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Guanidinium chloride and urea denaturations of β-Lactoglobulin A at pH 2.0 and 25 °C: The equilibrium intermediate contains non-native structures (helix, tryptophan and hydrophobic patches)

Tanveer Ali Dar [a], Laishram Rajendrakumar Singh [d], Asimul Islam [a,b], Farah Anjum [a], Ali Akbar Moosavi-Movahedi [c], Faizan Ahmad [a,b,*]

[a] *Department of Biosciences, Jamia Millia Islamia, Jamia Nagar, New Delhi-110025, India*
[b] *Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi-110 025, India*
[c] *Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran*
[d] *Division of Population Science, Fox Chase Cancer Center, 333, Cottman Avenue, Philadelphia, PA 19111, USA*

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Abstract

We have carried out guanidinium chloride (GdmCl) and urea denaturations of bovine β-lactoglobulin A (β-lgA) at pH 2.0 and 25 °C, using far-UV and near-UV circular dichroism, near-UV absorption and tryptophan fluorescence spectroscopies. The stable intermediate state that occurs during GdmCl denaturation has been characterized by the far- and near-UV circular dichroism, tryptophan difference absorption, tryptophan fluorescence and 8-anilino-1-naphthalene sulphonic acid binding measurements. Following conclusions have been reached. (a) Urea-induced denaturation is not a two-state process. (b) GdmCl-induced denaturation is composed of two distinct two-state processes. (c) α-Helical content, burial of tryptophan residues and burial of hydrophobic surface area are more in the GdmCl-induced stable intermediate than those originally present in the native protein.

Keywords: Protein folding; β-lactoglobulin; Non-native intermediate; Guanidinium chloride denaturation; Urea denaturation; Protein stability

1. Introduction

The α-helix to β-sheet transition of proteins is a key issue for understanding the folding and biological function of a number of proteins. For example, the α→β transition has been suggested to play an essential role in various conformational diseases, such as prion disease or Alzheimer disease where the α-helical forms are normal and the β-sheet forms are amyloidogenic [1–3]. Since the folding of β-lactoglobulin is accompanied by the α→β transition [4,5], it will, therefore, be a useful model for clarifying the mechanism of the α→β transition, which is responsible for conformational diseases.

Understanding the mechanism of protein folding is a major issue in structural biology. For most of the proteins, a collapsed state with native-like secondary structure is accumulated in the early steps of refolding [6]. In the case of small proteins, no collapsed state is accumulated and the formation of the native structure follows the nucleation process [7]. In both cases, folding is considered hierarchical, i.e., formation of the native structure without significant accumulation of non-native structures and interactions [8,9]. In the case of β-lactoglobulin, folding kinetic experiments show an α/β transition in which an intermediate with a non-native α-helix accumulates transiently in the early steps of the reaction, and the native β-sheet is formed in the subsequent steps [10]. Thus, β-lactoglobulin presents not only a case of a useful model for understanding

Abbreviations: β-lgA, β-lactoglobulin A; ε, molar absorption coefficient; $\Delta\varepsilon_{292}$, difference molar absorbance at 292 nm; $[\theta]_{222}$, mean residue ellipticity at 222 nm; ANS, 8-anilino-1-naphthalene sulphonic acid; GdmCl, guanidinium chloride; TFE, trifluoroethanol; y_N, optical property of the native state; y_D, optical property of the denatured state.

* Corresponding author. Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi-110 025, India.
E-mail address: faizan_ahmad@yahoo.com (F. Ahmad).

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various conformational diseases which are due to α/β transition, but also a case of the non-hierarchical protein folding, a striking contrast to the folding models suggested from studies of other proteins [11].

Preliminary investigations of Bigelow and co-workers [4,12], have suggested that the guanidinium chloride-(GdmCl-) induced denaturation of β-lactoglobulin A (β-lgA) is a three-step reversible process at pH 2.0 and 25 °C. However, they did not carry out the structural and thermodynamic characterization of this intermediate state. Interestingly, it has been shown recently that GdmCl-induced denaturation of β-lgA in the presence of 10% trifluoroethanol (TFE) is also a three-step process at pH 1.8 and 4 °C, and the thermodynamically stable intermediate state, which is richer in helical content than the native protein, resembles the kinetic folding intermediate [5]. It has also been suggested that this stable intermediate state induced by 2 M GdmCl possesses resemblance with the molten globule state [13]. On the contrary, urea-induced denaturation of β-lgA has been shown to be a two-state process [14,15]. Using model peptides, the differences in the GdmCl and urea denaturations of proteins have been extensively studied and discussed in terms of differences in the ionic character between GdmCl and urea [16] and references therein.

Although structural and thermodynamic features of the stable intermediate occurring during GdmCl-induced denaturation of β-lgA in the presence of 10% TFE is known at pH 2.0 and 4 °C, no conformational and thermodynamic data are available for the stable intermediate state that occurs during the denaturation of β-lgA by GdmCl in the absence of trifluoroethanol at pH 2.0 and 25 °C [4,12]. In the present study we have measured GdmCl-induced and urea-induced denaturations of the protein in the presence of different NaCl concentrations at pH 2.0 and 25 °C, and determined the structural features of the GdmCl-induced equilibrium intermediate state. We report that (a) urea-induced denaturation is not a two-state process, for the tertiary structure melts at lower concentration of the denaturant than the secondary structure; (b) GdmCl-induced denaturation is composed of two distinct two-state processes; (c) the formation of the stable intermediate state during GdmCl-induced denaturation is due to the intrinsic behavior of the salt denaturant, for the non-native helix is not observed during the urea-induced denaturation in the presence of different NaCl concentrations in the range 0–1 M; (d) the GdmCl-induced stable intermediate contains non-native helix, tryptophan and hydrophobic patches; and (e) the GdmCl-induced intermediate state does have characteristics of the molten globule state.

2. Materials and methods

Commercially lyophilized chromatographically purified bovine β-lactoglobulin A (β-lgA), was purchased from Sigma Chemical Co. Glycine and 8-anilino-1-naphthalene sulfonic acid (ANS) were also from Sigma Chemical Co. Guanidinium chloride and urea were ultra pure samples from MP Biomedicals, Inc., Ohio. NaCl was from Merck (India). These and other chemicals were analytical-grade reagents and used without further purification.

The concentration of β-lgA was determined experimentally using a value of 17,600 M^{-1} cm^{-1} for the molar absorption coefficient (ε) at 280 nm and pH 5.4 [14]. Concentration of the stock solution of ANS was determined spectrophotometrically using a value of 5000 M^{-1} cm^{-1} for ε at 350 nm [17]. For optical measurements all solutions were prepared in 0.05 M glycine-HCl buffer containing 0.1 M KCl at pH 2.0 and equilibrated overnight at room temperature.

Isothermal denaturation of β-lgA by GdmCl and urea at 25.0± 0.1 °C was measured in a Shimadzu 1601 UV/Vis spectrophotometer and in a Jasco spectropolarimeter (model J-715) equipped with a Peltier-type temperature controller (PTC-348 WI). Protein concentrations used for the absorption and circular dichroism (CD) measurements were in the ranges 7–10 μM and 18–20 μM

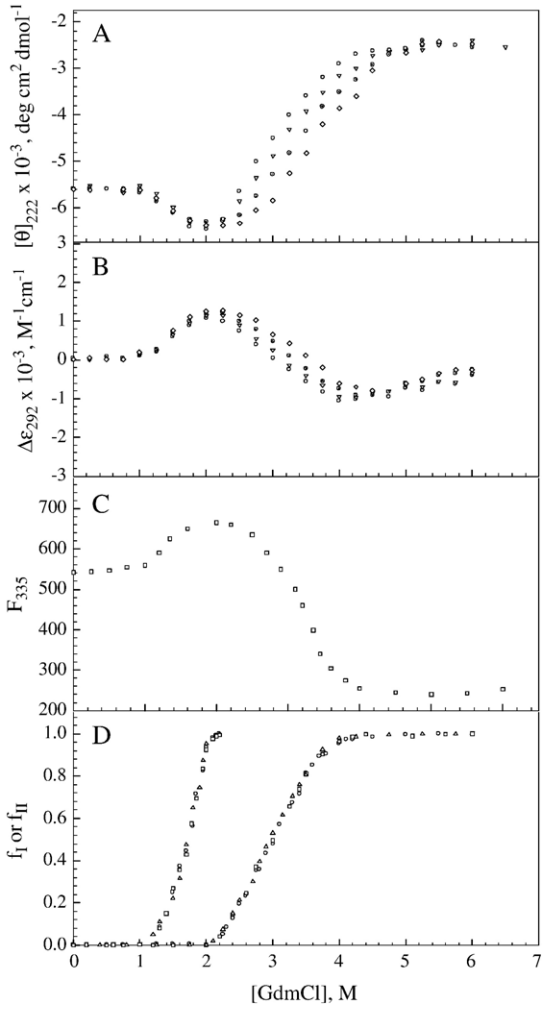

Fig. 1. The GdmCl-induced denaturation of β-lgA at pH 2.0 and 25 °C. (A) Transition curves followed by $[\theta]_{222}$ measurements at different concentrations of NaCl. O,▽, ⊙ and ◊ represent 0, 0.3, 0.6 and 1.0 M NaCl, respectively. (B) Transition curves followed by $\Delta\varepsilon_{292}$ measurements at different concentrations of NaCl. Symbols have the same meaning as in (A). It should be noted that Δε is the difference between ε of the protein solution containing given concentrations of GdmCl and NaCl and ε of the protein solution containing the same NaCl concentration but no GdmCl. (C) Transition curve followed by measuring fluorescence intensity at 335 nm. (D) The normalized transition curves in the absence of NaCl; O, Δ and □ represent values obtained from $[\theta]_{222}$, $\Delta\varepsilon_{292}$ and F_{335} measurements, respectively.

respectively. 0.1 and 1.0 cm pathlength cells were used for the measurements of the far- and near-UV CD spectra, respectively. Reversibility of the isothermal denaturation by GdmCl and urea was checked using the procedure described earlier [18]. CD instrument was routinely calibrated with D-10-camphorsulphonic acid. The results of all the CD measurements are expressed as mean residue ellipticity ($[\theta]_\lambda$) in deg cm^2 dmol^{-1} at a given wavelength λ (nm) using the relation,

$$[\theta]_\lambda = \theta_\lambda M_o/10cl \quad (1)$$

where θ_λ is the observed ellipticity in millidegrees at wavelength λ, M_o is the mean residue weight of the protein, c is the protein concentration (mg/cm^3), and l is the path length (cm). It should be noted that each observed θ_λ of the protein was corrected for the contribution of the solvent.

Fluorescence spectra were measured in a Perkin–Elmer L-5 spectroluminescencemeter in a 5 mm quartz cell at 25 °C, with both excitation and emission slits set at 7 nm. The protein concentration for all the experiments was in the range 7–10 μM. For the ANS fluorescence in ANS-protein binding experiments the excitation wavelength was 350 nm, and emission spectra were recorded from 600 to 400 nm. For tryptophan fluorescence measurements the excitation wavelength was 280 nm, and emission spectra were recorded in the wavelength region 300–400 nm.

3. Results

3.1. GdmCl-induced denaturation of β-lgA

To understand the unfolding behavior of β-lgA by GdmCl, we carried out the GdmCl-induced denaturation of the protein at pH 2.0 and 25 °C by observing changes in the difference absorption at 292 nm ($\Delta\varepsilon_{292}$), circular dichroism at 222 nm ($[\theta]_{222}$) and Trp-fluorescence emission intensity at 335 nm (F_{335}). Fig. 1(A–C) shows the unfolding profiles of the protein induced by GdmCl. It is seen in this figure that the denaturation induced by GdmCl is composed of two distinct processes. The first transition is centered in the [GdmCl], the molar concentration of GdmCl, range 0–2.0 M and is represented here by the reaction N↔X, where X is the thermodynamically stable intermediate state of the protein between its N (native) and D (denatured) states. The second transition occurs in the [GdmCl] range 2.0–6.0 M and is represented by the reaction X↔D. Fig. 1(A and B) also shows the GdmCl-induced denaturation of β-lgA at different concentrations of NaCl.

Assuming the process N↔X, designated here as transition I, follows a two-state mechanism, results shown in Fig. 1A, B and C were used to determine values of f_I (fraction of molecules in the intermediate state) and ΔG_I (Gibbs energy change associated with the transition I) using the relations,

$$f_I = (y - y_N)/(y_X - y_N) \quad (2)$$

$$\Delta G_I = -RT\ln[(y - y_N)/(y_X - y)] \quad (3)$$

Table 1
Thermodynamic parameters characterizing the denaturation of β-lgA by guanidinium chloride in the presence of different concentrations of NaCl at pH 2.0 and 25 °C

Probe	Transition	[NaCl]	$\Delta G_I^0/\Delta G_{II}^X$	m_I/m_{II}	C_{mI}/C_{mII}
		M	kJ mol^{-1}	kJ mol^{-1} M^{-1}	M
$[\theta]_{222}$	N↔X	0	30.07±0.55	17.70±0.71	1.70±0.05
	X↔D		20.80±0.59	15.80±0.59	3.25±0.09
	N↔X	0.3	29.90±0.50	17.64±0.80	1.69±0.06
	X↔D		21.80±1.00	16.40±0.50	3.35±0.11
	N↔X	0.6	30.20±0.46	17.60±0.67	1.71±0.08
	X↔D		24.15±1.22	16.40±0.71	3.52±0.13
	N↔X	1.0	30.20±0.50	17.64±0.84	1.68±0.09
	X↔D		26.50±1.47	15.80±0.63	3.75±0.18
$\Delta\varepsilon_{292}$	N↔X	0	29.80±0.97	19.90±0.92	1.50±0.13
	X↔D		19.20±1.13	15.90±0.80	3.20±0.10
	N↔X	0.3	29.90±1.05	19.80±1.10	1.48±0.13
	X↔D		21.90±0.97	15.80±0.80	3.29±0.12
	N↔X	0.6	30.00±0.50	19.90±1.22	1.53±0.10
	X↔D		23.50±1.30	15.41±0.92	3.55±0.15
	N↔X	1.0	30.00±0.63	19.90±1.26	1.57±0.09
	X↔D		26.00±1.47	15.20±0.84	3.75±0.18
F_{335}	N↔X	0	29.90±1.34	20.80±0.92	1.44±0.11
	X↔D		20.41±0.97	17.14±0.76	3.14±0.14

where R is universal gas constant, T is the temperature in Kelvin, y is the observed optical property corresponding to transition I and y_N and y_X are, respectively, optical properties of the native and intermediate states under the same experimental conditions in which y has been measured. Values of f_I and those of ΔG ($-5.5 \le \Delta G_I$, kJ mol$^{-1} \le 5.5$) were estimated as a function of [GdmCl]. Fig. 1D shows the normalized transition curve (plot of f_I versus [GdmCl]). ΔG_I versus [GdmCl] plots (not shown here) was analyzed for ΔG_I^0 (ΔG_I value at zero [GdmCl]) and m_I, the slope ($\delta\Delta G_I/\delta$[GdmCl]) using the relation,

$$\Delta G_I = \Delta G_I^0 - m_I[\text{GdmCl}] \quad (4)$$

Table 1 shows the values of ΔG_I^0, m_I and C_{mI}, the midpoint of the transition I ($=\Delta G_I^0/m_I$) at different NaCl concentrations.

Assuming that the process X↔D, designated here as transition II, is also of a two-state type, the results shown in Fig. 1A–C were used to determine values of f_{II} (fraction of molecules in the D state) and ΔG_{II} (Gibbs energy change associated with the transition II) using the relations,

$$f_{II} = (y - y_X)/(y_D - y_X) \quad (5)$$

$$\Delta G_{II} = -RT\ln[(y - y_X)/(y_D - y)] \quad (6)$$

where y is observed optical property corresponding to the transition II, and y_D is the optical property of the denatured protein molecule. Each transition II, shown in Fig. 1(A–C) was analyzed for f_{II} and ΔG_{II} using Eqs. (5) and (6) respectively. Values of ΔG_{II} in the range $-5.5 \le \Delta G_{II}$, kJ mol$^{-1} \le 5.5$ were plotted as a function of [GdmCl] (results not shown). A linear

least-squares analysis was used to obtain values of ΔG^0_{II} and m_{II} using the relation,

$$\Delta G_{II} = \Delta G^0_{II} - m_{II}[\text{GdmCl}] \quad (7)$$

where the subscript II represents the fact that these parameters correspond to the transition II, and superscript '0' represents the value at 0 M GdmCl. Values of ΔG^X_{II}, the value of ΔG_{II} at 2 M GdmCl, m_{II} and C_{mII} ($=\Delta G^0_{II}/m_{II}$) are given in Table 1.

Fig. 2A shows the far-UV CD spectra of β-lgA of the native (o) and 2 M GdmCl-induced intermediate (Δ) states. Fig. 2B shows the absorption spectrum of the native (−) and 2 M GdmCl-induced intermediate (– –) states. Fig. 2C shows ANS fluorescence spectra under different solvent conditions, which are given in the figure.

3.2. Urea-induced denaturation of β-lgA

In order to understand the difference between the equilibrium folding/unfolding behaviors of β-lgA in the presence of GdmCl and urea, urea-induced denaturation of the protein was also

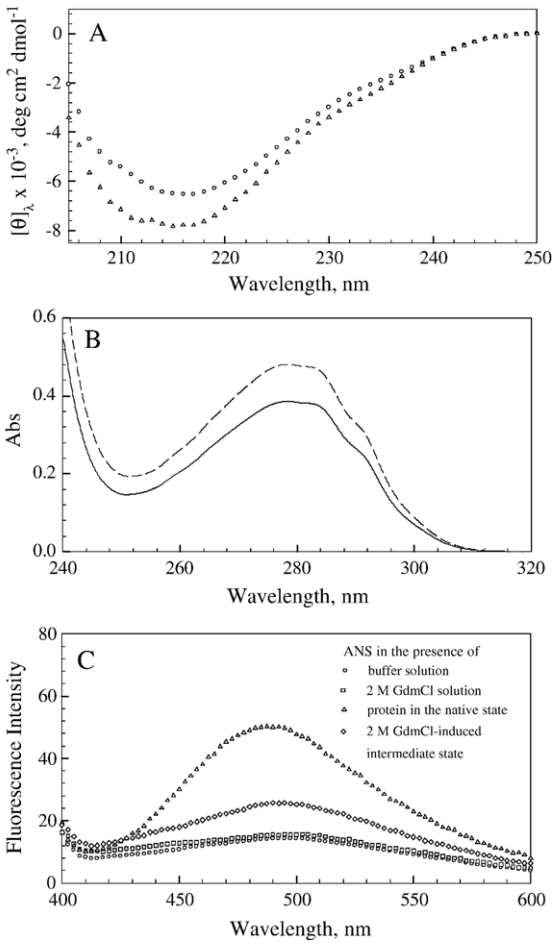

Fig. 2. Characterization of the equilibrium intermediate state of β-lgA at pH 2.0 and 25 °C. (A) The far-UV CD spectra of the native (O) and 2 M GdmCl-induced intermediate state (Δ). (B) The absorption spectra of the native (–) and 2 M GdmCl-induced intermediate state (– –) of the protein. (C) Fluorescence spectra of ANS in different solvent conditions shown in the panel.

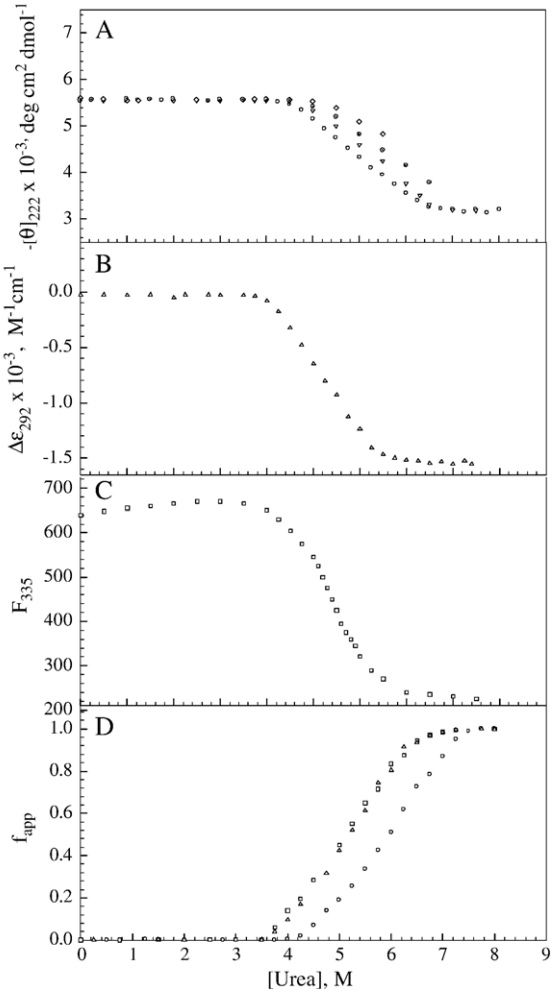

Fig. 3. Urea-induced denaturation of β-lgA at pH 2.0 and 25 °C. (A) Transition curves followed by measuring $[\theta]_{222}$ in the presence of 0, 0.3, 0.6 and 1.0 M NaCl. (B) and (C) transition curves followed by measuring $\Delta\varepsilon_{292}$ and fluorescence intensity at 335 nm in the absence of NaCl, respectively. (D) The normalized transition curves measured by $[\theta]_{222}$, $\Delta\varepsilon_{292}$, and F_{335}. Symbols have the same meaning as in Fig. 1(A–C).

carried out by observing changes in $[\theta]_{222}$, $\Delta\varepsilon_{292}$, and F_{335} at pH 2.0 and 25 °C. It is seen in Fig. 3(A–C) that the urea-induced unfolding of β-lgA, measured by each optical probe, is a one step process, and, however, C_m, the midpoint of denaturation, from the $[\theta]_{222}$ measurements is significantly larger than those from $\Delta\varepsilon_{292}$ and F_{335} measurements. Thus, the urea-induced denaturation of β-lgA is not a two-state process. However, for such a case, one can estimate the fraction of denatured protein, f_{app}, where the subscript 'app' represents the fact that all the thermodynamic parameters are apparent and not the true ones [19]. Results of each transition curve shown in Fig. 3(A–C) were used to determine values of f_{app} using the relation,

$$f_{app} = (y - y_N)/(y_D - y) \quad (8)$$

where y is observed optical property, y_N and y_D are, respectively, the optical properties of the N state (initial state) and the D state (final state) under the same experimental conditions in which y has been determined. The values of ΔG_{app} (apparent Gibbs

Table 2
Thermodynamic parameters characterizing the denaturation of β-lgA by urea in the presence of different concentrations of NaCl at pH 2.0 and 25 °C

Probe	Transition	[NaCl]	ΔG^0_{app}	m	C_m
		M	kJ mol^{-1}	kJ mol^{-1}.M^{-1}	M
$[\theta]_{222}$	N↔D	0	26.80±1.30	4.45±0.42	6.00±0.10
	N↔D	0.3	28.60±1.72	4.50±0.33	6.34±0.15
	N↔D	0.6	30.00±1.90	4.60±0.25	6.55±0.22
$\Delta\varepsilon_{292}$	N↔D	0	35.90±1.43	6.76±0.38	5.30±0.11
F_{335}	N↔D	0	35.20±1.39	6.51±0.46	5.42±0.13

energy change associated with the transition N state↔D state) in the range $-5.5 \le \Delta G_{app}$ (kJ mol^{-1}) ≤ 5.5 were determined using the relation,

$$\Delta G_{app} = -RT\ln(f_{app}/1-f_{app}) = -RT\ln[(y-y_N)/(y_D-y)] \quad (9)$$

A linear least-squares analysis was used to fit the (ΔG_{app}, [urea]) data obtained from each transition curve to the relation,

$$\Delta G_{app} = \Delta G^0_{app} - m[\text{urea}] \quad (10)$$

where ΔG^0_{app} is the value of ΔG_{app} at 0 M urea, and m gives the linear dependence of ΔG_{app} on the [urea]. Values of ΔG^0_{app}, m and C_m ($=\Delta G^0_{app}/m$) are given in Table 2.

Fig. 3A also shows denaturation curves of β-lgA induced by urea in the presence of 0.3, 0.6 and 1.0 M NaCl. It is seen in this figure that due to experimental constraints, measurements of $[\theta]_{222}$ of the protein in the presence of 0.6 and 1.0 M NaCl cannot be carried out at higher urea concentrations. However, we were able to estimate ΔG^0_{app}, m and C_m values in the presence of 0.6 M NaCl as well using Eqs. (8)–(10). These values are given in Table 2.

4. Discussion

In 1977 Bigelow and co-workers [12] reported their preliminary results of the GdmCl-induced denaturation of β-lgA at pH 2.0 and 25 °C. They showed that this denaturation is biphasic when followed by measuring $[\theta]_{222}$ as a function of [GdmCl]. However, they did not characterize the intermediate state of this denaturation. Twenty years later, Goto and Hamada [5] confirmed their findings. For the first time, they made a detailed study of characterization of this thermodynamically stable intermediate state induced by 2 M GdmCl in the presence of 10% TFE at pH 2.0 and 4 °C. Three important findings of their studies are: (i) the first transition curve (N↔X) observed at 25 °C (see Fig. 1A) is temperature sensitive, i.e., it disappears at 4 °C; (ii) the GdmCl-induced denaturation of the protein in the presence of 10% TFE at pH 2.0 and 4 °C is again bi-phasic, and the thermodynamically stable intermediate state contains 50% α-helix, and (iii) the transition N↔X is β→α transition. It may be noted that almost all earlier studies have focused on the kinetic characterization of the intermediate state(s) in the presence of TFE. In this study, we have obtained thermodynamic results to characterize the GdmCl-induced intermediate state in the absence of TFE (a helix-inducing solvent) at pH 2.0 and 25 °C, in terms of α-helical content, burial of Trp and burial of hydrophobic patches (see Figs. 1 and 2). These results are discussed below.

β-lgA, a 162-residue long protein, exists as a dimer above pH 3.0 [20–22] and the X-ray diffraction and NMR studies near neutral pH have revealed that the dimeric form of the protein is a predominantly β-protein; 7% α-helix and 51% β-sheet [23]. NMR studies have shown that the monomeric and dimeric forms of β-lgA have the same structure [24]. This predominantly β-sheet protein contains nine β strands (A-I), one major α-helix and 4 short helices [23]. The β-barrel assumes a flattened calyx (or cone) and is exposed to solvent. β-lgA contain two tryptophan residues; in the structure of the protein Trp19 is located in the first strand (βA) and is buried whereas Trp61 is exposed to solvent [23,25]. The native protein contains hydrophobic patches, for there exists a strong binding between the protein and ANS [26]. These structural information will be used to interpret our equilibrium results obtained from the measurements of denaturation of β-lgA by GdmCl at pH 2.0 and 25 °C.

Fig. 1A shows that the GdmCl-induced denaturation involves two steps. The first step (N↔X) involves the formation of the additional secondary structure and the second step (X↔D) represents the melting of all secondary structures in the protein. Fig. 2A compares the far-UV CD spectrum of the native protein with that of GdmCl-induced stable intermediate state. These spectra were used to estimate the helical content of the native and intermediate states. Instead of fitting the entire CD spectrum of each state of the protein for the estimation of various components of secondary structure, we have estimated only α-helical content in both states using $[\theta]_{222}$ values in the Eq. (2) given in Chen et al. [27]. The reason for using a single wavelength measurement of $[\theta]$ was that the CD spectrum of the intermediate state cannot be measured accurately below 205 nm due to strong absorption of 2 M GdmCl. We obtained a value of 9±1% for α-helix in the native protein which is very close to the value obtained from X-ray diffraction studies [23]. It should, however, be noted that different researchers have reported different values of α-helical content in the native protein from their far-UV CD spectral measurements; these values fall in the range 10–17% [28–30]. This discrepancy between our results and those reported earlier may, at least, be due to the different solvent conditions used during measurements of the far-UV CD spectrum of the native protein and methods used for the analysis of CD spectrum. Using the same method of analysis of the CD spectrum of the native β-lgA, we estimated a value of 15±1% for the α-helix content for the 2 M GdmCl-induced intermediate state of the protein at pH 2.0 and 25 °C. Thus, this equilibrium intermediate state of β-lgA contains about 6% additional α-helix called the non-native α-helix. A question arises: Where is this non-native α-helical segment in the protein sequence? Baldwin and Rose [8] simulated the folding of β-lgA and reached a conclusion that the two regions, i.e., the residues 19–27 of the first β strand (βA) and the residues 58–63, the loop between strands βC and βD, have high helix propensities. It has been suggested that the non-native α-helix is formed from the residues in the strand βA [10].

Combining ultra-mixing techniques with fluorescence detection and NMR based H-exchange labeling methods, Kuwata et al.

[31] have shown that the kinetic folding intermediate of β-lgA at pH 3.0 and 20 °C accumulates on the millisecond time scale, and there is a further solvent shielding of Trp19 in this kinetic intermediate. The latter finding is supported by the kinetic results obtained from the measurements of the β-lgA unfolding using fluorescence probe [10]. These results suggest that the kinetic intermediate on the folding pathway of the protein contains a non-native Trp as well. In order to see whether the thermodynamically stable intermediate state observed during the GdmCl-induced denaturation of β-lgA at pH 2.0 and 25 °C (Fig. 1A) contains, in addition to the non-native α-helix, a non-native Trp, we have followed this denaturation using two different probes namely $\Delta\varepsilon_{292}$ and Trp fluorescence emission (F_{335}), and these results are shown in Fig. 1B and C. It is seen in these figures that the N ↔ X transition involves the burial of Trp residue. The reason for saying this is that the transfer of Trp from a less non-polar medium to a more non-polar medium is accompanied by an increase in the absorption in the region 280–292 nm and in the fluorescence emission spectrum in the region 330–350 nm. It is also seen in Fig. 1B and C that the tertiary structure melts on the addition of GdmCl above 2 M, for both Trp absorption at 292 nm and fluorescence emission at 335 nm decrease on transferring Trp from a non-polar environment to a polar environment.

Kauffmann et al. [32] using a diffusion IR mixer have shown that there exists a kinetic intermediate, which is more compact than the native β-lgA. We, therefore, ask a question: Does the 2 M GdmCl-induced intermediate state have buried hydrophobic surface more than that of the native protein? In order to understand this question we measured the ANS fluorescence of the native protein and that of the 2 M GdmCl-induced intermediate state, for ANS shows an increase in fluorescence intensity with a blue-shift in the emission maximum on binding with the exposed hydrophobic clusters. In fact, β-lgA has two ANS binding sites in its native state [26]. We were, therefore, interested to see if more ANS is bound or released upon the formation of the thermodynamically stable intermediate induced by 2 M GdmCl at pH 2.0 and 25 °C. It is seen in Fig. 2C that the intermediate state has two-third lower fluorescence emission intensity than the native state with no shift in the emission maximum. This decrease in the fluorescence intensity of the intermediate state suggests that about two-third of the exposed hydrophobic clusters present in the native state is now buried in the intermediate state. It is tempting to conclude from all the measurements on the characterization of the X state presented above that the stable unfolding intermediate contains non-native α-helix, Trp and buried hydrophobic patches. Furthermore, our results do not support the earlier arguments that the intermediate state induced by 2 M GdmCl at pH 2.0 and 25 °C has molten globule-like characters [13]. It is noteworthy that GdmCl-induced denaturation of the genetic variant B of β-lactoglobulin is also biphasic when it is followed by $[\theta]_{222}$, $\Delta\varepsilon_{292}$ and F_{334} measurements (33). This suggests that the natural mutation has no effect on the thermodynamics of denaturation of β-lactoglobulins at least by GdmCl.

The most detailed study of thermodynamics of the denaturation of β-lgA by urea came from the laboratory of Tanford [14]. They have also obtained preliminary results on the kinetics of the urea-induced denaturation of this protein. One of the main conclusions of their studies is that the denaturation of β-lgA by urea is a two-state process. Another detailed study of the thermodynamics of denaturation of the B variant of β-lg was carried out by D' Alfonso et al. [33] and Ragona et al. [15]. They have found no evidence for the stable intermediate during denaturation of the protein by urea contrary to what has been detected during GdmCl denaturation (Figs. 1 and 2). Fig. 3 shows the results of the measurements of urea-induced denaturation of β-lgA at pH 2.0 and 25 °C. Two important conclusions can be drawn from these results. One is that the non-ionic denaturant (urea) induces one cooperative transition between N and D states. The second is that this denaturation is not a two-state process. The latter conclusion is drawn from the non-coincidence of the normalized denaturation curve measured by optical techniques monitoring the changes in the secondary and tertiary structures (see Fig. 3D). Indeed, it is seen in Fig. 3D that the secondary structure is more resistant to the urea denaturation than the tertiary structure. Results shown in Fig. 3 also suggest that the intermediate state(s) is (are) different from that induced by GdmCl, which has non-native α-helix, Trp and hydrophobic patches.

Urea and GdmCl, although leading to the same denatured state, [34,35 and this study] are found to unfold β-lgA differently, i.e., no evidence is found for the presence of stable non-native structure during denaturation by urea contrary to what has been detected during GdmCl-induced denaturation. Is this difference in β-lgA denaturation by the ionic denaturant and the non-ionic polar denaturant due to their difference in ionic character? The reason for asking this question is the argument put forward by Monera et al. [16] that GdmCl has ionic and denaturant components, and on the molar scale denaturing effect of Guanidinium (Gdm^+) and urea are equal. In order to see whether the occurrence of the non-native intermediate during the denaturation of β-lgA by GdmCl is due to chloride effect (electrostatic interaction), we have measured urea denaturation of the protein in the presence of different concentrations of a neutral salt sodium chloride (NaCl). These results are shown in Fig. 3A where it can be seen that no stable intermediate exists during denaturation of the protein by urea. This seems to suggest that 2 M GdmCl-induced intermediate (see Fig. 3) is not due to the effect of Cl^-. It is also seen in this figure that the urea denaturation of β-lgA shows strong dependence on the salt concentration. One possibility for the stabilization of β-lgA by NaCl against urea denaturation may be that the protein at pH 2.0 is positively charged and the neutral salt screens the electrostatic repulsion leading to the stabilization of the protein. In order to see that the latter possibility is a general salt effect, we have also measured GdmCl-induced denaturation of β-lgA at different concentrations of NaCl (see Fig. 1A and B). It is seen in Fig. 1A and B that the NaCl has no effect on the N ↔ X transition whereas, as observed in the case of urea denaturation (Fig. 3A), NaCl stabilizes the intermediate state against the denaturation by GdmCl. The latter result suggests that the stabilization of the protein by NaCl against urea and GdmCl cannot only be due to the screening of the electrostatic repulsion. The possible cause for this stabilization will be discussed below.

In order to estimate values of thermodynamic parameters from the analysis of the GdmCl-induced denaturation curves of β-lgA at pH 2.0 and 25 °C (Fig. 1) representing the reversible equilibria, N↔X and X↔D, two assumptions were made. First is that both of these transitions follow a two-state mechanism. The second is that ΔG_I and ΔG_{II} show linear dependence on [GdmCl]. One of the criteria to test the validity of a two-state denaturation is to see whether one gets comparable values of thermodynamic parameters associated with the transition curves monitored by different structural probes. It can be seen in Fig. 1D that values of f_I and f_{II} obtained from different optical methods fall on the same f_I versus [GdmCl] plot and the same f_{II} versus [GdmCl] plot, respectively. This suggests that N↔X and X↔D transitions follow a two-state mechanism. As expected for such a transition, values of ΔG^0_I, m_I, C_{mI}, ΔG^0_{II}, m_{II} and C_{mII} obtained from different optical probes are, within experimental errors, identical (see Table 1). As far as the second assumption is concerned, we do not have data to validate the assumption that ΔG of β-lgA associated with each transition is a linear function of [GdmCl]. It should, however, be noted that both theoretical and experimental considerations do suggest a linear dependence of ΔG on denaturant concentration [19,36–40].

Results shown in Table 1, suggest that ΔG^0_D ($=\Delta G^0_I+\Delta G^X_{II}$) of denaturation for the N↔D transition of β-lgA in the absence of NaCl is 50.4 kJ mol^{-1}. Since there is no report of the value of ΔG_D for the GdmCl-induced denaturation of β-lgA at pH 2.0 and 25 °C, a comparison of our results is therefore not possible. However, Pace [19] reported that goat β-lactoglobulin denaturation by GdmCl is a two-state process and that the stability of the protein at pH 2.0 and 25 °C is 50.4 kJ mol^{-1}. It has been argued that this value should be the same as for the β-lgA [19].

Contrary to a few equilibrium studies of the GdmCl-induced denaturation of β-lgA, the equilibrium studies of denaturation of this protein by urea have been carried extensively [14,15,33]. It has always been argued in these studies that urea-induced denaturation of β-lgA is a two-state process. If, for example, the mechanisms were actually N↔X↔D where X state has structural properties between those of N and D states, values of ΔG^0_D and m will be lower from those expected for a two-state transition [19,41]. Here we show for the first time that urea-induced denaturation of β-lgA at pH 2.0 and 25 °C is not a two-state process.

Each transition curve of β-lgA shown in Fig. 3 is analyzed assuming an apparent two-state transition. It is seen in Fig. 3D that when fraction of the denatured protein (f_{app}) is plotted as a function of [urea], it falls on two different curves. Two following important conclusions are drawn from such a plot. For sure, (i) urea-induced denaturation of the protein is not a two-state process, and (ii) secondary structure is more stable than the tertiary structure. Thus, all the thermodynamic parameters derived from optical measurements of denaturation are apparent i.e., they should be less than those expected for a two-state process. Table 2 shows ΔG^0_{app} values for N↔D transition induced by urea, which is in the range of 25.2–37.8 kJ mol^{-1} in absence of NaCl. Different values of ΔG^0_{app} from urea denaturation of monomeric bovine β-lactoglobulin have been reported in the literature; ΔG^0_{app} (kJ mol^{-1}) are 42 [19], 33.6 [15], 34 [42], 32.8 [33]. Thus, values of ΔG^0_{app} found in the present work are in agreement with those reported earlier.

Using models of randomly coiled proteins and their known native crystal structures, following empirical relation describing the dependence of m values, obtained from the two-state urea denaturation of proteins, on the change in accessible surface area on denaturation (ΔASA) has been reported [43],

$$m = 0.11\ \Delta\mathrm{ASA} + 373 \qquad (11)$$

using a value of 23,000 Å^2 for ΔASA [44] in Eq. (11), we estimated a value of 12.22 kJ mol^{-1} for m. This value is significantly higher than those we estimated in this study. This discrepancy seems to further support our conclusion that the urea-induced denaturation of β-lgA is not a two-state process.

Contrary to the urea-induced denaturation (Fig. 3A), GdmCl induces a transition, N↔X (Fig. 1A), which most probably represents β↔α transition [25]. As discussed above, this stable intermediate is not due to the general salt screening effect of Cl^-. Our results suggest that the intermediate state X is due to the intrinsic effect of Gdm^+, and that the β↔α transition observed during GdmCl-induced denaturation may be not intrinsically related to the protein folding pathway, since it is not detected during denaturation by urea.

It is seen in Fig. 3A that NaCl stabilizes the positively charged β-lgA against denaturation by urea (also see Table 2). Is this stabilization due to a general salt effect? If this is the case, NaCl should have no effect on the denaturation of β-lgA by GdmCl, for the ionic nature of this denaturant masks electrostatic interaction in the proteins [16]. However, Fig. 1A and B show two interesting observations. One, NaCl has no effect on the transition N↔X, and second, it affects the transition X↔D in such a manner that the state X gets stabilized against denaturation by GdmCl. Interestingly, in order to understand the role of salts on the protein stability, Robinson and Jencks [45] have measured the solubility of a model peptide in a series of salts and concluded that NaCl should salt-out (stabilize) proteins. This effect is also observed when it is measured in terms of the preferential hydration of proteins or preferential exclusion of salts from the protein domain [46,47]. It has been shown that both Na^+ and Cl^- have an exclusion capability mainly due to the unfavorable interaction between protein backbone and NaCl [45]. It is expected that this exclusion effect will be more on the denatured protein than that on the native protein [48]. This could be the reason for the observation that NaCl has no effect on N and X states, which are very compact [10]. It is noteworthy that the stabilization of β-lgA by NaCl is not very significant during the thermal denaturation of the protein [49]. One possible explanation for the discrepancy between the effect of NaCl on the urea (or GdmCl) denaturation and that on the heat denaturation is the observation that thermally denatured β-lgA is still compact at pH 2.0 [49]; judging by the observed and calculated heat capacity increment, the increase in the solvent accessible surface area upon denaturation by heat is only 37% of that which could have been upon complete unfolding.

In summary, our studies of denaturation of the monomeric β-lgA by GdmCl and urea led to two definite conclusions. (i) The intermediate state observed during GdmCl-induced denaturation has not only non-native α-helix but also non-native tryptophan and non-native hydrophobic patches. (ii) Mechanism of denaturation by urea is not a two-state process.

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