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Determination of heat capacity of unfolding for marginally stable proteins from a single temperature induced protein unfolding profile

Komal Saini, Unnati Ahluwalia, Shashank Deep*

Department of Chemistry, Indian Institute of Technology, Delhi 110016, India

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ABSTRACT

A reliable estimation of heat capacity of denaturation (ΔC_p) is necessary to calculate the free energy of unfolding of proteins. For marginally stable proteins, such as mutants of a protein or proteins at low pH or under denaturating conditions, the pre-transition region is not fully populated by the native state. Analysis of differential scanning calorimeter (DSC) data under such conditions may not yield a reliable value of ΔC_p and other associated thermodynamic parameters of unfolding. Analysis of denaturation profiles of (a) cytochrome c at pH 2.5, 3 and 8 and (b) myoglobin at pH 4, show that an accurate value of ΔC_p can be extracted from a single unfolding profile obtained spectroscopically by including low temperature data.

1. Introduction

Stability of proteins is generally quantified in terms of ΔG_u^0, the free energy change for the native to unfolded transition. ΔG_u^0 is estimated from the protein unfolding data monitored by calorimetric or spectroscopic methods [1–3]. In order to calculate ΔG_u^0 from calorimetric/spectroscopic data, an accurate estimation of T_m, ΔH_m and ΔC_p is essential.

For a protein with typical free energy of denaturation (>2.5 kcal/mol) between 273 K and 298 K, conventional analysis of DSC data yields an accurate value of these thermodynamic parameters. However, there are the cases where the proteins are only marginally stable. Mutant proteins, obtained through site-directed mutagenesis, generally have low intrinsic thermal stability. Similarly, intermediates like molten globules are only marginally stable. In addition, protein folding/unfolding experiments are often conducted under conditions where free energy of denaturation is positive but small. For example, at low pH or with denaturants, the onset of cold denaturation prevents the pre-transition region to be fully populated by the native state. Under such conditions, extracted thermodynamic parameters obtained from the conventional analysis of DSC data may not be accurate [4–6].

T_m and ΔH_m can be readily determined from thermal denaturation curves obtained using spectroscopic methods. Analysis of single unfolding profile is often not been found to give a reliable value of ΔC_p. Therefore, single unfolding profile of denaturation is rarely been used to measure ΔC_p and even not mentioned as a method to determine ΔC_p [7]. Instead, other methods, which make use of several denaturation profiles, have been proposed to determine ΔC_p. The first one involves the determination of ΔH as a function of temperature from van't Hoff analysis of thermal unfolding curves at different pH values or denaturant concentrations [8–12]. ΔC_p is then calculated using Kirchoff equation. A second approach involves the estimation of ΔC_p from the plot of ΔH_m and corresponding T_m obtained from thermal transition curves at different pH values [1,13]. A third method developed by Pace and Laurent [7] involves the measurement of ΔG_{H_2O} (the value of ΔG in the absence of urea) by analysis of the urea unfolding curve. Determining ΔG_{H_2O} as function of temperature yields $\Delta G(T)$ (change in the free energy of unfolding at temperature T) which is used with ΔH_m and T_m in the Gibb's Helmholtz equation to calculate ΔC_p. All these methods rely on the premise that the ΔC_p does not change with change in conditions used to alter ΔH_m and T_m of proteins. This may not be true in the case of marginally stable proteins. For example, ΔC_p for molten globules of cytochrome c at different pH is different.

It would have been ideal if we could extract the ΔC_p from single thermal denaturation profile for these marginally stable proteins. Keeping these in mind, we decided to revisit at the analysis of single thermal denaturation profile of marginally stable proteins. In this paper, simulation of protein unfolding profiles as a function of ΔC_p was attempted to understand the effect of heat capacity on them. Based on these simulations and experimental unfolding data on some proteins, we show that for marginally stable protein,

* Corresponding author. Tel.: +91 11 26596596; fax: +91 11 26581102.
E-mail address: sdeep@chemistry.iitd.ac.in (S. Deep).

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an accurate value of ΔC_p can be extracted from a single unfolding profile by including low temperature data.

2. Experimental

2.1. Materials and methods

Horse cytochrome c and myoglobin were purchased from Sigma. The concentration of cyt c and myoglobin samples was determined spectrophotometrically using an extinction coefficient of $10.6 \times 10^4\ M^{-1}\ cm^{-1}$ at 409 nm and $18.6 \times 10^4\ M^{-1}\ cm^{-1}$ at 409.5 nm, respectively. Thermal denaturation studies of cytochrome c in 50 mM phosphate buffer (pH 8.0) and myoglobin in 50 mM acetate buffer (pH 4.0) were carried out on a Varian Cary eclipse fluorescence spectrophotometer with peltier based temperature controller. Excitation and emission wavelengths were kept at 295 nm and 350 nm, respectively, whereas a slit width of 10 nm (both for excitation and emission) was used for all these experiments. All the data were normalized to 1.0 with respect to maximum fluorescence intensity before being used for fitting.

DSC measurements of cytochrome c at pH 2.5 and 3.0 in 50 mM phosphate buffer were performed on Microcal VP-DSC microcalorimeter with a cell volume of 0.52 ml, under a constant pressure of 30 psi, in order to avoid the formation of gas bubbles during the experiment. The calorimeter was operated at a scan rate of $1\ K\ min^{-1}$ with sample concentration of 100 μM. Samples were gently degassed for 2–3 min prior to the experiments. The concentration of degassed cyt c sample was determined spectrophotometrically using an extinction coefficient of $10.6 \times 10^4\ M^{-1}\ cm^{-1}$ at 409 nm and this concentration was used in the analysis of DSC data. All the scans were found to be completely reversible on rescan. Thermograms were corrected for instrumental baseline, using control buffer:buffer scans under identical conditions, and analyzed using standard MicroCal ORIGIN software.

2.2. Thermodynamic models used to fit protein denaturation profiles obtained by fluorescence

For thermally induced two-state transitions, the following equations were used to simulate or for a nonlinear least square fit of signal vs. temperature profile.

$$y(T) = \frac{(y_N^0 + m_N \Delta T) + (y_D^0 + m_D \Delta T) e^{-\Delta G/RT}}{1 + e^{-\Delta G/RT}} \quad (1)$$

$\Delta G(T)$ is given by

$$\Delta G(T) = \Delta H_m \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left((T_m - T) + T \ln\left(\frac{T}{T_m}\right)\right) \quad (2)$$

where $y(T)$ is the experimentally observed spectroscopic property of protein at temperature T, $\Delta G(T)$ is the free energy of denaturation at temperature T, ΔC_p is the specific heat capacity of denaturation and ΔH_m is the enthalpy change at T_m, the mid-point of the thermal denaturation, y_N^0 and y_D^0 are the spectroscopic value for the native and denatured state at 298 K, $\Delta T = T - 298$. m_N is the slope of the linear plot of signal from native vs. temperature, $(\partial y/\partial T)_N$, and m_D is the slope of the linear plot of signal from denatured state vs. temperature, $(\partial y/\partial T)_D$.

A family of protein denaturation profiles were simulated using Eqs. (1) and (2) for different values of T_m, ΔC_p and ΔH_m. To see the effect of a small change in heat capacity on the thermal profile of a protein, the deviation of the simulated real intensity data (I_i^{sim1}) at temperature "i" from the one simulated at same temperature with slightly higher/lower value of heat capacity (I_i^{sim2}) than the heat capacity value taken for real data was calculated in terms of sum of square of residuals $(SSR)_{sim}$ using Eq. (3).

$$(SSR)_{sim} = \sum_i (I_i^{sim1} - I_i^{sim2})^2 \quad (3)$$

Best-fit values of parameters were obtained by analyzing three different experimental denaturation profiles. For nonlinear fitting of experimentally obtained denaturation profile using Eqs. (1) and (2), a good estimate of initial value of various thermodynamic parameters are needed. Initial value of T_m, y_N^0, and y_D^0 was easy to guess whereas the initial value of m_N and m_D was calculated from the slopes of the pre-transition (excluding the pre-transition data showing abrupt increase/decrease) and post-transition data, respectively. Simulations were carried out to generate a family of curves and get a better feel of how ΔH_m and ΔC_p affect the denaturation profiles. Simulated and experimental data were plotted in the same plot and initial values of ΔH_m, ΔC_p, y_N^0, y_D^0, m_N and m_D were chosen from a simulation profile which shows a good overlap with experimental profile and a very small *SSR* was observed between simulated and experimental values. Finally, the seven parameter nonlinear regression analysis was carried out to calculate the best-fit values of ΔC_p, ΔH_m, T_m, y_N^0, y_D^0, m_N and m_D. Root mean sum of square of residuals, *RM-SSR*, which is the best-fit estimate of standard deviation of curve, was calculated from *SSR*, number of data point (N) and number of variable parameters (P).

$$(RM\text{-}SSR) = \sqrt{\frac{SSR}{N - P}}$$
$$(SSR) = \sum_i (I_i^{\exp} - I_i^{fit})^2 \quad (4)$$

where $I^{\exp}$ and I^{fit} are experimental and the best-fit value of intensity at temperature i.

Confidence interval for the fit was calculated by Monte-Carlo simulation. An ideal data set was generated using best-fit parameters. To each data point, random error was added from a Gaussian distribution with a mean of zero and the standard deviation equal to 1.5 times *RM-SSR* obtained from the nonlinear regression of the experimental data. Nonlinear regression of simulated data was carried out to get best-fit value of each parameter. The exercise of generating new random error was repeated 1000 times and 1000 new simulated data were generated. Nonlinear regression of 1000 different simulated profiles yielded 1000 best-fit values of various parameters. To obtain 95% confidence interval, these 1000 best-fit values of a parameter were sorted and 25 values from each ends were removed. The range of the rest is the 95% confidence interval of that parameter.

3. Results

3.1. Simulation of protein denaturation profiles

To examine the effect of ΔC_p, denaturation curve was simulated in the temperature range 298–373 K using a typical value of thermodynamic parameters for marginally stable proteins, $\Delta H_m = 52$ kcal/mol, $T_m = 330$ K and $\Delta C_p = 1.5$ kcal/mol/K (see caption for Fig. 1), as input for Eq. (1). Unfolding curves were further simulated for several different values of ΔC_p keeping other inputs same. The sum of the square of the residuals $(SSR)_{sim}$ was calculated to see the deviation between these simulated curves against the one simulated with experimental ΔC_p (1.5 kcal/mol/K). There was no remarkable change in simulated data on varying ΔC_p values from 0 kcal/mol/K to 2.5 kcal/mol/K, with $(SSR)_{sim}$ values increasing only at higher ΔC_p value (Fig. 1) indicating that ΔC_p cannot be accurately determined by fitting the spectroscopically obtained unfolding curve. Now, the low temperature data (283–297 K) were simulated

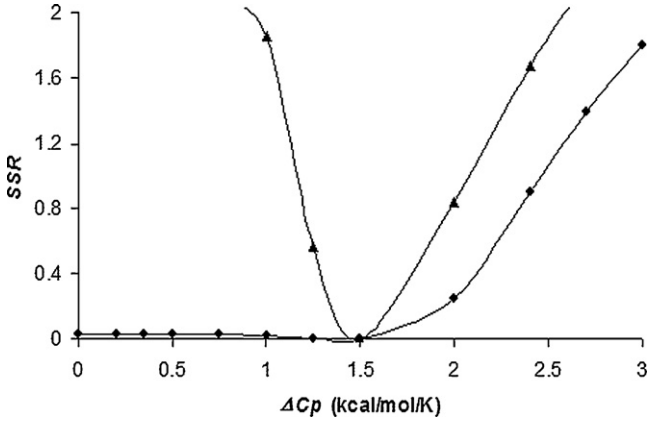

Fig. 1. Sum of square of residuals (*SSR*) for the simulated data with different heat capacity of denaturation (ΔC_p) against the one simulated with experimental one as a function of ΔC_p in two different temperature ranges: (i) 273–373 K (▲) and (ii) 298–373 K (●). Other inputs are kept same during simulation, e.g. enthalpy of denaturation (ΔH_m) = 52 kcal/mol, denaturation temperature (T_m) = 330 K and slopes of pre- and post-transition baselines (m_N and m_D) = 0.

and $(SSR)_{sim}$ was recalculated after including them. Recalculated $(SSR)_{sim}$ vs. ΔC_p curve is also shown in Fig. 1. It was interesting to find out that low temperature data differ significantly with change in heat capacity of denaturation. Consequently, on the inclusion of low temperature data in the analysis, $(SSR)_{sim}$ changes significantly with the change in the input value of ΔC_p (higher/lower than 1.5 kcal/mol/K). This suggests that the accuracy of ΔC_p extracted will increase with the inclusion of the low temperature data during analysis.

Now, we simulated the protein denaturation profiles for ΔC_p = 1.5 kcal/mol/K and various combinations of ΔH_m and T_m and $(SSR)_{sim}$ was calculated against the simulated curve with same ΔH_m and T_m but different values of ΔC_p. During the $(SSR)_{sim}$ calculation, data in the range 283–373 K were employed. Three-dimensional plots with ΔC_p as *x*-axis, $(SSR)_{sim}$ as y axis and ΔH_m as z axis at various T_m are shown in Fig. 2. It is evident from Fig. 2(a–c) that for proteins with low ΔC_p, $(SSR)_{sim}$ is high only for proteins with smaller ΔH_m and against a simulated curve with ΔC_p higher than experimental. Fig. 2(d–f) shows that, for proteins with high T_m, the $(SSR)_{sim}$ is high even for proteins with high ΔH_m making extraction of ΔC_p easy. However, for proteins with low T_m and high ΔH_m, the exact heat capacity can be extracted only when proteins has high ΔC_p. Entire $(SSR)_{sim}$ calculation was repeated for the data in the temperature range 278–363 K (data not shown). $(SSR)_{sim}$ is high for the data range 278–363 K in almost every case, indicating that inclusion of the low temperature data increases the accuracy of ΔC_p extracted. In summary, an exact value of ΔC_p cannot be extracted for proteins with higher stability between 273 K and 310 K, an accurate value of ΔC_p can be extracted only for proteins with high T_m/large ΔC_p/small ΔH_m, i.e. proteins with marginal stability in low temperature range (273–310 K).

We tested these observations by analyzing the thermal denaturation curves of proteins which are known to undergo cold denaturation above 273 K like cytochrome c (cyt c) and myoglobin. We also analyzed the thermal denaturation of cytochrome c at low pH where it is known to exist as molten globule.

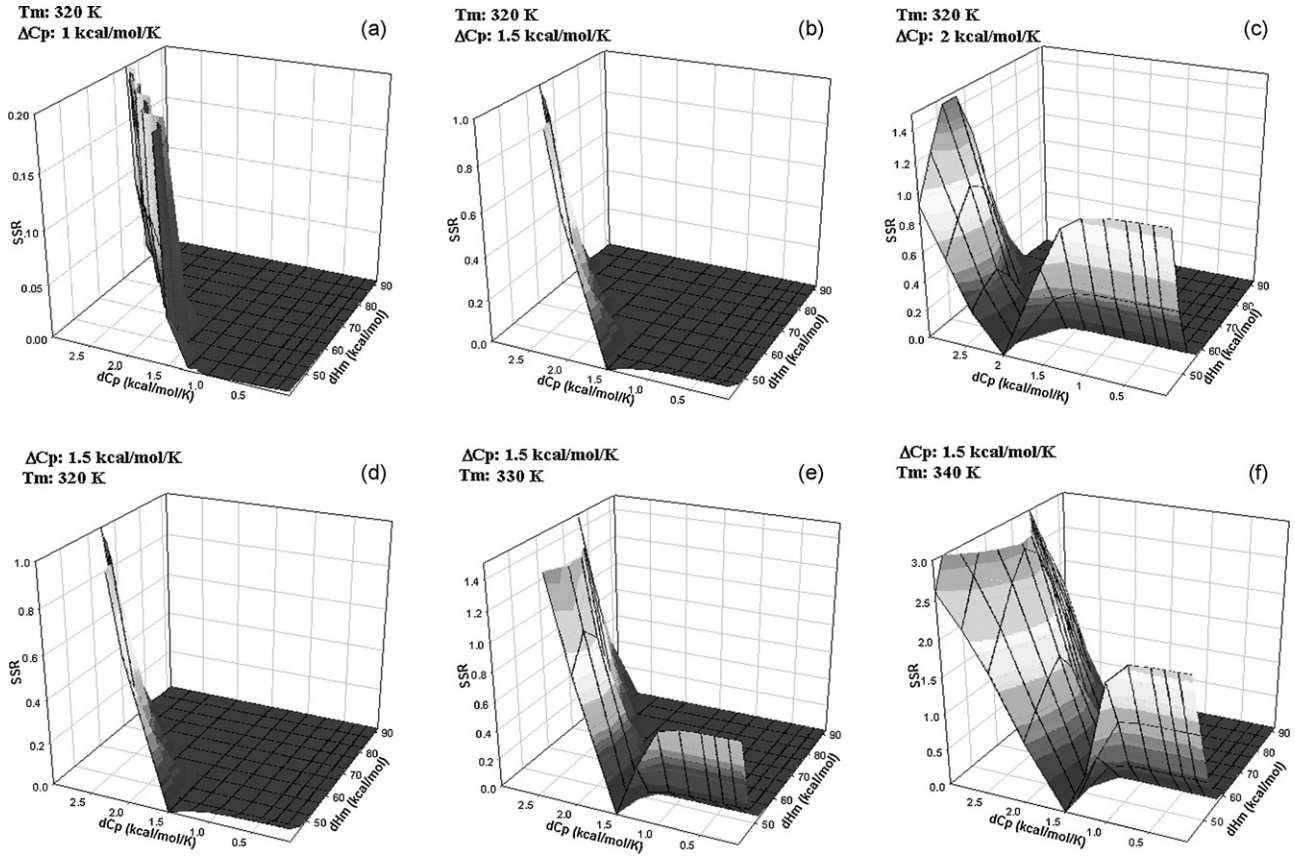

Fig. 2. Sum of square of residuals (*SSR*) for the simulated data against the one simulated with experimental ΔC_p as a function of variation in heat capacity of denaturation (ΔC_p), enthalpy of denaturation (ΔH_m) and denaturation temperature (T_m). Panels (a–c) show the effect of change in experimental ΔC_p (written at the top of the panel) at T_m = 320 K whereas panels (d–f) show the effect of change in T_m at ΔC_p = 1.5 kcal/mol/K. These simulations included low temperature range (273–373 K) data. Slope of the pre- and post-transition baselines (m_N and m_D) = 0 for all simulations.

Table 1
The extracted thermodynamic parameters with confidence interval (CI) from nonlinear fitting of denaturation profiles of cytochrome c (10 μM, pH 8.0) and myoglobin (10 μM, pH 4.0).

Thermodynamic parameter	Fluorescence data	
	Cytochrome c	Myoglobin
T_m (K) (±CI)[a]	361.0 (0.6)	321.3 (0.7)
ΔH_m (kcal/mol) (±CI)[a]	74.97 (4.53)	37.05 (4.86)
ΔC_p (kcal/mol/K) (±CI)[a]	1.57 (0.15)	1.936 (0.20)
RM-SSR	0.0079	0.0110

[a] Confidence interval (CI) was calculated through Monte-Carlo simulation.

3.2. Analysis of cytochrome c fluorescence data

Fluorescence spectra of 10 μM cytochrome c (pH 8) as a function of temperature were collected. Fluorescence intensity at 350 nm increases on denaturation since tryptophan fluorescence is quenched in the native state of cytochrome c. Nonlinear fitting of the denaturation profile is carried out using Eq. (1) to get the best-fit value of different thermodynamic parameters. Confidence interval (95%) for various thermodynamic parameters, as calculated from Monte-Carlo simulation, is reported in Table 1 along with the best-fit values of the parameters. Small values of confidence interval for various parameters indicate a good fitting. However, confidence limit changes from 0.15 to 0.5 if the low temperature data is excluded (303–370 K). ΔC_p extracted from nonlinear fitting of data in the temperature range 278–370 K is 1.57 kcal/mol/K which is quite similar to the ΔC_p extracted from other methods [14,15]. Fitted denaturation profile for cytochrome c and two simulated profiles with slightly different ΔC_p are shown in Fig. 3a. Fluorescence spectra of 10 μM cytochrome c at pH 4 (Suppl. Fig. 1) as a function of temperature were also collected. The nonlinear fitting of data yields ΔH_m = 92 kcal/mol, ΔC_p = 1.86 kcal/mol/K and T_m = 347 K. However, confidence interval of ΔC_p is large (0.6 kcal/mol/K), indicating that ΔC_p can only be extracted for marginally stable protein using this method.

3.3. Analysis of myoglobin denaturation data

Fluorescence spectra of 10 μM myoglobin at pH 4 as a function of temperature were collected. Best-fit parameters and their confidence intervals were given in Table 1. It is quiet evident from the confidence interval that a reliable and accurate value of ΔC_p can be extracted for myoglobin. Fitted denaturation profile for myoglobin and two simulated profiles with slightly different ΔC_p are shown in Fig. 3(b). The figure clearly shows that the low temperature pre-transition data differ with the change in ΔC_p value and hence provide information about ΔC_p. Thus an accurate value of ΔC_p can be extracted for proteins with small ΔH_m (37.05 kcal/mol), T_m = 321.3 K and intermediate value of ΔC_p (1.94 kcal/mol/K). Using DSC data, Hallerbach and Hinz [16] reported ΔH_m = 50.6 kcal/mol, ΔC_p = 1.7 kcal/mol/K and T_m = 333.4 K for myoglobin at pH 4.3, whereas Kelly et al. reported ΔC_p to be 1.86 kcal/mol/K [17]. A lower value of ΔC_p is obtained from the analysis of DSC data which is not surprising since native state is not fully populated in the pre-denaturation temperature range of DSC thermograms. Similarly, the ΔH_m obtained from the analysis of DSC data is small as compared to the expected ΔH_m at T = 333.4 K from the spectroscopically obtained thermodynamic parameters. With ΔH_m = 37.05 kcal/mol at T_m = 321.3 K [16], we expect $\Delta H_m = 37.05 + (333.4 - 321.3) \times 1.94 = 60.5$ kcal/mol to be at 333.4 K assuming that ΔC_p is constant with the change in pH and equal to 1.94 kcal/mol/K.

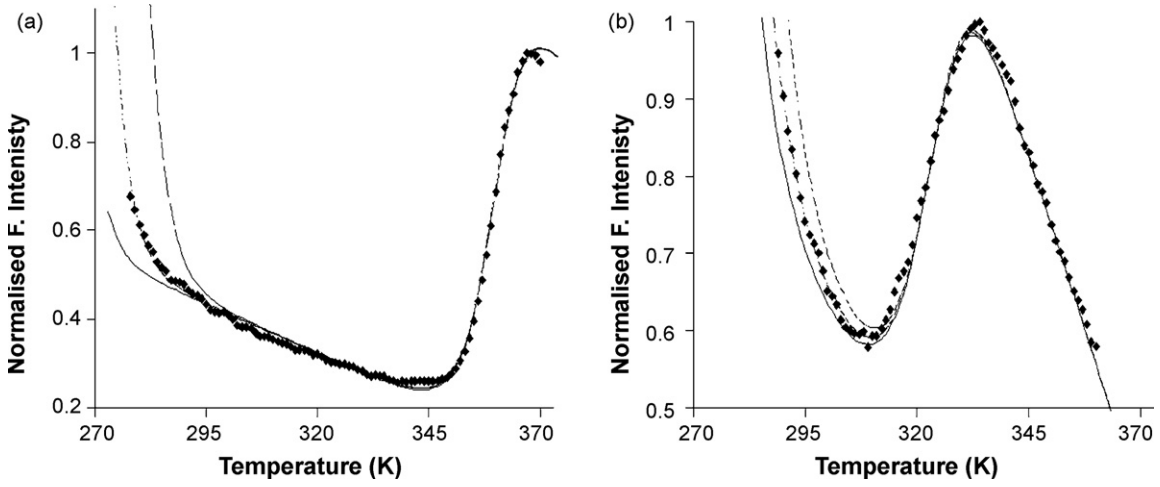

Fig. 3. Panel (a) shows normalized fluorescence (F.) intensity of cytochrome c (10 μM, pH 8.0) (♦) as a function of temperature. The lines (—), (–·–) and (– –) are two state fit with heat capacity of denaturation (ΔC_p) = 1.4 kcal/mol/K, 1.57 kcal/mol/K and 1.7 kcal/mol/K, respectively. Panel (b) shows thermal denaturation profile of myoglobin (10 μM, pH 4.0) (♦). The lines (—), (–·–) and (– –) are two state fit with ΔC_p = 1.8 kcal/mol/K, 1.94 kcal/mol/K and 2.1 kcal/mol/K, respectively.

Table 2
The extracted thermodynamic parameters with confidence interval (CI) from nonlinear fitting of denaturation profiles of molten globule of cytochrome c (pH 2.5 and 3.0).

Thermodynamic parameter	pH 2.5		pH 3.0	
	Fluorescence data	DSC data	Fluorescence data	DSC data
T_m (K) (±CI)[a]	313.0 (0.4)	314.65 ± 0.02	329.6 (0.2)	330.67 ± 0.01
ΔH_m (kcal/mol) (±CI)[a]	36.6 (4.13)	36.5 ± 0.02	53.0 (3.44)	57.5 ± 0.08
ΔC_p (kcal/mol/K) (±CI)[a]	0.86 (0.19)	–	1.06 (0.20)	–
RM-SSR	0.0005		0.0006	

[a] Confidence interval (CI) was calculated through Monte-Carlo simulation.

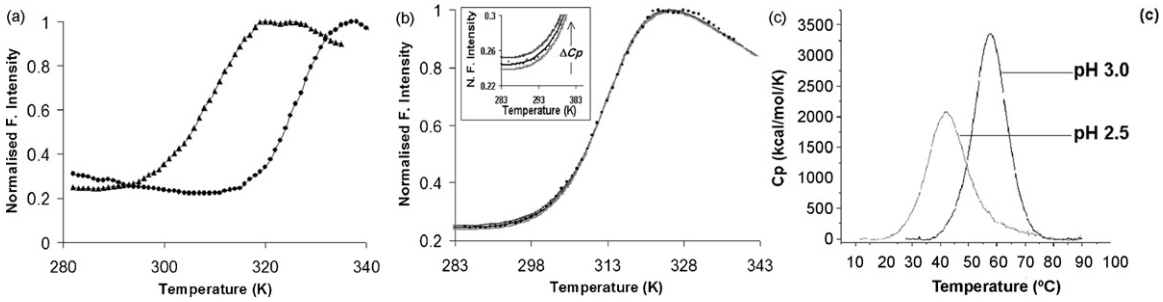

Fig. 4. Panel (a) shows normalized (N.) fluorescence (F.) intensity of molten globule of cyt c as a function of temperature at pH 2.5 (▲) and 3.0 (●). Panel (b) shows fluorescence thermal profile of cyt c at pH 2.5, (●) along with three lines (—), (ЖЖ) and (¤¤) which are simulated profiles with heat capacity of denaturation (ΔC_p) = 0.86 kcal/mol/K, 0.66 kcal/mol/K and 1.06 kcal/mol/K. Inset presents the better view of the pre-transition region. (c) DSC profile (excess heat capacity (C_p) vs. temperature profile) of cyt c at pH 2.5 and 3.0.

3.4. Analysis of cytochrome c molten globule denaturation data

Fluorescence spectra of 10 μM cytochrome c (pH 2.5, 3.0) as a function of temperature were collected. Extracted values of ΔH_m, ΔC_p and T_m, along with their confidence interval, are listed in Table 2. Small value of confidence interval indicates that ΔC_p can be easily extracted. With increase in pH, T_m of cyt c increases with a concomitant increase in ΔH_m and ΔC_p as seen from Fig. 4(a). Fitted denaturation profile for cytochrome c at pH 2.5 and two simulated profiles with slightly different ΔC_p are shown in Fig. 4(b). Inset of Fig. 4(b) shows clearly the difference between these three profiles in the pre-transition region. Since DSC data for cytochrome c at pH 2.5 was not available, DSC thermograms were also obtained for these samples and shown in Fig. 4(c). Extracted ΔH_m and T_m from fluorescence data is similar to that obtained from analysis of DSC data.

4. Discussion

To understand the dependence of protein unfolding profile on the heat capacity of denaturation, we calculated the changes in spectroscopic signal with change in ΔG using Eq. (1). The plot of spectroscopic signal vs. ΔG for several temperatures is shown in Suppl. Fig. 2(a and b). At 303 K, the signal is almost constant for ΔG greater than 2.5 kcal/mol, decreases/increases slightly between 2.5 kcal/mol and 2 kcal/mol, and there is sharp decrease/increase below 2 kcal/mol. Therefore, a change in signal is expected in the temperature range where ΔG is below 2.5 kcal/mol. This is not surprising considering the fact that for $\Delta G > 2.5$, D/N ratio is too low and contribution of D to signal is negligible. With an increase in the temperature, a sharp change in the signal value is seen at somewhat lower value of ΔG.

Suppl. Fig. 2(c) shows that at higher temperatures, the effect of ΔC_p on ΔG is not significant. However at lower temperatures, the ΔG is quite sensitive to ΔC_p. Since the signal changes are only significant when ΔG is low, the ΔC_p extracted will be accurate only for the proteins with marginal stability in the pre-transition region. For the particular case of Suppl. Fig. 2(c), ΔG is 2 kcal/mol at temperature 277 K, 284 K and 290 K for ΔC_p values of 2.4 kcal/mol/K, 2.7 kcal/mol/K and 3.0 kcal/mol/K, respectively. Therefore for a very small change (~0.3 kcal/mol/K) in ΔC_p, we see a temperature difference of 6–7 K where signal shows an abrupt change and thus a very accurate value of ΔC_p can be determined. Analysis of cyt c at pH 8, myoglobin at pH 4 signifies the importance of inclusion of low temperature data for the extraction of ΔC_p.

Fig. 5 shows various combinations of ΔH_m, ΔC_p and T_m at which the ΔG is equal or less than 2.5 kcal/mol above 273 K. It is evident from the figure that there are two temperature ranges for

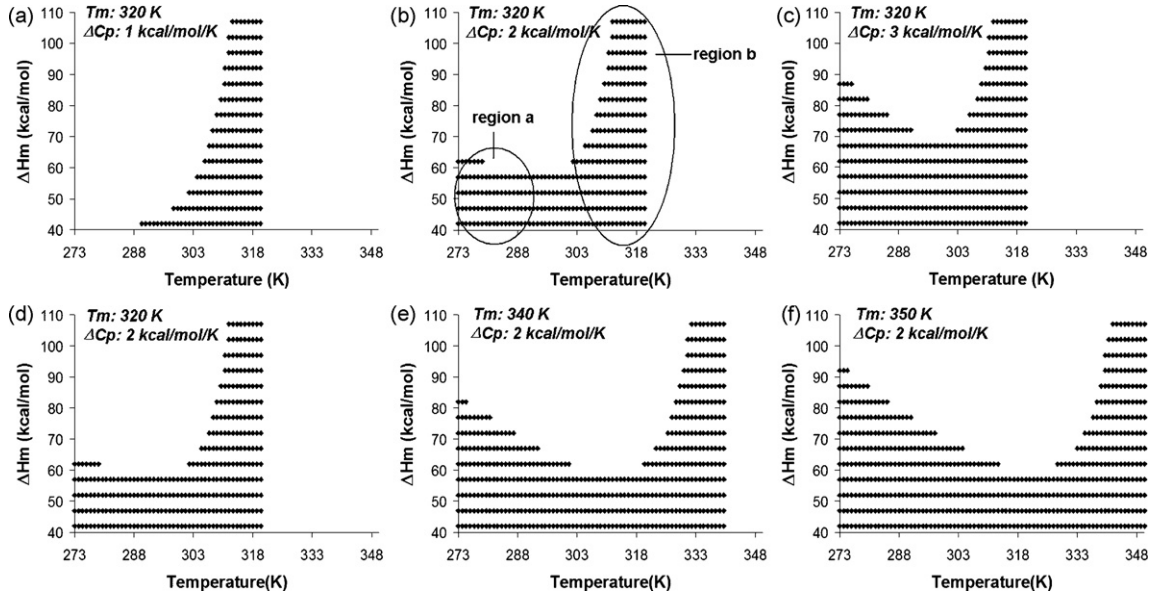

Fig. 5. Each panel shows temperature range in which free energy (ΔG) of the unfolding = 0–2.5 kcal/mol as a function of denaturation enthalpy (ΔH_m). Heat capacity of denaturation (ΔC_p) and denaturation temperature (T_m) are kept constant during calculation of temperature range for each panel and their values are mentioned at the top of the panel. Panels (a–c) show the effect of ΔC_p and panels (d–f) show the effect of T_m on the temperature range. Region a shows the foot of cold denaturation region where ΔG is less than 2.5 kcal/mol/K whereas region b shows the pre-transition region close to denaturation temperature where ΔG is less than 2.5 kcal/mol/K.

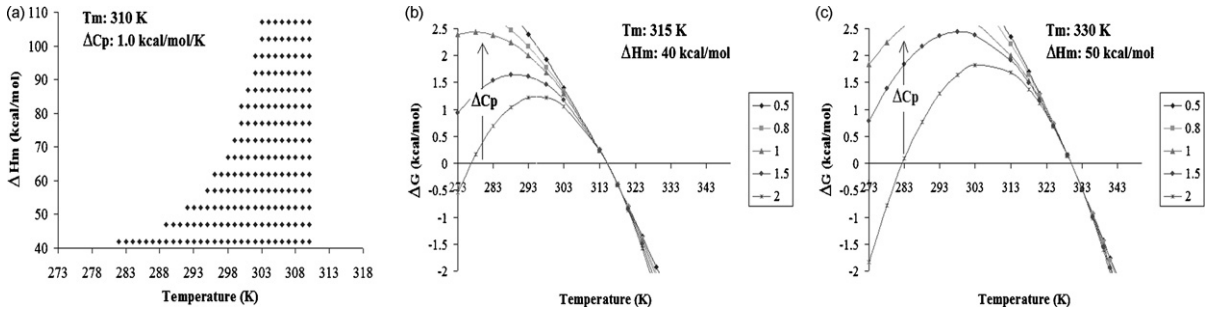

Fig. 6. Panel (a) shows temperature range in which free energy (ΔG) of the unfolding = 0–2.5 kcal/mol for different value of enthalpy of denaturation (ΔH_m) at denaturation temperature (T_m) = 310 K and heat capacity of denaturation (ΔC_p) = 1 kcal/mol/K. Panels (b) and (c) show variation in free energy (ΔG) of protein unfolding with temperature for different value of heat capacity. Other inputs are ΔH_m = 40 kcal/mol and T_m = 315 K for panel (b) and ΔH_m = 50 kcal/mol and T_m = 330 K for panel (c).

proteins in pre-transition region (shown in Fig. 5(b)) where ΔG is equal or less than 2.5 kcal/mol for any protein, one near the foot of cold denaturation region (region a) and another near the transition temperature (region b).

As evident from Fig. 5(a–c), the range of ΔH_m and T_m at which region a is above 273 K is larger for proteins with high ΔC_p. For proteins with smaller ΔC_p, region a is below 273 K for large range of ΔH_m and T_m whereas the region b is at high temperature making it impossible to extract ΔC_p with reasonable accuracy.

Fig. 5 shows the effect of T_m and ΔH_m on the region a and region b. For proteins with low T_m and high ΔH_m, the region a is below 273 K and region b is at high temperature. However, with decrease in ΔH_m, the region a shifts to high temperature and region b shifts to low temperature. At smaller values of ΔH_m, both regions get mixed. For a protein with high T_m, both region a and region b shifts to higher temperature and it becomes easier to extract heat capacity even for proteins with high ΔH_m since the data in region a is sensitive to ΔC_p. Confidence interval was large for ΔC_p extracted from cytochrome c unfolding profile at pH 4 because of combination of high ΔH_m and small T_m. We were able to extract a good-fit of ΔC_p from cytochrome c unfolding profile at pH 8 because of high T_m and low ΔH_m, making it marginally stable at low temperature. Similarly, we were able to extract ΔC_p from myoglobin unfolding profile at pH 4. A good fit value of ΔC_p could be extracted from these unfolding profile because region a was above 273 K.

A reasonable value of ΔC_p for cytochrome c was extracted at acidic pH. At pH 2.5 and 3, low T_m ensures that region b is at low temperature (Fig. 6(a)) and thus, the signal in region b is sensitive to ΔC_p (Fig. 6(b and c)), allowing extraction of a reliable value of ΔC_p. At acidic pH, cyt c is known to exist as molten globule like structure [18]. From the extracted thermodynamic data for cyt c unfolding at low pH, it is evident that the heat capacity is a function of pH. Therefore, it is essential to extract heat capacity from a single profile.

5. Conclusion

In summary, spectroscopic methods can work as complementary to DSC as far as the measurement of ΔC_p is concerned. A reliable value of ΔC_p can be easily extracted from DSC thermogram of more stable proteins where spectroscopic methods fail. Whereas ΔC_p for a marginally stable protein can be extracted from a single thermal denaturation profile obtained by spectroscopic method. For proteins with low ΔH_m and high T_m, even the smaller value of ΔC_p can be easily extracted since the data in region a is sensitive to ΔC_p. ΔC_p extraction is also easy for proteins with low T_m although for a different reason. For proteins with low T_m, the region b is more sensitive to ΔC_p. However, for a protein with intermediate T_m (>320 K) and high ΔH_m, the exact heat capacity can be extracted only when the protein has high ΔC_p. Inclusion of low temperature data generally increases the accuracy of extracted ΔC_p.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tca.2010.04.010.

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