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Biophysical Chemistry 133 (2008) 81 – 89

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

A new method for determining the constant-pressure heat capacity change associated with the protein denaturation induced by guanidinium chloride (or urea)

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Received 2 November 2007; received in revised form 18 December 2007; accepted 18 December 2007
Available online 27 December 2007

Abstract

Differential scanning calorimetry (DSC) provides authentic and accurate value of ΔC_p^X, the constant-pressure heat capacity change associated with the N (native state)↔X (heat denatured state), the heat-induced denaturation equilibrium of the protein in the absence of a chemical denaturant. If X retains native-like buried hydrophobic interaction, ΔC_p^X must be less than ΔC_p^D, the constant-pressure heat capacity change associated with the transition, N↔D, where the state D is not only more unfolded than X but it also has its all groups exposed to water. One problem is that for most proteins D is observed only in the presence of chemical denaturants such as guanidinium chloride (GdmCl) and urea. Another problem is that DSC cannot yield authentic ΔC_p^D, for its measurement invokes the existence of putative specific binding sites for the chemical denaturants on N and D. We have developed a non-calorimetric method for the measurements of ΔC_p^D, which uses thermodynamic data obtained from the isothermal GdmCl (or urea)-induced denaturation and heat-induced denaturation in the presence of the chemical denaturant concentration at which significant concentrations of both N and D exist. We show that for each of the proteins (ribonuclease-A, lysozyme, α-lactalbumin and chymotrypsinogen) ΔC_p^D is significantly higher than ΔC_p^X. ΔC_p^D of the protein is also compared with that estimated using the known heat capacities of amino acid residues and their fractional area exposed on denaturation.

Keywords: Protein folding; Protein denaturation; Heat capacity

1. Introduction

Differential scanning calorimetry (DSC) yields authentic and accurate value of the constant-pressure heat capacity change ΔC_p^X, associated with the N (native state)↔X (heat denatured state), the heat-induced denaturation equilibrium of the protein in the absence of a chemical denaturant [1]. If such heat-induced denaturation is a two-state process, optical methods also provide authentic and accurate ΔC_p^X of the protein [2,3]. On the contrary, if X is less unfolded than D, the GdmCl-induced (or urea-induced) denatured state, no authentic value of ΔC_p^D associated with the equilibrium N↔D in the absence of the chemical denaturant, is available from either DSC or optical measurements. There are a few reasons for saying this. First, in order to obtain ΔC_p^D, DSC measurements were carried out in the presence of predenaturation concentration range of GdmCl, and the observed ΔH_m, the enthalpy change at the midpoint of the denaturation (T_m) is corrected for the contribution due to preferential interaction of the chemical denaturant with the native and denatured protein. There are two problems with this

Abbreviations: CD, circular dichroism; $[\theta]_\lambda$, mean residue ellipticity at wavelength, λ nm; RNase-A, ribonuclease-A; apo-La, apo-α-lactalbumin; ctg, chymotrypsinogen A; GdmCl, guanidinium chloride.

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doi:10.1016/j.bpc.2007.12.006

procedure. (i) Optical methods suggest that a transition between N and X is observed in the presence of the predenaturation concentrations of GdmCl, which by themselves do not induce protein denaturation near room temperature (Singh and Ahmad, manuscript in preparation; for other cases see Ref. [4]). So the observed thermodynamic parameters are not for the transition N↔D but are actually for the N↔X transition. (ii) The correction for the preferential interaction contribution to the observed ΔH_m (see Eq. (34) in Ref. [1]) uses Δn (number of the 'specific' binding site for GdmCl exposed on denaturation) obtained from the analysis of the GdmCl-induced denaturation (N↔D) curves measured by optical techniques using the binding model [5]. In fact, we have shown earlier that the dependence of the unfolding Gibbs energy change of lysozyme and other proteins on the GdmCl concentration is linear throughout the denaturant concentration range [6], i.e., there exists no specific binding site for GdmCl on the protein. This observation supports the earlier arguments against the presence of specific binding sites on the protein for GdmCl [7]. The second reason is that there is a problem with the method used to obtain ΔC_p^D from optical denaturation curves as well. The observed equilibrium constant (K_D) for the reaction, N↔D is written as a product of three equilibrium constants, i.e., $K_D = K_D(T).\ K_D(\text{GdmCl}).\ K_D(\text{pH})$, where K_D depends only on the experimental variable given in the parenthesis, e.g., $K_D(T)$ depends on temperature only, i.e., it does not depend on either pH or GdmCl concentration [8,9]. If it is so, then this relation also implies that the enthalpy change on denaturation is independent of the denaturant concentration. In fact, it has been shown that this assumption is not valid [1,10]. Thus, the authenticity of ΔC_p^D obtained from the temperature dependence of K_D, which assumed that $K_D(T)$ is independent of the denaturant concentration at a given pH, is questionable.

Pace and Laurent [11] have developed a method for the determination of ΔC_p^D from the measurements of thermal and urea denaturation curves monitored by conformational techniques. This method involves (i) measurements of ΔH_m^X and T_m^X from the heat-induced transition curves in the absence of the chemical denaturant, where superscript X represents the fact that quantities are associated with N↔X reaction; (ii) measurements of the values of ΔG_D^0, the Gibbs energy change (ΔG_D) in the absence of the chemical denaturant for the reaction, N↔D at several temperatures (at least 25 °C below T_m) from the urea-induced denaturation curves at the same pH as the thermal denaturation experiments; and (iii) estimation of the constant-pressure heat capacity change from ΔH_m^X and T_m^X and ΔG_D^0 values at different temperatures by using Gibbs–Helmholtz equation. This method should give authentic value of the heat capacity change if both the heat and urea give the same denatured state of the protein, and both heat and urea induce a two-state transition.

In this study we describe a method for measuring ΔC_p^D, which is based on results from the isothermal GdmCl (or urea)-induced denaturation curves, and heat-induced denaturation curves in the presence of different concentrations of the chemical denaturant, which by themselves induce N↔D transition (i.e., detectable amounts of folded and unfolded proteins are in equilibrium) at the temperature as the GdmCl (or urea)-induced denaturation experiment. It has been observed that for a protein ΔC_p^D is significantly larger than ΔC_p^X. Furthermore, ΔC_p^D of the protein is also estimated using the known heat capacities of amino acid residues reported by Privalov et al. [12] and the known fractional area of each residue exposed on denaturation. It has been observed that for a protein the measured ΔC_p^D is very close to the predicted value for the transition between the folded native and the theoretical compact denatured states [13].

2. Materials and methods

Hen egg-white lysozyme (lot 111H-7010), α-lactalbumin from bovine milk (Type I, lot 92H-7015), bovine pancreas ribonuclease-A (Type III A, lot 41K-7651), and bovine pancreas α-chymotrypsinogen A (Type II, 16H-7075) were purchased from Sigma Chemical Co. Analytical grade EGTA (ethylene glycol-bis (β-aminethylether) *N*, *N*, *N*′, *N*′-tetra acetic acid) and sodium cacodylate were also purchased from Sigma Chemical Co. GdmCl and urea were ultrapure grade samples purchased from ICN Biomedical, Inc. KCl, glycine and other chemicals were of reagent grade and were used without further purification.

Lysozyme, ribonuclease-A (RNase-A), α-lactalbumin and α-chymotrypsinogen A (ctg) were dialyzed in cold (at ~4 °C) against several changes of 0.1 M KCl (pH 7.0). Apo-α-lactalbumin (apo-La) was prepared by adding 4 mM EGTA to the solution of the holoprotein (with ca^{2+} bound). Protein concentration of the stock solutions were determined experimentally using ε, the molar absorption coefficient ($M^{-1}\ cm^{-1}$) values of 39,000 at 280 nm for lysozyme [14], 9800 at 277.5 nm for RNase-A [15], 29,210 at 280 nm for α-lactalbumin [16], and 50,562 at 280 nm for α-chymotrypsinogen A [17]. Concentrations of stock solutions of GdmCl and urea in a given buffer were determined refractometrically using tabulated values of the solution refractive indices [18].

The buffers used throughout the denaturation studies were 0.1 M KCl–HCl buffer (pH 2.0) for lysozyme, 0.1 M glycine–HCl buffer containing 0.1 M KCl of pH 2.2 for RNase-A and pH 2.0 for ctg, and 0.05 M cacodylic acid buffer (pH 7.0) containing 0.1 M NaCl and 4 mM EGTA for apo-La.

Far-UV circular dichroism (CD) measurements were carried out in Jasco Spectropolarimeter (model J-715) equipped with the peltier-type temperature controller (PTC-348). Thermal denaturation curves were obtained at three different scan rates, namely, 0.5, 1 and 1.5 °C/min. It has been observed that the thermodynamic parameters obtained at different rates of scan were constant. This observation indicates that the denaturation process does not occur as a kinetically controlled process, and each scan rate provided an adequate time for equilibration [19]. However we have shown denaturation curves obtained at a heating rate of 1 °C in the temperature range 20–85 °C. Protein concentrations used for CD measurements were in the range of 0.2–0.4 mg/ml, and 0.1 cm path length cell was used. CD instrument was routinely calibrated with D-10-camphorsulfonic acid. Results of all CD measurements are expressed as mean

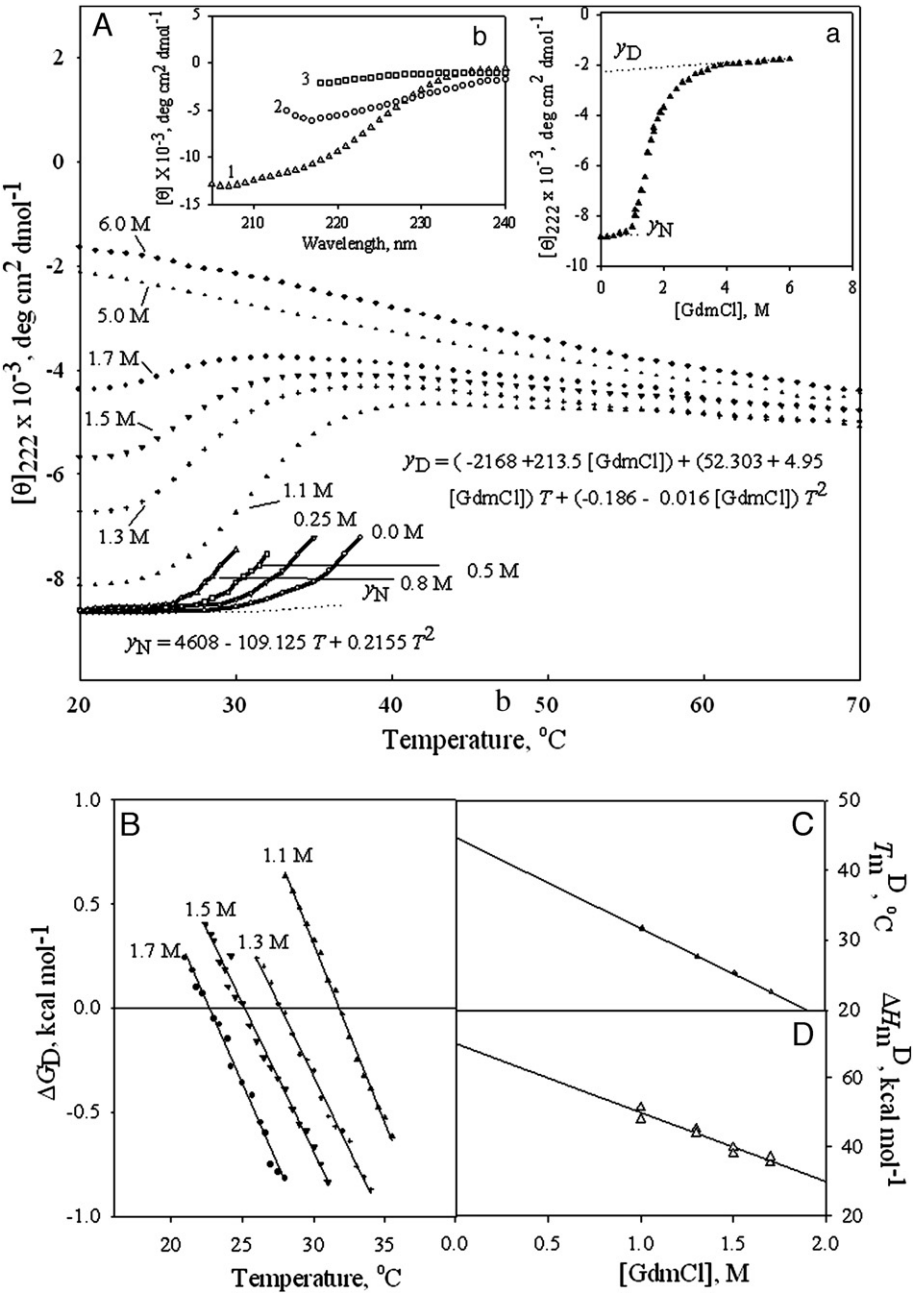

Fig. 1. Denaturation of RNase-A. Thermal denaturation curves of RNase-A in the presence of 1.1, 1.3, 1.5, 1.7, 5.0 and 6.0 M GdmCl at pH 2.2 (A). In order to maintain clarity all data points are not shown. y_N and y_D describes dependencies of the native and denatured proteins, respectively. Inset (a) in Panel A shows the GdmCl-induced transition between N and D of the protein at 25 °C, and inset (b) shows the far-UV CD spectra of the protein in the native state (curve 1), heat denatured state (curve 2) and GdmCl denatured state (curve 3). Plots of ΔG_D versus temperature at 1.1, 1.3, 1.5 and 1.7 M GdmCl (B). Plot of T_m^D versus [GdmCl] (C) and that of ΔT_m^D versus [GdmCl] (D).

residue ellipticity ($[\theta]_\lambda$) in ° cm^2 $dmol^{-1}$ at a given wavelength, λ (nm) using the relation,

$$[\theta]_\lambda = \theta_\lambda M_0 / 10cl \quad (1)$$

where θ_λ is the observed ellipticity in millidegrees at λ, M_0 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.

Details of the preparation of protein solutions for isothermal denaturation by GdmCl and urea and renaturation experiments were reported earlier [20]. Reversibility of the GdmCl (or urea) denaturation was completely established as revealed by the identical CD value at a given denaturant concentration during denaturation and renaturation experiments. Reversibility of the heat-induced denaturation was checked by measuring the CD spectrum of the renatured protein after heating, and comparing it with that of the unheated protein sample. For this purpose, (i) CD spectrum of the native protein (unheated sample) was

measured at 20 °C, (ii) the protein sample was heated and the scan was terminated at 85 °C, (iii) the denatured protein sample was cooled immediately to 20 °C, and (iv) the CD spectrum of the renatured protein was determined at 20 °C. Reversibility of thermal denaturation was confirmed by the identical CD spectra of the protein before heating and after cooling of the denatured protein solution.

Calculations of accessible surface area (ASA) of the native protein were performed by the software DSSP [21] using coordinate data from the Protein Data Bank [22]. For the denatured protein the extended state ASA values of the tripeptide Ala-X-Ala were used. The extended state coordinates correspond to the ECEPP/2 algorithm [23] with the dihedral angles reported by Oobatake and Ooi [24]. The values of extended state ASA (in $Å^2$) are given in Ahmad et al. [25].

3. Results

GdmCl-induced and urea-induced denaturations at 25 °C and the heat denaturation in the presence of different concentrations of the chemical denaturants (GdmCl and urea), were followed by observing changes in the far-UV CD of RNase-A (pH 2.2), lysozyme (pH 2.0) and apo-La (pH 7.0) as it serves as an excellent probe for measuring changes in the conformation of

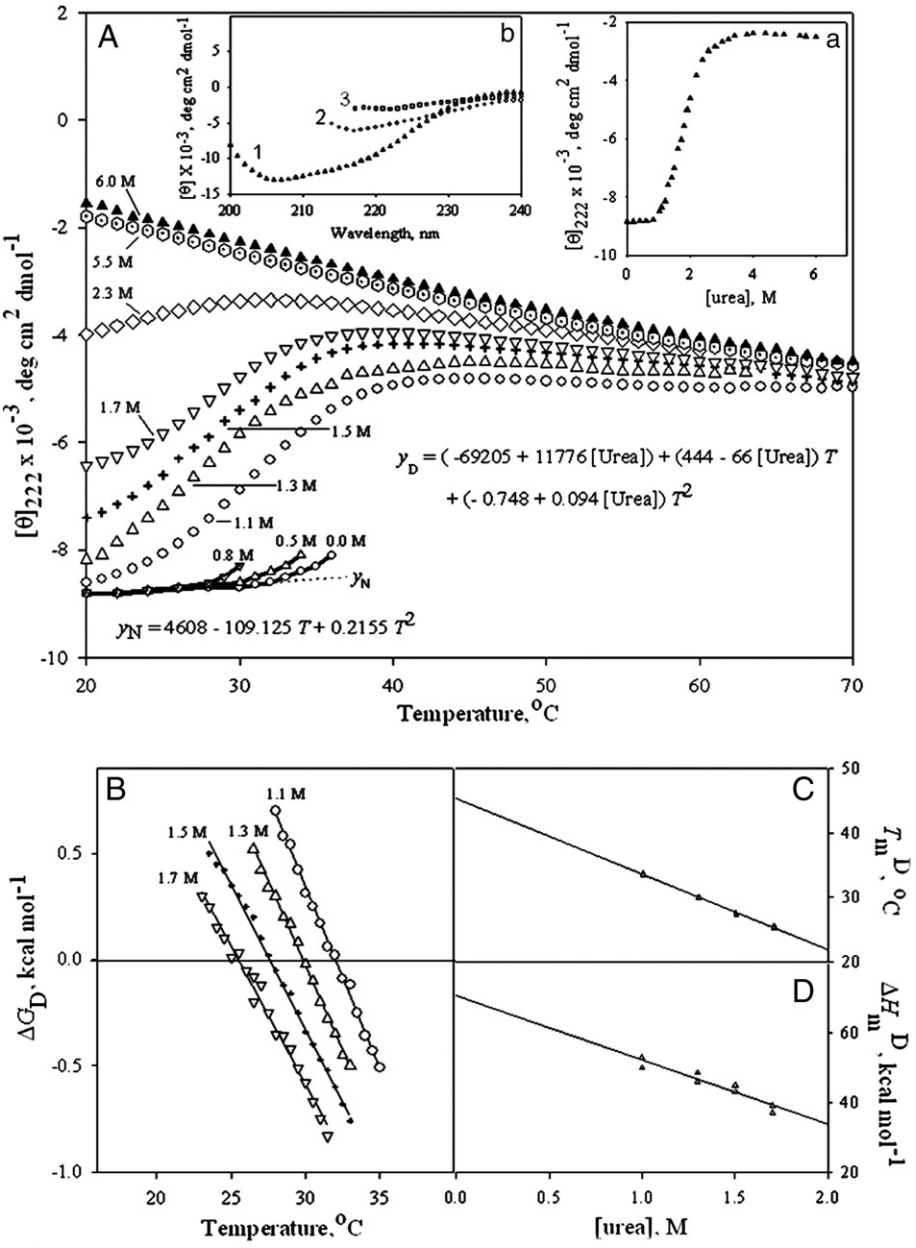

Fig. 2. Denaturation of RNase-A. Thermal denaturation curves of RNase-A in the presence of 1.1, 1.3, 1.5, 1.8, 5.5 and 6.0 M urea at pH 2.2 (A). In order to maintain clarity all data points are not shown. y_N and y_D describes the dependencies of the native and denatured proteins. Inset (a) in Panel A shows the urea-induced transition between N and D states at 25 °C, and inset (b) shows the far-UV CD spectra of the protein in the native state (curve 1), heat denatured state (curve 2) and urea denatured state (curve 3). Panel B shows plots of ΔG_D versus temperature at 1.1, 1.3, 1.5 and 1.7 M urea. (C) Plot of T_m^D versus [urea] and that of ΔH_m^D versus [urea] (D).

the entire polypeptide backbone [26], and in the near-UV CD of ctg (pH 2.0). All measurements were done at least in triplicate. It has been observed that denaturation of each protein is reversible. All optical transition data were converted into thermodynamic parameters using appropriate relations given below.

Inset (a) in Figs. 1A and 2A show the GdmCl-induced and urea-induced denaturations of RNase-A monitored by $[\theta]_{222}$, respectively. Such transition curves for lysozyme, apo-La and ctg are given in the Supplementary materials. Assuming that the chemical denaturant-induced unfolding follows a two-state mechanism, the entire data of each transition curve were analyzed for ΔG^0_D using a nonlinear least-squares relation [27],

$$y([d]) = \frac{y_N([d]) + y_D([d])\ \mathrm{Exp}\left[-\Delta G^0_D + m_d[d]/RT\right]}{1 + \mathrm{Exp}\left[-\Delta G^0_D + m_d[d]/RT\right]} \quad (2)$$

where $y([d])$ is the observed optical property at $[d]$, the molar concentration of the chemical denaturant, and $y_N([d])$ and $y_D([d])$ are, respectively, optical properties of the native and denatured states of protein molecules under the same experimental conditions in which $y([d])$ was measured. In this equation ΔG^0_D is the value of Gibbs energy change (ΔG_D) in the absence of the chemical denaturant, R is the universal gas constant, and T is the temperature in Kelvin. (It should be noted that T is in Kelvin throughout the text unless otherwise indicated.) It has been observed that for all proteins the dependencies of y_N and y_D on $[d]$ are linear (i.e., $y_N([d]) = a_N + b_N[d]$ and $y_D([d]) = a_D + b_D$ [d], where a and b are, respectively, intercept and slope of the baseline). Values of ΔG^0_D obtained from the GdmCl-induced and urea-induced denaturation curves of all proteins are given in Table 1. ΔG^0_D values obtained from both chemical denaturations are, within experimental errors, identical (see Table 1). It should be noted that the GdmCl-induced denaturation curve for ctg could not be measured due to aggregation in the presence of low denaturant concentrations. It has been observed that the ΔG^0_D values are in agreement with those reported earlier [6,30–33]. This led us to believe that our measurements and analysis of the transition curves are accurate. It may be noted that GdmCl is an ionic denaturant and has the ability to shield all kinds of electrostatic interactions whereas urea cannot, for it is an uncharged molecule. However, the agreement of ΔG^0_D values from GdmCl unfolding with ΔG^0_D from urea unfolding is an expected result as 0.1 M KCl is sufficient to shield all the electrostatic effect [34].

Figs. 1A and 2A show the thermal denaturation curves of RNase-A in the presence of different concentrations of GdmCl and urea, respectively (such transition curves for other proteins are given as Supplementary materials). It may be noted that the denaturant concentration chosen for all proteins lies above the pretransition region, i.e., these concentrations by themselves induce a transition between N and D at 25 °C (e.g., see inset of Figs. 1A and 2A in the case of RNase-A). In order to convert these data into values of ΔG_D it is necessary to know y_N and y_D as a function of composition variables ($[d]$ and T). The prerequisite data for y_N were obtained from measurements of the heat-induced denaturation curves of the protein in the presence of the chemical denaturant concentration lying in the pretransition region. It has been observed that the temperature dependence of y_N of each protein is independent of [GdmCl] and [urea]. Such dependencies for RNase-A are given in Figs. 1A and 2A, which were assumed to apply within the transition region as well. It should be noted that, for each protein, the heat-induced denaturation curves in the presence of predenaturation concentration range of the chemical denaturant are not displayed over the whole transition. In order to obtain the dependence of y_D of a protein on both $[d]$ and T, the composition variables, we have used all data in the posttransition region of the thermal denaturation curves as well as results from the measurements on the denatured protein outside the transition (posttransition). These data for a protein were fitted to a nonlinear equation. The temperature dependence of y_D of RNase-A on [GdmCl] and [urea] are shown in Figs. 1A and 2A, respectively. Dependencies for other proteins are given as Supplementary materials. It has been assumed that the observed dependence of y_D of a protein on the composition variables is applicable in the transition region as well.

Assuming that the heat-induced transition of each protein in the presence of GdmCl and urea follows a two-state mechanism, values of $\Delta G_D(T,[d])$ in the range $-5.4 \le \Delta G_D\ (T,\ [d])$, kJ mol^{-1} ≤ 5.4 were estimated from the transition curve using the relation,

$$\Delta G_D(T,[d]) = -RT\ \ln\left[\frac{y(T,[d]) - y_N(T,[d])}{y_D(T,[d]) - y(T,[d])}\right] \quad (3)$$

where $y(T,[d])$ is the observed optical property at a given T and $[d]$; and $y_N(T,[d])$ and $y_D(T,[d])$ are, respectively, the optical properties of the N and D molecules at the same T and $[d]$ at

Table 1
Values of thermodynamic parameters of RNase-A, lysozyme, apo-La and ctg

	GdmCl				Urea				ΔC_p^D predicted [b]		(ΔC_p^X) [c]
	ΔG^0_D kJ mol^{-1}	$T^D_m(0)$ °C	$\Delta H^D_m(0)$ kJ mol^{-1}	(ΔC_p^D) [a] kJ mol^{-1} K^{-1}	ΔG^0_D kJ mol^{-1}	$T^D_m(0)$ °C	$\Delta H^D_m(0)$ kJ mol^{-1}	(ΔC_p^D) [a] kJ mol^{-1} K^{-1}	Lower bound kJ mol^{-1} K^{-1}	Upper bound kJ mol^{-1} K^{-1}	kJ mol^{-1} K^{-1}
RNase-A	11.37±0.75	44.9±0.4	293±8.4	10.45	11.58±0.33	45.3±0.3	293±10.4	11.07	11.37	13.92	4.81
Lysozyme	17.35±0.33	59.3±0.5	376±9.2	11.33	17.55±0.50	60.0±0.4	372±11.3	11.24	14.09	16.43	5.39
apo-La	10.07±0.33	49.3±0.3	230±8.4	8.99	9.82±0.71	48.4±0.3	234±20.9	8.36	18.10	19.86	4.89
ctg [d]	–	–	–	–	11.07±0.84	49.2±0.3	376±12.5	18.90	26.38	30.26	10.78

[a] ΔC_p^D values were estimated with the help of Eq. (4). Possible errors in the estimation of ΔC_p^D are given in the text.
[b] ΔC_p^D predicted values are estimated from Eq. (5) using the lower bound and upper bound models [13].
[c] Values of ΔC_p^X of RNase-A and lysozyme and those of Apo-La and Ctg were taken from Refs. [1,28,29], respectively.
[d] GdmCl-induced denaturation could not be measured due to aggregation.

which y(T, [d]) has been measured. For a protein stability curve at a given [GdmCl] (e.g., see Panel B of Fig. 1) and [urea] (e.g., see panel B of Fig. 2) were constructed, and a procedure described earlier [35] was used to obtain the values of ΔH_m^D and T_m^D. The values of ΔH_m^D and T_m^D of all the proteins in the presence of GdmCl and urea are given in Table 2. For a protein, plots of ΔH_m^D and T_m^D versus [GdmCl] (e.g., see Fig. 1C and D), and plots of ΔH_m^D and T_m^D versus [urea], (e.g., see Fig. 2C and D), which were linear, were used to obtain values of ΔH_m^D (0) and T_m^D (0), the values of thermodynamic parameters in the absence of the chemical denaturants. Such plots for lysozyme, apo-La and ctg are given as Supplementary materials. The values of ΔH_m^D (0) and T_m^D (0) of all the proteins are given in Table 1. Value of ΔC_p^D of a protein was estimated using a form of Gibbs–Helmholtz equation with values of ΔH_m^D (0) and T_m^D (0) from thermal denaturation measurements in the presence of GdmCl (or urea), and ΔG_D^0 from the isothermal denaturation at 25 °C,

$$\Delta C_p^D = \frac{\Delta G_D^0(298.15) - \Delta H_m^D(0)\left(\frac{T_m^D(0)-298.15}{T_m^D(0)}\right)}{\left[\left(T_m^D(0) - 298.15\right) + 298.15\ \ln\left(\frac{298.15}{T_m^D(0)}\right)\right]} \quad (4)$$

ΔC_p^D values for all proteins thus obtained are given in Table 1.

ΔC_p^D for the transition between the folded native and the theoretical denatured states can also be predicted using the relation,

$$\Delta C_p^D = \sum_{i=1}^{n} C_{p,i} \cdot f_i \quad (5)$$

where n is the total number of residues in the protein polypeptide chain, $C_{p,i}$ is the partial molar heat capacity of the ith residue (see Table 3 in Ref. [12]) and f_i is the fractional increase in the accessible surface area of the ith residue when protein unfolds. The value of f_i is estimated using the relation,

$$f_i = \frac{\mathrm{ASA}_{U,i} - \mathrm{ASA}_{F,i}}{\mathrm{ASA}_{U,i}} \quad (6)$$

where $\mathrm{ASA}_{U,i}$ and $\mathrm{ASA}_{F,i}$ are the accessible surface areas of the ith residue in the unfolded and the folded states, respectively. Calculation of $\mathrm{ASA}_{F,i}$ was performed by the software DSSP [21] using coordinate data from the Protein Data Bank [22]. For the $\mathrm{ASA}_{U,i}$ values, both the lower bound and upper bound models (see Table 1 in Ref. [13]) were used and the values of ΔC_p^D for RNase-A, lysozyme, apo-La and ctg were predicted with the help of Eq. (5). These predicted ΔC_p^D values in cases of upper bound and lower bound models are given in Table 1.

Table 2
Values of ΔH_m^D and T_m^D of proteins characterizing the transition N↔D induced by GdmCl and urea [a]

	GdmCl			Urea		
	M	T_m^D °C	ΔH_m^D kJ mol^{-1}	M	T_m^D °C	ΔH_m^D kJ mol^{-1}
RNase-A	1.0	31.5	217	1.1	33.5	230
	1.3	27.5	188	1.3	30.3	205
	1.5	25.5	167	1.5	27.7	175
	1.7	22.5	146	1.7	25.3	159
Lysozyme	1.5	38.5	201	2.2	38.5	272
	1.7	36.1	171	2.5	35.4	259
	2.0	33.5	146	2.8	32.6	238
	2.2	31.7	121	3.2	28.6	217
apo-La	1.2	34.7	125	1.8	32.2	167
	1.3	32.5	113	2.0	30.3	159
	1.5	30.4	96	2.5	27.7	129
	1.8	26.9	79	3.0	24.1	104
ctg [b]	–	–	–	1.8	35.0	293
				2.2	32.2	288
				2.5	29.7	276
				3.0	25.6	255

[a] From triplicate measurements values of maximum errors from the mean are 1% and 9% in T_m^D and ΔH_m^D, respectively. It should be noted that the standard error in the estimation of a thermodynamic parameter by fitting a set of data to an appropriate equation is less than the mean error from three independent experiments.

[b] GdmCl-induced denaturation could not be measured due to aggregation.

4. Discussion

We already have given reasons why earlier methods [1,9,11] will not provide an accurate value of ΔC_p^D, if the thermally denatured protein is less unfolded than the GdmCl (or urea) denatured state (see Introduction). We begin by explaining why our non-calorimetric method will yield a reliable estimate of ΔC_p^D, i.e., heat capacity change associated with the transition between N and D (which is more unfolded than X).

Our main aim is to estimate ΔC_p^D values of four model proteins, namely, lysozyme, RNase-A, apo-La and ctg for the transition, N↔D. It is possible only when the values of ΔG_D^0 (Gibbs energy change in the absence of the denaturant at 25 °C), ΔH_m^D (0) (enthalpy change in the absence of the denaturant) and T_m^D (0) (midpoint of denaturation in the absence of the denaturant) for each protein are accurately known (see Eq. (4)). These thermodynamic parameters are obtained from the measurements of ΔG_D from the conformational transition curves. Estimation of ΔG_D from an optical transition curve is possible if the protein operates under certain constraints. A few comments are therefore necessary. First, the chemical denaturant-induced and heat-induced transitions between N and D are reversible. It has been observed that GdmCl-induced and urea-induced denaturations of each protein at 25 °C and its heat-induced denaturation in the presence of different concentrations of GdmCl and urea are reversible as judged by the identical values of the optical property during denaturation and renaturation experiments.

Second, in order to estimate ΔG_D value from denaturation curves a two-state mechanism has been assumed under all experimental conditions. All available data strongly support that this assumption is valid in case of GdmCl-induced and urea-induced denaturations of lysozyme [1,36], RNase-A [1,37], apo-La [38] and ctg [33,39].

Third, for estimating the ΔG_D^0 of proteins from the GdmCl (or urea)-induced denaturation curves at 25 °C, we have analyzed our data using nonlinear least-squares method given by Santoro and Bolen [27], which assumes a linear dependence of ΔG_D on the denaturant concentration. Our earlier studies on four proteins, namely, RNase-A, lysozyme, cytochrome-c and myoglobin have shown that ΔG_D^0 is linear in the entire concentration

range of both denaturants [6,30,34]. Although such data are not available in the cases of ctg and apo-La, it has been assumed that ΔG_D of these two proteins shows a linear dependence on [denaturant]. This assumption is supported by Schellman [7] who, using a thermodynamic approach, has shown that ΔG_D of dilute protein solution is a linear function of [denaturant].

Fourth, in the derivation of Eq. (4), it has been assumed that ΔC_p is independent of temperature. ΔC_p must vary with temperature [40], but the dependence is too small to measure [11].

Are ΔG^0_D, ΔH^D_m (0) and T^D_m (0) the properties of the protein alone, not of the protein and the chemical denaturant? In order to answer this question, we estimated these quantities for two chemical denaturants, GdmCl and urea, which are known to induce the same N↔D transition [41]. Since the ΔG^0_D values obtained from the urea-induced and GdmCl-induced curves for RNase-A, lysozyme and apo-La are, within experimental errors, identical (see Table 1), we therefore conclude that ΔG^0_D is the property of the protein alone. In case of ctg, we could not obtain the ΔG^0_D value from GdmCl-induced denaturation due to its aggregation in the presence of low [GdmCl] up to 1.7 M.

ΔH^D_m and T^D_m can be obtained from the measurements of the effect of temperature on K_D, the equilibrium constant associated with the transition, N↔D. This equilibrium exists only in the transition region of the chemical-induced denaturation but not in the absence or presence of denaturant concentrations corresponding to the pre and posttransition regions [5]. We have therefore measured the denaturation curves of the protein in the presence of transition region concentration of the chemical denaturant by following changes in the optical property as a function of temperature (e.g., see Figs. 1A and 2A). Each transition at a chemical denaturant concentration was analyzed for ΔG_D values as a function of temperature. Stability curve at a given [d] was constructed (e.g., see Panel B of Figs. 1 and 2). Following the earlier procedure [35] values of T^D_m and ΔS^D_m ($=-(\delta\Delta G_D/\delta T)_p$, where p is pressure), the entropy change at T^D_m were determined. The product of ΔS^D_m and T^D_m gave ΔH^D_m, the enthalpy change at T^D_m. These values of ΔH^D_m and T^D_m for all the four proteins in the presence of different concentrations of the chemical denaturants are given in Table 2. It is seen in this table that ΔH^D_m shows strong dependence on [urea] and [GdmCl]. These findings are in agreement with the recent DSC and equilibrium measurements [10,42,43]. On the contrary, in order to estimate ΔC^D_p almost all analyses of heat-induced transition curves followed by various conformational properties of proteins in the presence of urea and GdmCl have assumed that ΔH^D_m is independent of the chemical denaturant concentration [5,44,45].

In order to estimate ΔC^D_p for the N↔D transition in the absence of a chemical denaturant, we require ΔH^D_m and T^D_m values in the absence of the denaturant. These values were obtained from the plots of T^D_m versus [denaturant] (e.g., see Panel C of Figs. 1 and 2) and ΔH^D_m versus [denaturant] (e.g., see Panel D of Figs. 1 and 2) using values of ΔH^D_m and T^D_m at different GdmCl and urea concentrations given in Table 2; the linear extrapolation of these plots to 0 M denaturant gave values of ΔH^D_m (0) and T^D_m (0) for all the proteins (see Table 1). Since values of ΔH^D_m (0) and T^D_m (0) from the heat denaturations are, with in experimental errors, identical (see Table 1), these thermodynamic quantities are the properties of the protein alone.

Using values of ΔH^D_m (0) and T^D_m (0) along with the ΔG^0_D values, ΔC^D_p values were estimated for all the proteins with the help of Eq. (4). The estimated values of ΔC^D_p from this method for all the proteins are given in Table 1. It can be seen in this table that both urea and GdmCl denaturation gave, within experimental errors, identical values of ΔC^D_p. This is an expected result because both urea and GdmCl induce the same conformational transition between N and D [41]. For each protein, a comparison of ΔC^D_p with ΔC^X_p from the DSC measurements in the absence of the chemical denaturant (see Table 1) suggests that the heat denatured protein retains residual native structure. This is supported by the CD spectra of N, X and D states of all proteins studied here (see inset (b) in Figs. 1A, 2A, Figs. S1–S5); the thermally denatured protein (curve 2) has more structure than the GdmCl or urea denatured protein (curve 3). This could be a most probable cause for the difference in ΔC^D_p and ΔC^X_p of a protein [46].

How do errors in the measured values of ΔG^0_D, ΔH^D_m (0) and T^D_m (0) affect the ΔC^D_p values of proteins, which are estimated with the help of Eq. (4)? Our estimate of the upper limits of the probable errors are ±0.8 kJ mol^{-1} for ΔG^0_D, ±12.5 kJ mol^{-1} for ΔH^D_m (0) and ±0.3 °C for T^D_m (0). Following the procedure of Pace and Laurent [11], these errors lead, respectively, to errors in ΔC^D_p of ±12.5, 12 and 1% for RNase-A; ±4, 6 and 0.2% for lysozyme; ±10, 10 and 0.01% for apo-La; and ±4.8, 5.4 and 0.6% for ctg. It has been shown that the error involved in ΔC_p from the DSC measurements is in the range 10–12% [40]. It is then concluded that our method measures ΔC^D_p with the same accuracy as in the DSC.

Privalov [1] have used a different method for determining ΔC^D_p. The observed calorimetric ΔH_m of the protein is corrected for the contribution due to the denaturant binding. This correction uses Δn (number of specific binding site exposed for the denaturant molecules on denaturation). A question arises: are there specific binding sites for the denaturant on proteins? The calorimetric binding studies show that there is binding between the protein and GdmCl, as well as urea [43]. As pointed out by Makhatadze and Privalov [43], "…if protein interaction with these denaturant can be regarded as a simple [specific] binding, the total Gibbs energy of binding, and thus Gibbs energy of protein unfolding, would not be a linear function of the denaturant concentration". Since dependence of ΔG_D of lysozyme and other proteins on [GdmCl], as well as [urea], is linear [6], the protein interaction with the denaturants is, therefore, not specific binding [7,43]. Hence, in the absence of any evidence for the presence of specific binding sites for GdmCl and urea on the protein, the correction for the contribution due to specific denaturant binding to the calometric ΔH_m is not justified.

We have also attempted to predict ΔC^D_p for the transition between the folded native and the theoretical denatured states with the help of the lower bound and the upper bound models [13]. The two models mentioned here establish limits on the accessible surface area of unfolded proteins without disulfide cross-links. Backbone and side chain ASA values (see Table 1 in Ref. [13]) were summed up for each model, and f_i was

determined for lower and upper bound models with the help of Eq. (6). For each denatured state model ΔC_p^D was predicted with the help of Eq. (5) for RNase-A, lysozyme, apo-La and ctg. These predicted values of ΔC_p^D for both lower bound and upper bound models are given in Table 1. This table also shows ΔC_p^X values of proteins obtained from DSC measurements. It is seen in Table 1 that for each protein ΔC_p^D observed here is significantly larger than ΔC_p^X. This comparison clearly shows that the heat denatured proteins are less unfolded than that obtained during denaturation by GdmCl (or urea), and are stabilized by hydrophobic interactions, for ΔC_p is approximately proportional to the non-polar surface area exposed to water upon unfolding [47].

Table 1 also shows that for each protein ΔC_p^D observed here is significantly smaller than those predicted for the lower and upper bound models of the linear random coil. One reason for this discrepancy is that any analysis based on a tripeptide model overestimates the backbone ASA of a linear random coiled protein polypeptide chain [13,48]. Another reason for this discrepancy is the fact that GdmCl (or urea) gives cross-linked denatured states of all proteins used in this study, due to the presence of intrachain disulphide bonds. These disulphide cross-links will reduce the solvent accessibilities of the protein groups in the denatured proteins. In fact, it has been shown that such cross-links reduce the dimensions of the linear random coiled (without disulphide bonds) RNase-A, ctg, lysozyme and apo-La by 42, 59, 62 and 70%, respectively (see Table 4 in Ref. [49]). It is interesting to note that the difference between the observed and predicted ΔC_p^D values is smallest for RNase-A, and largest for apo-La. It is expected that the discrepancy between the observed and predicted ΔC_p^D will vanish for a protein without disulphide bonds. The work is under progress for such proteins to test which theoretically denatured model (lower or upper bound) is best suited to describe the experimentally observed denatured state.

Acknowledgements

This work was supported by the Council of Scientific and Industrial Research, India (Grant No. 37(1237)/05/EMR-II) and the Department of Science and Technology, India (Grant No. SR/SO/BB-80/2004).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2007.12.006.

References

[1] P.L. Privalov, Stability of proteins: small globular proteins, Adv. Protein Chem. 33 (1979) 167–241.
[2] A. Sinha, S. Yadav, R. Ahmad, F. Ahmad, A possible origin of differences between calorimetric and equilibrium estimates of stability parameters of proteins, Biochem. J. 345 (2000) 711–717.
[3] S. Yadav, F. Ahmad, A new method for the determination of stability parameters of proteins from their heat-induced denaturation curves, Anal. Biochem. 283 (2000) 207–213.
[4] F. Ahmad, C.C. Bigelow, The denaturation of ribonuclease-A by combinations of urea and salt denaturants, J. Mol. Biol. 131 (1979) 607–617.
[5] C. Tanford, Protein denaturation. C. Theoretical models for the mechanism of denaturation, Adv. Protein Chem. 24 (1970) 1–95.
[6] F. Ahmad, S. Yadav, S. Taneja, Determining stability of proteins from guanidinium chloride transition curves, Biochem. J. 257 (1992) 481–485.
[7] J.A. Schellman, Solvent denaturation, Biopolymers 17 (1978) 1305–1322.
[8] K.C. Aune, C. Tanford, Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. II. Dependence on denaturant concentration at 25 °C, Biochemistry 8 (1969) 4586–4590.
[9] C. Tanford, K.C. Aune, Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. III. Dependence on temperature, Biochemistry 9 (1970) 206–211.
[10] B. Moza, S.H. Qureshi, F. Ahmad, Equilibrium studies of the effect of difference in sequence homology on the mechanism of denaturation of bovine and horse cytochrome-*c*, Biochim. Biophys. Acta 1646 (2003) 49–56.
[11] C.N. Pace, D.V. Laurent, A new method for determining the heat-capacity change from protein folding, Biochemistry 28 (1989) 2520–2525.
[12] P.L. Privalov, E.I. Tiktopulo, S. Venyaminov, Yu-V. Griko, G.I. Makhatadze, N.N. Khechinashvili, Heat capacity and conformation of proteins in the denatured state, J. Mol. Biol. 205 (1989) 737–750.
[13] T.P. Creamer, R. Srinivasan, G.D. Rose, Modeling unfolded states of proteins and peptides. II. Backbone solvent accessibility, Biochemistry 36 (1997) 2832–2835.
[14] K. Hamaguchi, A. Kurono, Structure of muramidase (lysozyme). I. The effect of guanidine hydrochloride on muramidase, J. Biochem. (Tokyo) 54 (1963) 111–122.
[15] C.C. Bigelow, Difference spectra of ribonuclease and two ribonuclease derivatives, C. R. Trav. Lab. Carlsberg. 31 (1960) 305–324.
[16] S. Sugai, H. Yashiro, K. Nitta, Equilibrium and kinetics of the unfolding of alpha-lactalbumin by guanidine hydrochloride, Biochim. Biophys. Acta 328 (1973) 35–41.
[17] C.N. Pace, Evaluating contribution of hydrogen bonding and hydrophobic bonding to protein folding, Methods Enzymol. 259 (1995) 538–554.
[18] C.N. Pace, Determination and analysis of urea and guanidine hydrochloride denaturation curves, Methods Enzymol. 131 (1986) 266–289.
[19] J.M. Sturtevant, Biochemical applications of differential scanning calorimetry, Annu. Rev. Phys. Chem. 38 (1987) 463–488.
[20] F. Ahmad, C.C. Bigelow, Estimation of the free energy of stabilization of ribonuclease A, lysozyme, alpha-lactalbumin, and myoglobin, J. Biol. Chem. 257 (1982) 12935–12938.
[21] W. Kabsch, C. Sander, Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features, Biopolymers 22 (1983) 2577–2637.
[22] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, The protein data bank, Nucleic Acids Res. 28 (2000) 235–242.
[23] F.A. Momany, R.F. Mc Guire, A.W. Burgess, H.A. Scheraga, Energy parameters in polypeptides. VII. Geometric parameters, partial atomic charges, nonbonded interactions, hydrogen bond interactions, and intrinsic torsional potentials for the naturally occurring amino acids, J. Phys. Chem. 79 (1975) 2361–2381.
[24] M. Oobatake, T. Ooi, Hydration and heat stability effects on protein unfolding, Prog. Biophys. Mol. Biol. 59 (1993) 237–284.
[25] S. Ahmad, M.M. Gromiha, A. Sarai, Real value prediction of solvent accessibility from amino acid sequence, Proteins 50 (2003) 629–635.
[26] F. Ahmad, Protein stability from denaturation transition curves, Ind. J. Biochem. Biophys. 28 (1991) 168–173.
[27] M.M. Santoro, D.W. Bolen, Unfolding free energy changes determined by the linear extrapolation method. I. Unfolding of phenylmethanesulfonyl a-chymotrypsin using different denaturants, Biochemistry 27 (1988) 8063–8068.
[28] Y.V. Griko, E. Freire, P.L. Privalov, Energetics of the alpha-lactalbumin states: a calorimetric and statistical thermodynamic study, Biochemistry 33 (1994) 1889–1899.
[29] Y. Fujita, Y. Noda, Effect of reductive alkylation on thermal stability of ribonuclease A and chymotrypsinogen A, Int. J. Pept. Protein Res. 38 (1991) 445–452.

[30] R. Gupta, F. Ahmad, Protein Stability: Functional dependence of denaturational Gibbs energy on urea concentration, Biochemistry 38 (1999) 2471–2479.
[31] G. Velicelebi, J.M. Sturtevant, Thermodynamics of the denaturation of lysozyme in alcohol–water mixtures, Biochemistry 18 (1979) 1180–1186.
[32] M. Ikeguchi, M. Fujino, M. Kato, K. Kuwajima, S. Sugai, Transition state in the folding of {alpha}-lactalbumin probed by the 6–120 disulfide bond, Protein Sci. 7 (1998) 1564–1574.
[33] M. Jackson, J.F. Brandts, Thermodynamics of protein denaturation. A calorimetric study of the reversible denaturation of chymotrypsinogen and conclusions regarding the accuracy of the two-state approximation, Biochemistry 9 (1970) 2294–2301.
[34] R. Gupta, S. Yadav, F. Ahmad, Protein stability: urea-induced versus guanidine-induced unfolding of metmyoglobin, Biochemistry 35 (1996) 11925–11930.
[35] S. Taneja, F. Ahmad, Increased thermal stability of proteins in the presence of amino acids, Biochem. J. 303 (1994) 147–153.
[36] B. Ibarra-Molero, J.M. Sanchez-Ruiz, A model-independent, nonlinear extrapolation procedure for the characterization of protein folding energetics from solvent-denaturation data, Biochemistry 35 (1996) 14689–14702.
[37] M. Yao, D.W. Bolen, How valid are denaturant-induced unfolding free energy measurements? Level of conformance to common assumptions over an extended range of ribonuclease-A stability, Biochemistry 34 (1995) 3771–3781.
[38] W. Pfeil, M.L. Sadowski, A scanning calorimetric study of bovine and human apo-a-lactalbumin, Stud. Biophys. 109 (1985) 163–170.
[39] T.V. Chalikian, J. Voelker, D. Anafi, K.J. Breslauer, The native and the heat-induced denatured states of alpha-chymotrypsinogen A: thermodynamic and spectroscopic studies, J. Mol. Biol. 274 (1997) 237–252.
[40] W.J. Becktel, J.A. Schellman, Protein stability curves, Biopolymers 26 (1987) 1859–1877.
[41] C.N. Pace, The stability of globular proteins, CRC Crit. Rev. Biochem. 3 (1975) 1–43.
[42] M.M. Santoro, D.W. Bolen, A test of the linear extrapolation of unfolding free energy changes over an extended denaturant concentration range, Biochemistry 31 (1992) 4901–4907.
[43] G.I. Makhatadze, P.L. Privalov, Protein interactions with urea and guanidinium chloride. A calorimetric study, J. Mol. Biol. 226 (1992) 491–505.
[44] F. Ahmad, P. McPhie, Thermodynamics of the denaturation of pepsinogen by urea, Biochemistry 17 (1978) 241–246.
[45] F. Ahmad, A. Salahuddin, Reversible unfolding of the major fraction of ovalbumin by guanidine hydrochloride, Biochemistry 15 (1976) 5168–5175.
[46] S. Robic, M. Guzman-Casado, J.M. Sanchez-Ruiz, S. Marqusee, Role of residual structure in the unfolded state of a thermophillic protein, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 11345–11349.
[47] N. Muller, Hydrophobicity and stability for a family of model proteins, Biopolymers 33 (1993) 1185–1193.
[48] J.K. Myers, C.N. Pace, Hydrogen bonding stabilizes globular proteins, Biophys. J. 71 (1996) 2033–2039.
[49] F. Ahmad, C.C. Bigelow, Estimation of the stability of globular proteins, Biopolymers 25 (1986) 1623–1633.