8.0 and various temperatures. The absorbances were measured at  $\lambda_{\text{max}}$ .

TABLE 1. Coefficients of the concentration dependencies of  $y_N$  and  $y_D$ .

$T/K$	$a_N/(M^{-1} \cdot cm^{-1})$	$b_N/(M^{-2} \cdot cm^{-1})$	$a_D/(M^{-1} \cdot cm^{-1})$	$b_D/(M^{-2} \cdot cm^{-1})$
298	-416.1	$2.0 \cdot 10^8$	135,548	$9.0 \cdot 10^8$
308	-4287.4	$8.0 \cdot 10^8$	135,158	$1.0 \cdot 10^9$
318	-2041.5	$1.0 \cdot 10^9$	118,920	$1.0 \cdot 10^9$
328	-3704.6	$2.0 \cdot 10^9$	152,828	$1.0 \cdot 10^9$
338	-3624.4	$1.0 \cdot 10^9$	165,610	$7.0 \cdot 10^8$
348	-1771.7	$9.0 \cdot 10^8$	145,733	$1.0 \cdot 10^9$
358	-1368.9	$4.0 \cdot 10^8$	56,825	$1.0 \cdot 10^9$

Figure 3 shows the Gibbs free energy of denaturation,  $\Delta G_D$  (which is calculated from equation (2) based on the data of figure 2), which varies linearly with CPC concentration. The  $\Delta G_D$  was plotted against [CPC] and a linear least-squares analysis was used to fit the  $\Delta G_D$  and [CPC] data to the following relation [24]:

$$\Delta G_D = \Delta G_D(H_2O) - m[CPC], \quad (5)$$

where  $\Delta G_D(H_2O)$  is the value of  $\Delta G_D$  at 0 M denaturant and  $m$  is the slope of the curves which gives the linear dependence of  $\Delta G_D$  on CPC concentration. In addition, at the transition concentration,  $[S]_{1/2}$ , we have:

$$[N] = [D], \quad F_D = 0.5, \quad K = 1 \quad \text{and} \quad \Delta G_D = 0.$$

Thus, at the midpoint of the transition we get:

$$\Delta G_D(H_2O) = m[S]_{1/2}. \quad (6)$$

Unfolding of BLG in an isothermal chemical denaturation process can be divided into thermodynamically defined stages according to the scheme below [14].

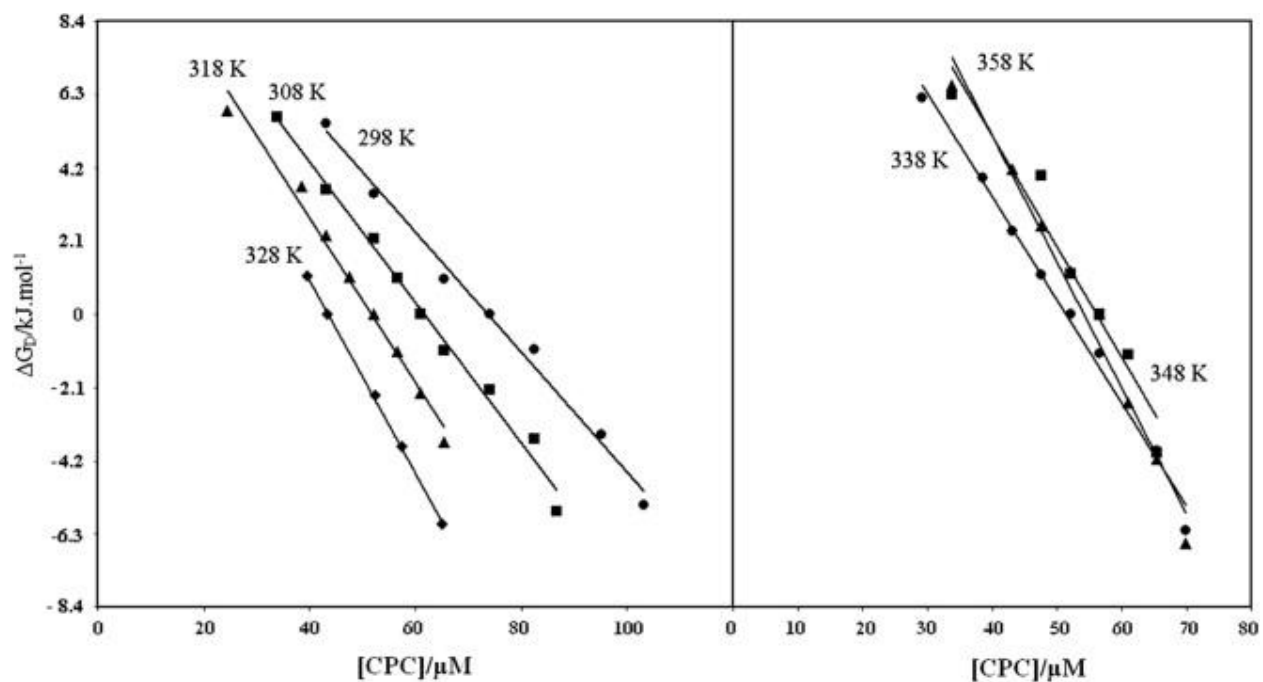
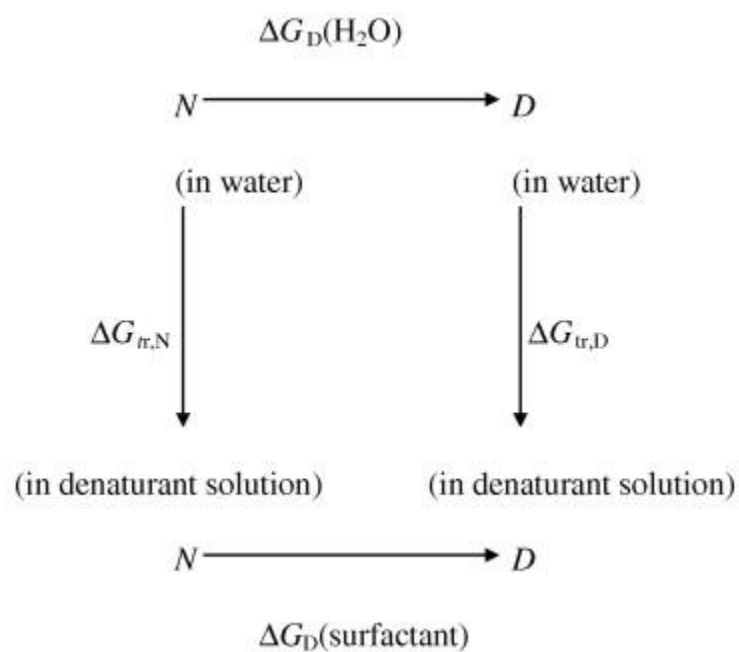


FIGURE 3. Plot of  $\Delta G_D$  versus CPC concentration at various temperatures.



It follows that  $\Delta G_D$ , for example, for the process of BLG (in water)  $\rightarrow$  BLG (in surfactant) is given by the relationship:  $\Delta \diamond_{tr,N} + \Delta \diamond_D(\text{surfactant}) = \Delta \diamond_D(\text{H}_2\text{O}) + \Delta \diamond_{tr,D}$ ,



$$\Delta G_{tr,N} + \Delta G_D(\text{surfactant}) = \Delta G_D(\text{H}_2\text{O}) + \Delta G_{tr,D},$$

or

$$\Delta G_{tr} = \Delta G_D(\text{surfactant}) - \Delta G_D(\text{H}_2\text{O}) = \Delta G_{tr,D} - \Delta G_{tr,N}, \quad (7)$$

where  $\Delta G_{tr,N}$  and  $\Delta G_{tr,D}$  represent the transfer Gibbs free energies of native and denatured states of BLG from water to surfactant solution at a given concentration of surfactant. The  $\Delta G_D(\text{surfactant})$  is the Gibbs free energy change for this process:

Native BLG(in surfactant solution)  $\rightarrow$  Denatured BLG(in surfactant solution).

Figure 4 shows the linearity of the plot of  $\Delta G_D$  versus  $\ln[\text{CPC}]$ . From the least-squares analysis of these plots, the Gibbs free energy in the surfactant solution,  $\Delta G_D(\text{surfactant})$ , was obtained at  $\ln[\text{CPC}] = 0$ . A concentration of CPC of 1 M is in good agreement for the transition of the native state of protein (from water) to the denatured state of protein (to surfactant solution).

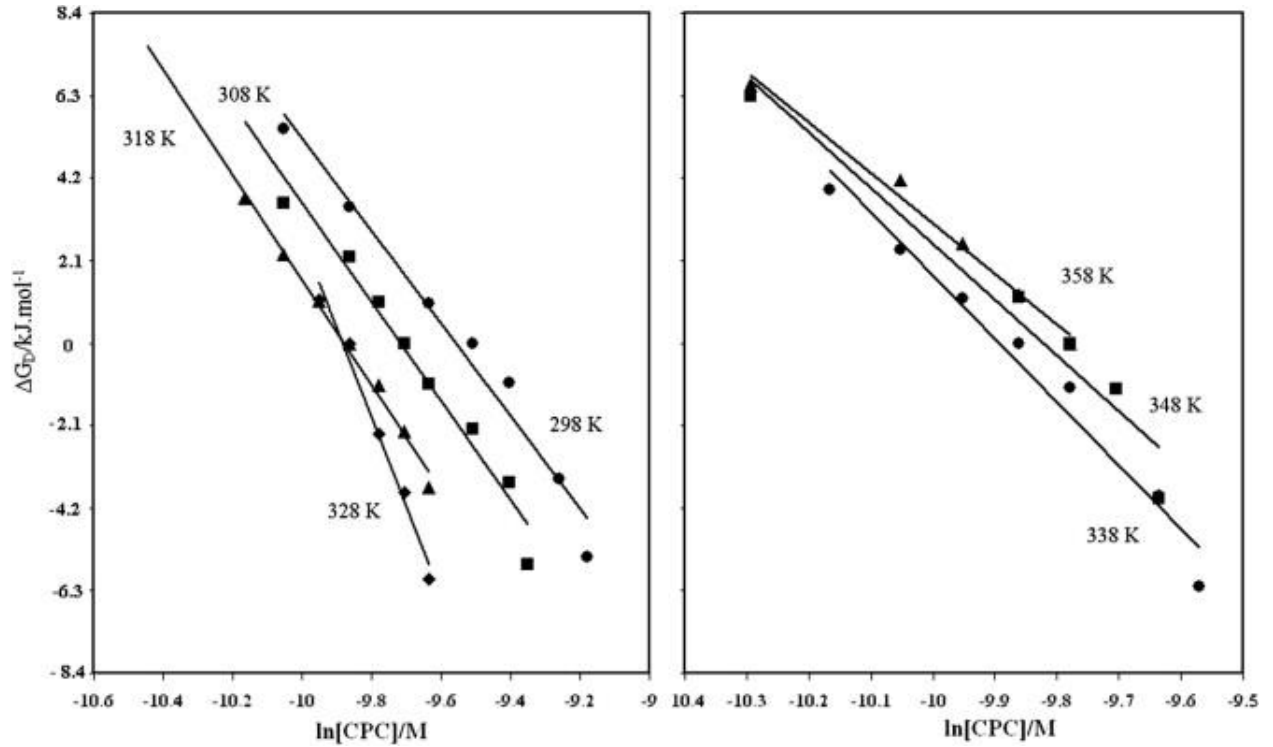


FIGURE 4. Plot of  $\Delta G_D$  versus  $\ln[\text{CPC}]$  at various temperatures.

We can use the Gibbs–Helmholtz equation to calculate the  $\Delta H_D$  and then  $\Delta S_D$ :

$$(\partial(\Delta G_D/T)/\partial(1/T))_P = \Delta H_D, \quad (8)$$

$$(\partial\Delta G_D/\partial T)_P = -\Delta S_D. \quad (9)$$

FIGURE 5, FIGURE 6 show the variation of  $\Delta G_D/T$  versus  $1/T$  and the  $\Delta G_D$  versus  $T$ , respectively. The values of  $\Delta H_D$  and  $\Delta S_D$  were obtained from the slope of curves in FIGURE 5, FIGURE 6, respectively. As the protein denaturation is an endothermic process, so  $\Delta H_D$  increases with increasing concentration of surfactant. The intramolecular bonds in the protein break on interaction with the surfactant. Increasing the surfactant concentration causes more intermolecular interactions and higher values of  $\Delta H_D$  [25]. The  $\Delta H_D(\text{H}_2\text{O})$  and  $\Delta S_D(\text{H}_2\text{O})$  are obtained from extrapolating the  $\Delta H_D$  and  $\Delta S_D$  to  $[\text{CPC}] = 0$ . The values of  $18.54 \text{ kJ} \cdot \text{mol}^{-1}$  and  $18.32 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$  were obtained for  $\Delta H_D(\text{H}_2\text{O})$  and  $\Delta S_D(\text{H}_2\text{O})$ , respectively. These values represent the enthalpy term dominates the entropy term.

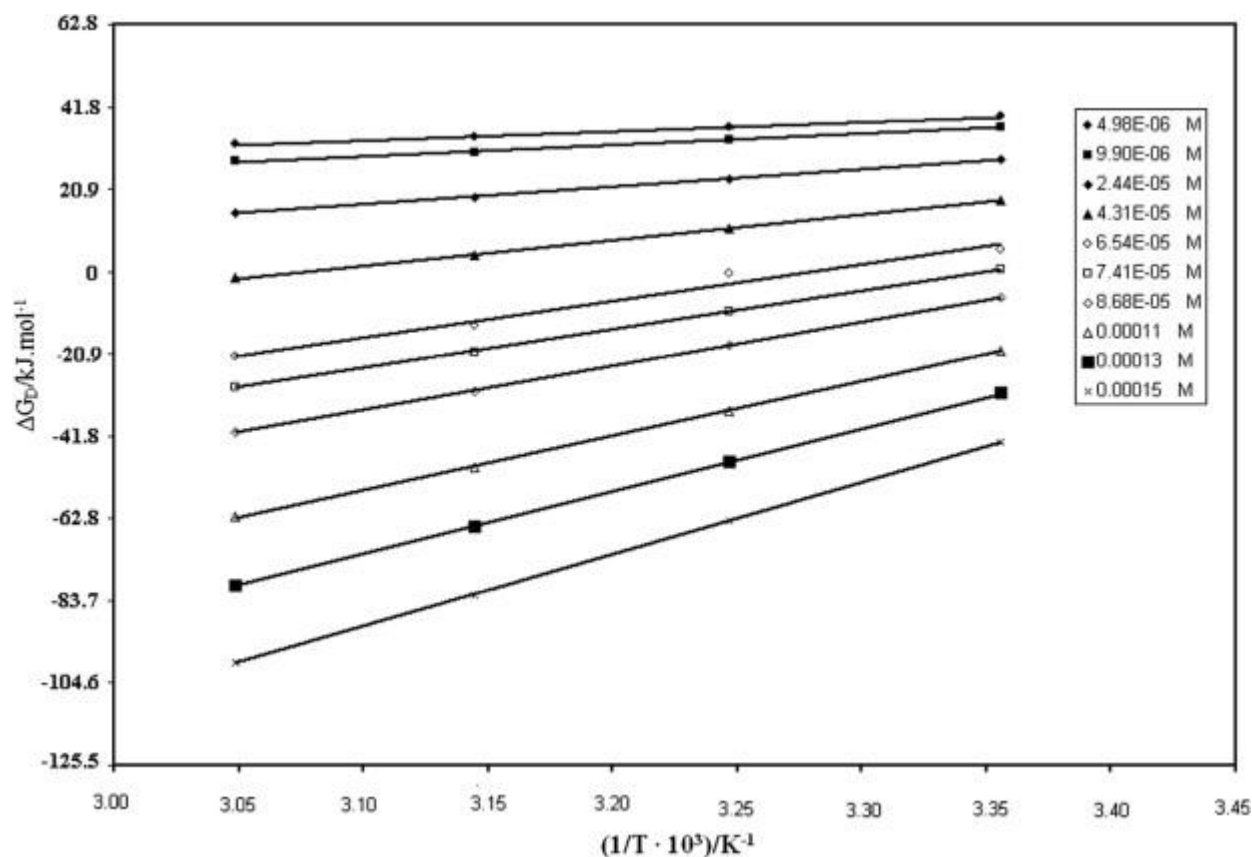


FIGURE 5. Plot of  $\Delta G_D/T$  versus  $1/T$  at various CPC concentrations.

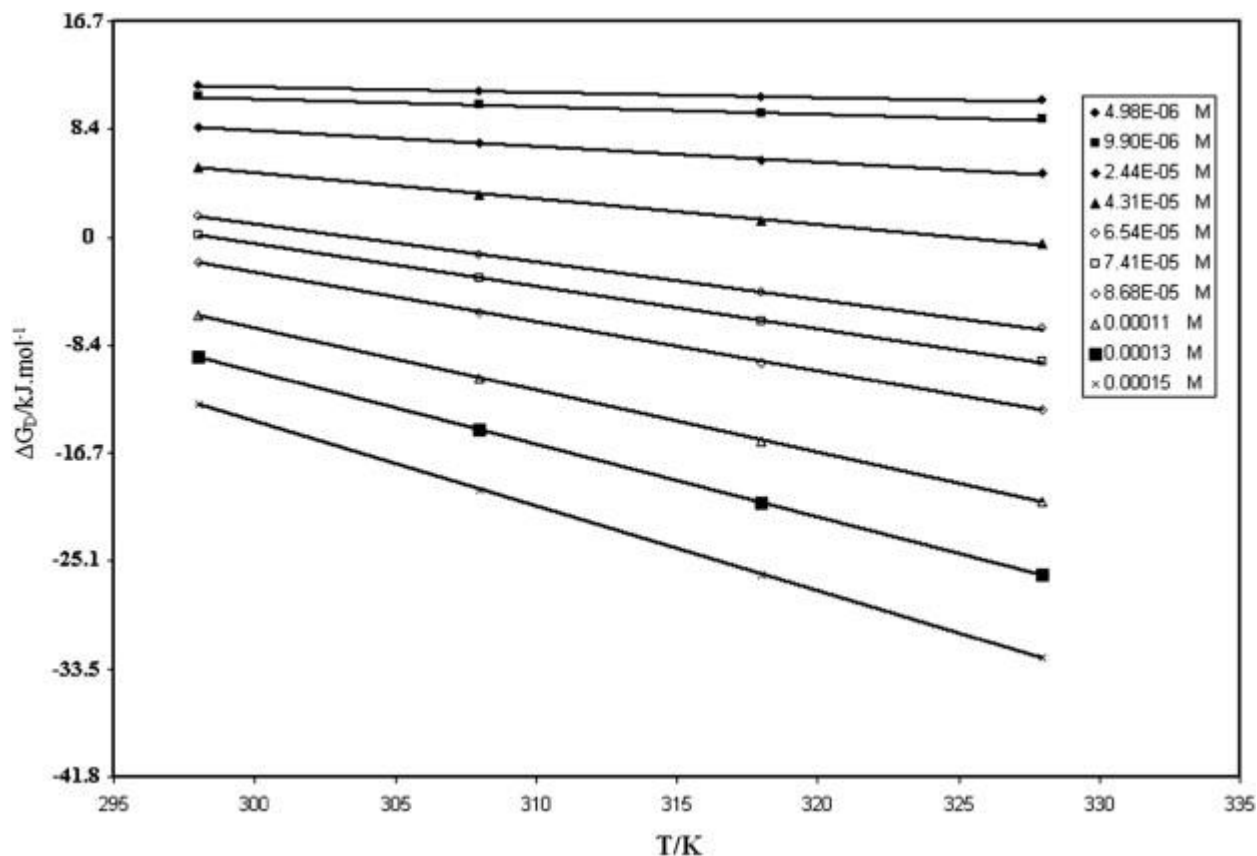


FIGURE 6. The variation of  $\Delta G_D$  versus  $T$  at various CPC concentrations.

The estimates of  $\Delta G_D(\text{surfactant})$ ,  $\Delta G_D(\text{H}_2\text{O})$ ,  $\Delta G_{tr}$  and  $[S]_{1/2}$  are shown in table 2. The  $\Delta G_D(\text{H}_2\text{O})$  has positive values but  $\Delta G_D(\text{surfactant})$  and  $\Delta G_{tr}$  have negative values. The  $\Delta G_D(\text{H}_2\text{O})$  shows unusual changes that have not been reported for any other proteins before. The maximum in  $\Delta G_D(\text{H}_2\text{O})$  occurs at  $T = 358 \text{ K}$  after the minimum at  $T = 328 \text{ K}$ , *i.e.* minimum stability is at  $T = 328 \text{ K}$ . Upon heating, BLG undergoes intramolecular and intermolecular changes. Raising the temperature shifts the BLG (monomer + dimer) equilibrium at BLG concentrations below  $10 \text{ g} \cdot \text{dm}^{-3}$  toward monomers [26], [27]. Upon heating above  $T = 333 \text{ K}$ , the molecule undergoes conformational changes and partially unfolds (*i.e.* it denatures); the nonpolar groups and the thiol group become exposed [28]. Denaturation can be followed by an irreversible aggregation reaction so that the whole heat-induced process becomes irreversible [29]. So, above  $T = 328 \text{ K}$  in the presence of CPC, aggregation of the protein molecules causes increasing of stability up to  $T = 358 \text{ K}$ . Thus BLG has minimum stability at  $T = 328 \text{ K}$  in the absence of CPC. The values of  $\Delta G_D(\text{surfactant})$  show that the minimum stability at the presence of surfactant occurs at  $T = 328 \text{ K}$  (the protein is completely surrounded by denaturant). The  $\Delta G_{tr}$  is also less negative at this temperature which is the best state for a denatured protein.

TABLE 2. Parameters characterizing the CPC denaturation for  $\beta$ -lactoglobulin at various temperatures.

$T/K$	$m/(kJ \cdot mol^{-1} \cdot M^{-1})$	$\Delta G_D(H_2O)/(kJ \cdot mol^{-1})$	$\Delta G_D(\text{surfactant at 1 M of CPC})/(kJ \cdot mol^{-1})$	$\Delta G_{tr}/(kJ \cdot mol^{-1})$	$[CPC]_{1/2}/M$
298	167.36	12.59	-112.13	-124.72	$7.53 \cdot 10^{-5}$
308	209.20	12.34	-122.05	-134.39	$5.90 \cdot 10^{-5}$
318	251.04	12.09	-131.80	-143.89	$4.82 \cdot 10^{-5}$
328	292.88	12.05	-222.67	-234.76	$4.12 \cdot 10^{-5}$
338	292.88	15.15	-159.20	-174.30	$5.17 \cdot 10^{-5}$
348	334.72	17.78	-138.36	-156.15	$5.31 \cdot 10^{-5}$
358	376.56	19.54	-124.56	-144.10	$5.19 \cdot 10^{-5}$

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