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On the Estimation of Stability Parameters from Heat-Induced Conformational Transition Curves of Proteins

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A method is suggested to determine valid and authentic values of thermodynamic stability parameters of proteins from their heat-induced conformational transition curves. We show (a) that the estimate of ΔH_m^{van}, the enthalpy change on denaturation at T_m, the midpoint of denaturation, is significantly less than ΔH_m^{cal}, the value obtained by the calorimetric measurements, if the analysis of the conformational transition curve uses the conventional method which assumes a linear temperature-dependence of the pre- and post-transition baselines; and (b) that there exists an excellent agreement between ΔH_m^{van} and ΔH_m^{cal} values of proteins, if the analysis of thermal denaturation curves assumes that the temperature-dependence of pre- and post-transition baselines is described by a parabolic function. The latter analysis is supported by our observations that the temperature-dependencies of the absorption and circular dichroism properties of protein groups are indeed nonlinear. It is observed that the estimate of ΔC_p, the constant-pressure heat capacity change is independent of the model used to describe the temperature-dependence of the pre- and post-transition baselines. An important conclusion is that for proteins which exhibit a two-state character, all stability parameters are measured with the same error as that observed with a calorimeter.

Keywords: Protein stability, Thermal denaturation, Van't Hoff analysis, Enthalpy change, Heat capacity change, Gibbs energy change, Ribonuclease, Lysozyme

INTRODUCTION

Denaturation of many proteins is reversible, so the three-dimensional structure must be determined by the primary structure [1]. How this occurs has come to be known as 'the protein folding problem'. This problem can be broken down into several related questions. Some of these are: What are the physical factors responsible for the stability of folded proteins, what is the mechanism of folding of an unfolded protein, and why is it that only one particular folded structure rather than some other is coded by the primary structure. One of the prerequisites for answers to these questions is the knowledge of protein stability, ΔG_D^o, the Gibbs energy change associated with the process, native (N) conformation $\leftrightarrow$ denatured (D) conformation, occurring under physiological conditions usually taken as dilute buffer (or water) at 25 °C.

Two different approaches have been used to obtain almost all estimates of protein stability (ΔG_D^o) from the thermodynamic study of denaturation by heat and strong chemical denaturants such as guanidinium chloride (GdmCl). These are microcalorimetric and equilibrium methods. The differential scanning calorimetry (DSC) provides direct estimates of denaturational enthalpy change (ΔH_D) and the constant-pressure heat capacity change (ΔC_p). In the second

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approach, equilibrium constant is measured from the denaturant-induced conformational transition curve representing the equilibrium between N and D states. The latter method is hence called equilibrium method. DSC measurements of the heat-induced denaturation of the native protein in the absence and presence of low concentrations of GdmCl, isothermal calorimetric measurements of the GdmCl-induced denaturation of the native and heat denatured proteins, and heat capacity measurements of protein groups (N- and C-termini, amino acids and peptide), led Makhatadze and Privalov [2] to two definite conclusions. The first is the values for enthalpy and entropy of their thermal denaturation are the same as those for GdmCl-induced denaturation if the latter process is properly corrected for solvation effect. The second is the correspondence of the heat capacity of the denatured protein with the heat capacity expected for the unfolded polypeptide chain, which can be accurately calculated using the known heat capacities of the amino acid residues, appears to be one of the strongest criteria for the completeness of unfolding.

A survey of literature on the structural characterization of the species involved in the heat and GdmCl denaturations and on the measurements of thermodynamic parameters from the heat-induced and GdmCl-induced conformational transition curves has revealed (a) that for a protein, the GdmCl denatured state at 25 ^{o}C is structurally more unfolded than the heat denatured state [3]; (b) that the heat/acid denatured state contains residual structure that can be removed by the addition of GdmCl [4-7]; and (c) that with a few exceptions [8], ΔG_D^o for heat denaturation is less than that for GdmCl denaturation [9-11]. Furthermore, a comparison of the equilibrium ΔG_D^o from GdmCl-induced denaturation [12-15] with the calorimetric ΔG_D^o for heat denaturation of the same protein [16] suggests that the former is significantly less than the latter; for instance, the equilibrium and calorimetric ΔG_D^o values are respectively, 37.2 and 60.7 kJ mol^{-1} for lysozyme, 33.5 and 50.2 kJ mol^{-1} for myoglobin; 36.4 and 44.3 kJ mol^{-1} for ribonuclease-A (RNase-A), and 31.8 and 37.7 kJ mol^{-1} for cytochrome-c. These conclusions and those arrived from the calorimetric measurements mentioned in the preceding paragraph, are not only controversial but they also lead researchers to question the validity of the equilibrium method for the determination of protein stability parameters [16,17] and vice versa [18,19]. It is interesting to note that Privalov et al. [17] draw our attention by stating "... we do not have reliable procedure for evaluating the thermodynamic parameters of conformational transitions caused by denaturants. We do not know how to take into account the denaturant solvation effect and, even more importantly, we do not know what kind of reaction we are analyzing and usually only assume for simplicity that it is a two-state transition." On the other hand, Franks [19] states "...Makhatadze and Privalov (1992) have reported that the states of a protein subjected to different denaturing treatments are enthalpically identical. If that is indeed the case, then calorimetry may not be the best diagnostic tool for a study of protein stability, because other physical techniques, especially nuclear magnetic resonance (NMR), circular dichroism (CD), and optical rotary dispersion (ORD) have revealed quite distinct differences in the structures of unfolded states of proteins produced by different treatments...". In short, although the history of evaluation of ΔG_D^o spans more than thirty years, it seems that the procedures for the estimation of protein stability lack confidence.

The statement made by Privalov *et al*. [17] is very puzzling. If their view is the case, it then means that the estimated protein stability from the equilibrium method has no validity. This review addresses specifically this issue, namely, whether estimates of thermodynamic parameters from the heat-induced conformational transition curves of proteins are valid.

Analysis of Transition on Curves

The basic observation of a two-state heat-induced conformational transition curve of a protein is a sigmoid-shaped curve (*e.g*., see Figs. 1 and 2). This type of transition curves can be divided into three regions: (a) pre-transition region in which protein exists predominately in N state, (b) the transition region in which equilibrium between N and D states exists, and (c) the posttransition region in which protein exists predominately in D state. There are several methods that have been used to determine ΔH_m^{van}, the van't Hoff enthalpy change on denaturation at T_m, the midpoint of denaturation and T_m from a conformational transition curve induced by heat. The earlier procedure [20-24] involves the estimation of K_D, the equilibrium constant of denaturation (N conformation $\leftrightarrow$ D

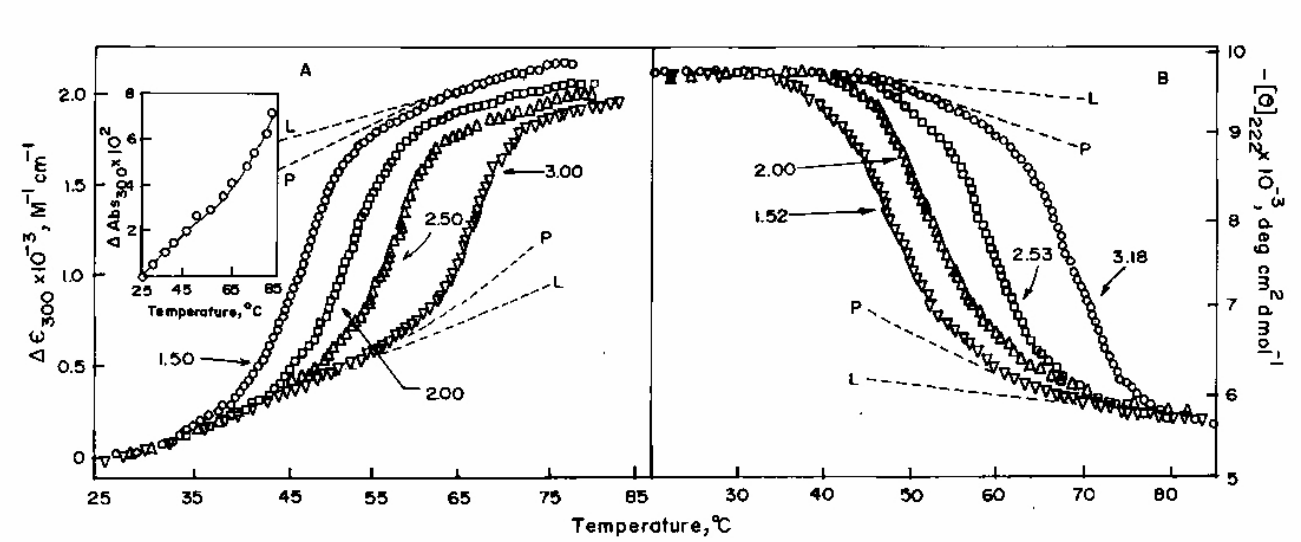

Fig. 1. Thermal denaturation curves of lysozyme followed by measurements of $\Delta\varepsilon_{300}$, the difference molar absorption coefficient at 300 nm (A) and $[\theta]_{222}$, the mean residue ellipticity at 222 nm (B) at different pH values. In order to maintain clarity, all data points are not shown on the transition curves. The dashed lines at a given pH represent the extrapolated baselines assuming that the temperature-dependence of the optical property is linear (L) and parabolic (P). The inset in (A) shows the thermal perturbation of the absorption of tryptophane at 300 nm. Buffers used were 0.05 M KCl-HCl, 0.05 M glycine-HCl and 0.03 M cacodylate for pH ranges 1.50-2.45, 2.50-3.20 and 4.48-6.00, respectively, and all the buffers contained 0.1 M KCl.

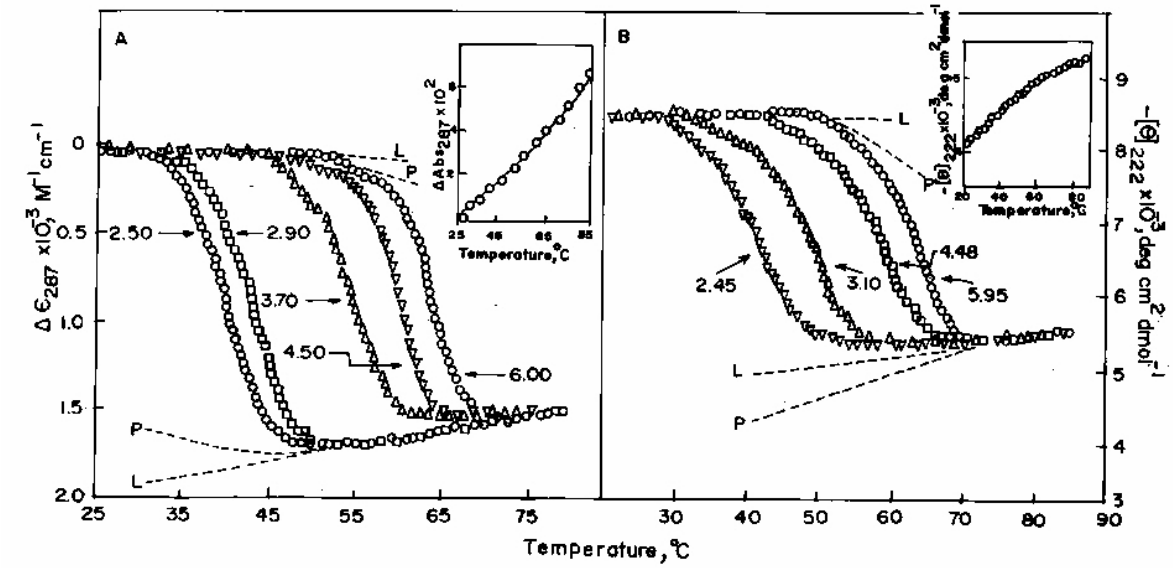

Fig. 2. Heat-induced denaturation of RNase-A measured by $\Delta\varepsilon_{287}$ (A) and $[\theta]_{222}$ (B) at different pH values. P and L have the same meanings as in Fig. 1. The insets show the thermal perturbations of $\Delta\varepsilon_{287}$ of tyrosine (A) and that of $[\theta]_{222}$ of the denatured RNase-A (B). Buffers used were the same as shown in Fig.1.

conformation), in the range 0.1-10 using equation (1) and fitting the entire data ($\ln K_D$ (or ΔG_D), 1/T (or T)) according to van't Hoff equation (or Gibbs-Helmoltz equation). In another procedure developed by Santoro and Bolen [25] all the transition data (y, T) are fitted to an equation that gives the dependence of the optical property, *y* on temperature (see eqns. 2 and 3 in [26]). Taneja and Ahmad [27] have recently proposed a simple method for the determination of ΔH_m^{van}, the value of ΔH_m from equilibrium method. In this procedure stability curve ($\Delta G_D(T)$ versus T plot) is constructed to choose a range of $\Delta G_D(T)$ values around T_m that fall on a straight line. A linear least-squares fit of the data ($\Delta G_D(T)$, T) in this range gives the value of ΔS_m, the entropy change at T_m (= -$(\delta \Delta G_D(T)/\delta T)_p$), which is then multiplied by T_m to get the value of ΔH_m^{van}. The advantage of this procedure is that it uses $\Delta G_D(T)$ values in the vicinity of T_m, which are not only more accurate [28] but also better representative of the equilibrium between N and D states. All these procedures have one thing in common, namely, the assumption that the temperature-dependencies of the pre- and post-transition baselines are linear.

$$\Delta G_D(T) = -RT\ln K_D(T) = -RT\ln\left(\frac{y(T) - y_N(T)}{y_D(T) - y(T)}\right) \quad (1)$$

$$y(T) = \frac{y_N(T) + y_D(T)\mathrm{Exp}\left[\frac{-\Delta H_m^{van}}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)\right]}{1 + \mathrm{Exp}\left[\frac{-\Delta H_m^{van}}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)\right]} \quad (2)$$

where y(T) is the experimentally observed optical property of the protein at temperature T(K), $y_N(T)$ and $y_D(T)$ are the optical properties of the native and the denatured molecules at *T*(K), respectively, R is the gas constant, ΔH_m^{van} is the van't Hoff enthalpy change at T_m.

We have measured thermal denaturations of several proteins at different pH values using absorption and far-uv circular dichroism. Each transition curve of a protein was

Table 1. Thermodynamic Parameters of Lysozyme Using Different Baseline models[a-c]

pH	T_m (K)	ΔH_m^{van} (kJ mol^{-1})	ΔH_m^{cal} (kJ mol^{-1})
	Linear model		
3.00	340.0 ± 0.4 (339.9)	402 ± 8 (397 ± 17)	489
2.50	331.4 ± 0.3 (331.2)	360 ± 8 (372 ± 12)	434
2.00	326.3 ± 0.5 (326.0)	326 ± 8 (335 ± 12)	402
1.50	320.9 ± 0.5 (320.5)	293 ± 4 (293 ± 12)	368
	Mixed linear/parabolic model		
3.00	340.2 ± 0.2	349 ± 8	490
2.50	331.7 ± 0.2	393 ± 4	436
2.00	327.0 ± 0.4	368 ± 8	406
1.50	321.8 ± 0.4	326 ± 8	373
	Parabolic model		
3.00	340.9 ± 0.3	481 ± 8	495
2.50	331.6 ± 0.2	439 ± 8	436
2.00	327.1 ± 0.3	402 ± 12	407
1.50	321.9 ± 0.4	377 ± 12	374

[a] ΔH_m^{cal} values were estimated using results given by Privalov and Gill [29]. [b] Valuse of thermodynamic parameters given in parentheses were obtained using the procedure of Taneja and Ahmad, [27]. [c] The estimated error in ΔH_m^{cal} is ± 20 kJ mol^{-1} [30].

analysed according to equation (2) using the procedure developed by Santoro and Bolen [25] and used by Swint and Robertson [26] who used the linear model which assumes linear temperature-dependence of $y_N(T)$ and $y_D(T)$ (*i.e.*, $y_N(T) = a_N + b_NT$ and $y_D(T) = a_D + b_DT$, where a and b are temperature-independent parameters and subscript N and D represent native and denatured states, respectively). This analysis gave values of ΔH_m^{van} and T_m. These values of two proteins are given in Tables 1 and 2. It is seen in Table 1 that although ΔH_m^{van} values of lysozyme are determined with an accuracy of 2-5%, which is the same as observed in the DSC measurements [30], they are significantly less than the corresponding ΔH_m^{cal} values. Table 2 shows the results of similar analysis of (y, T) data of RNase-A at different pH values, assuming the linear dependence of both baselines. An identical value of ΔH_m from equilibrium and DSC measurements of a protein is, however, expected for the two-state heat-induced denaturation of a protein.

It has been shown earlier that the estimate of ΔH_m^{van} from a two-state heat-induced conformational transition curve will considerably depend on the function(s) by which the extrapolations of the pre- and post-transition baselines into the transition region are carried out [6,31]. In fact, Tiktopulo and Privalov [31] have shown that the most symmetrical sigmoidal normalized transition curve, a characteristic of a two-state process, is obtained only when a parabolic function (linear for the first derivative) and a linear function are, respectively, used for the extrapolations of the pre- and post-transition baselines of the heat-induced denaturation of RNase-A into the transition region. A comparison of ΔH_m^{van} thus obtained with the ΔH_m^{cal} suggested that the agreement between them is within 10% for RNase-A [31]. Using the same procedure, the optical transition of lysozyme at pH 3.0 was analyzed for ΔH_m^{van}, which was found to be in good agreement with the calorimetric value [6]. All transition curves such as those shown in Figures 1 and 2, were analysed according to equation (2) using the mixed/parabolic model, *i.e.*, the temperature-dependencies of $y_N(T)$ is described by a second degree polynomial ($y_N(T) = a_N + b_NT + c_NT^2$, where c is also a temperature-independent parameter) and that of $y_D(T)$ is

Table 2. Thermodynamic Parameters of RNase-A Using Different Baseline Models[a-c]

pH	T_m (K)	ΔH_m^{van} (kJ mol^{-1})	ΔH_m^{cal} (kJ mol^{-1})
	Linear model		
6.00	338.9 ± 0.1 (338.9)	481 ± 8 (494 ± 8)	519
4.50	335.4 ± 0.1 (335.3)	452 ± 8 (460 ± 8)	502
3.70	325.3 ± 0.2 (325.3)	397 ± 8 (389 ± 17)	448
2.90	318.1 ± 0.2 (317.7)	356 ± 8 (364 ± 8)	402
2.50	315.2 ± 0.2 (315.0)	343 ± 4 (343 ± 8)	385
	Mixed linear/parabolic model		
6.00	339.0 ± 0.1	502 ± 4	519
4.50	335.5 ± 0.3	481 ± 4	502
3.70	325.5 ± 0.2	423 ± 8	448
2.90	318.4 ± 0.2	377 ± 8	402
2.50	315.6 ± 0.2	364 ± 4	389
	Parabolic model		
6.00	339.1 ± 0.1	515 ± 8	519
4.50	336.0 ± 0.2	498 ± 4	502
3.70	325.3 ± 0.2	444 ± 8	448
2.90	318.3 ± 0.2	389 ± 8	402
2.50	315.5 ± 0.2	372 ± 8	389

[a-c]Have the same meaning as in Table 1.

described by a linear function ($y_D(T) = a_D + b_DT$). Such an analysis gave unique values of ΔH_m^{van} and T_m (*e.g.*, see Tables 1 and 2). It is seen in Tables 1 and 2 that although the agreement between ΔH_m^{van} and ΔH_m^{cal} of a protein at a pH is better than that between ΔH_m^{cal} and ΔH_m^{van} obtained from the analysis of the same set of data using the linear model, ΔH_m^{cal} is still significantly higher.

It is known that the temperature-dependencies of optical properties of a protein in the pre- and post-transition regions measure the thermal perturbations of protein groups. Ahmad and coworkers [32,33] have shown that the change in the optical properties of protein groups with temperature is nonlinear and is adequately described by an equation of polynomial of second degree in temperature. Taking this observation as a justification for analyzing heat-induced denaturation curves according to eqn. 2 with the parabolic model (*i.e.*, $y_N(T) = a_N + b_NT + c_NT^2$ and ($y_D(T) = a_D + b_DT + c_DT^2$) they analyzed the heat-induced denaturation curves of several proteins and observed an excellent agreement between ΔH_m^{van} and ΔH_m^{cal} of a protein (*e.g.*, see Tables 1-3 in ref. [32] and Tables 1 and 2 in ref. [33]). Results of two proteins are reproduced in Tables 1 and 2.

Several methods have been used to determine ΔC_p from the measurements of a conformational transition curve. The earlier method involves the estimation of the values of ΔH_m^{van}, as a function of temperature from a van't Hoff analysis of thermal denaturation curves measured at different pH values or chemical denaturant concentrations [21,34-38]. Once ΔH_m^{van} is measured as a function of temperature, ΔC_p is determined using Kirchoff equation. A second approach involves the measurements of ΔH_m^{van} and T_m from thermal transition curves obtained at different pH values and estimation of ΔC_p from the ΔH_m^{van} versus T_m plot [16,29]. It should be noted that this method assumes that ΔH_m^{van} and ΔC_p do not depend on pH. A third method developed by Pace and Laurent [39] involves (a) measurement of a thermal transition curve in the native buffer to estimate ΔH_m^{van} and T_m; (b) measurements of isothermal urea-induced denaturation to estimate $\Delta G_D(T)$ values in absence of urea at several temperatures; and (c) calculation of ΔC_p from the known values of ΔH_m^{van}, T_m and $\Delta G_D^o(T)$ by using Gibbs-Helmoltz equation. It should be noted that this procedure [39] assumes that urea and heat denatured states are identical. In another approach according to Swint and Robertson [26], ΔC_p is determined from a global fit of entire transition data (y, T) to an equation that includes the temperature-dependence of ΔH_D (see eqn. 3 in [26]).

DSC measurements of ΔC_p of several proteins have shown that it is independent of pH and temperature between 20-80 ^oC [16,22,26,39-41]. From their equilibrium measurements Ahmad and coworkers [32,33] determined ΔC_p from the slope of the plot of ΔH_m^{van} obtained at different pH values versus corresponding T_m It has been observed that for a protein, ΔC_p is independent of the model used to describe the pre- and post-transition baselines of the thermal transition curve; averaged values of ΔC_p are 6.07 ± 0.79 kJ mol^{-1} K^{-1} for lysozyme, 5.69 ± 0.37 kJ mol^{-1} K^{-1} for RNase-A, 6.82 ± 0.71 kJ mol^{-1} K^{-1} for cytochrome-c, and 11.38 ± 0.63 kJ mol^{-1} K^{-1} for myoglobin. These values of ΔC_p are not only in excellent agreement with those obtained calorimetrically [31] but also the error involved in determining ΔC_p from the conformation transition curve induced by heat is same as observed with a calorimeter [29, 39]. The following method is recommended for determining the accurate and authentic thermodynamic parameters from the two-state heat-induced optical transition curves. (a) Measure thermal denaturation curves at different pH values, (b) analyze each optical transition curve according to eqn. (2) using parabolic model to determine ΔH_m^{van} and T_m values, and (c) estimate ΔC_p from the ΔH_m^{van} versus T_m plot. ΔG_D^o is then estimated from the known values of ΔH_m^{van}, T_m and ΔC_p using eqn. (3), the Gibbs-Helmoltz equation.

CONCLUSIONS

DSC is the method of choice for the determination of ΔH_D and ΔC_p associated with the heat-induced denaturation of proteins. The reason for saying this is that both thermodynamic parameters can be determined directly and no assumption regarding the mechanism of denaturation is required for the analysis of the endotherm. Optical methods will also give accurate and authentic values of these stability parameters only for a protein undergoing a two-state and reversible thermal denaturation. There are, however, a few advantages in using the latter technique. (a) Protein concentration is not required in the determination of stability parameters. (b) Changes in the different parts of the protein molecules may be monitored by working at different relevant

wavelengths of the absorption and CD spectra. (c) Optical methods give more reliable values of K_D and the corresponding ΔG_D at a fixed temperature.

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