A possible origin of differences between calorimetric and equilibrium estimates of stability parameters of proteins

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To test the validity of thermodynamic parameters from the equilibrium method, we have studied the reversible heat-induced denaturations of lysozyme, ribonuclease A, cytochrome *c* and myoglobin at various pH values, using absorption spectral measurements. For each protein, if a linear temperature-dependence of the pre- and post-transition baselines is assumed for the analysis of the conformational-transition curve, 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 thermal-denaturation curves assumes that the temperature-dependence of pre- and post-transition baselines is described by a parabolic function, there exists an excellent agreement between ΔH_m values of all proteins obtained from equilibrium and calorimetric methods. The latter analysis is supported by the studies on model compounds, for measurements of absorption properties of tyrosine, tryptophan and haem as a function of temperature suggested that the temperature-dependencies of the optical properties are indeed non-linear. We have observed that for each protein the constant-pressure heat-capacity change on denaturation (ΔC_p) determined from the plots of ΔH_m^{van} versus T_m is not only independent of the method of analysis of the transition curve, but it is also in excellent agreement with calorimetric ΔC_p. An important conclusion of this study is that for these proteins that exhibit two-state character, all stability parameters are measured with the same error as that observed with a calorimeter.

Key words: cytochrome *c*, lysozyme, myoglobin, protein stability, ribonuclease A.

INTRODUCTION

One of the estimates of protein stability is ΔG_D^0, the Gibbs energy change associated with the following process: native (N) conformation ↔ randomly coiled denatured (D) conformation, occurring under physiological conditions usually taken as dilute buffer (or water) at 25 °C [1]. Almost all estimates of ΔG_D^O come from the study of denaturation by heat and strong chemical denaturants such as guanidinium chloride (GdmCl) using microcalorimetric and equilibrium methods. 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 approach, the 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 the equilibrium method [1]. Calorimetric measurements of heat-induced denaturation of the native protein in the presence and absence of GdmCl, GdmCl-induced denaturation of the native and heat-denatured proteins, and heat capacity of protein groups (N- and C-termini, amino acids and peptide) led Makhatadze and Privalov [2] to two definite conclusions. (i) "… 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 …", and (ii) "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 denaturations by heat and GdmCl, and on thermodynamic parameters from heat-induced and GdmCl-induced conformational-transition curves, has revealed that, for a protein, the GdmCl-denatured state at 25 °C is structurally more unfolded than the heat-denatured state (see the review by Ptitsyn [3]). In fact, the heat/acid-denatured state contains residual structure that can be removed by the addition of GdmCl [4–7] and, with a few exceptions [8], ΔG_D^0 for heat denaturation is less than that for GdmCl denaturation [9–11]. Furthermore, a comparison between equilibrium [12–15] and calorimetric [16] ΔG_D^0 values associated with the heat denaturation of the same protein suggests that the former is significantly less than the latter. Equilibrium and calorimetric ΔG_D^O values ($kJ \cdot mol^{-1}$) are, respectively, 37.2 and 60.7 for lysozyme, 33.5 and 50.2 for myoglobin (Mb), 36.4 and 44.3 for RNase A and 31.8 and 37.7 for cytochrome *c* (cyt *c*). These conclusions and those arrived at from the calorimetric measurements mentioned in the preceding paragraph are not only controversial, they have also led researchers to question the validity of the equilibrium [17] and calorimetric [18] methods for the determination of protein stability.

Privalov et al. [17] attracted 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 analysing and usually only assume for simplicity that it is a two-state transition." On the other hand, Franks [18] 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,

Abbreviations used: Mb, myoglobin; cyt *c*, cytochrome *c*; DSC, differential scanning calorimetry; GdmCl, guanidinium chloride.

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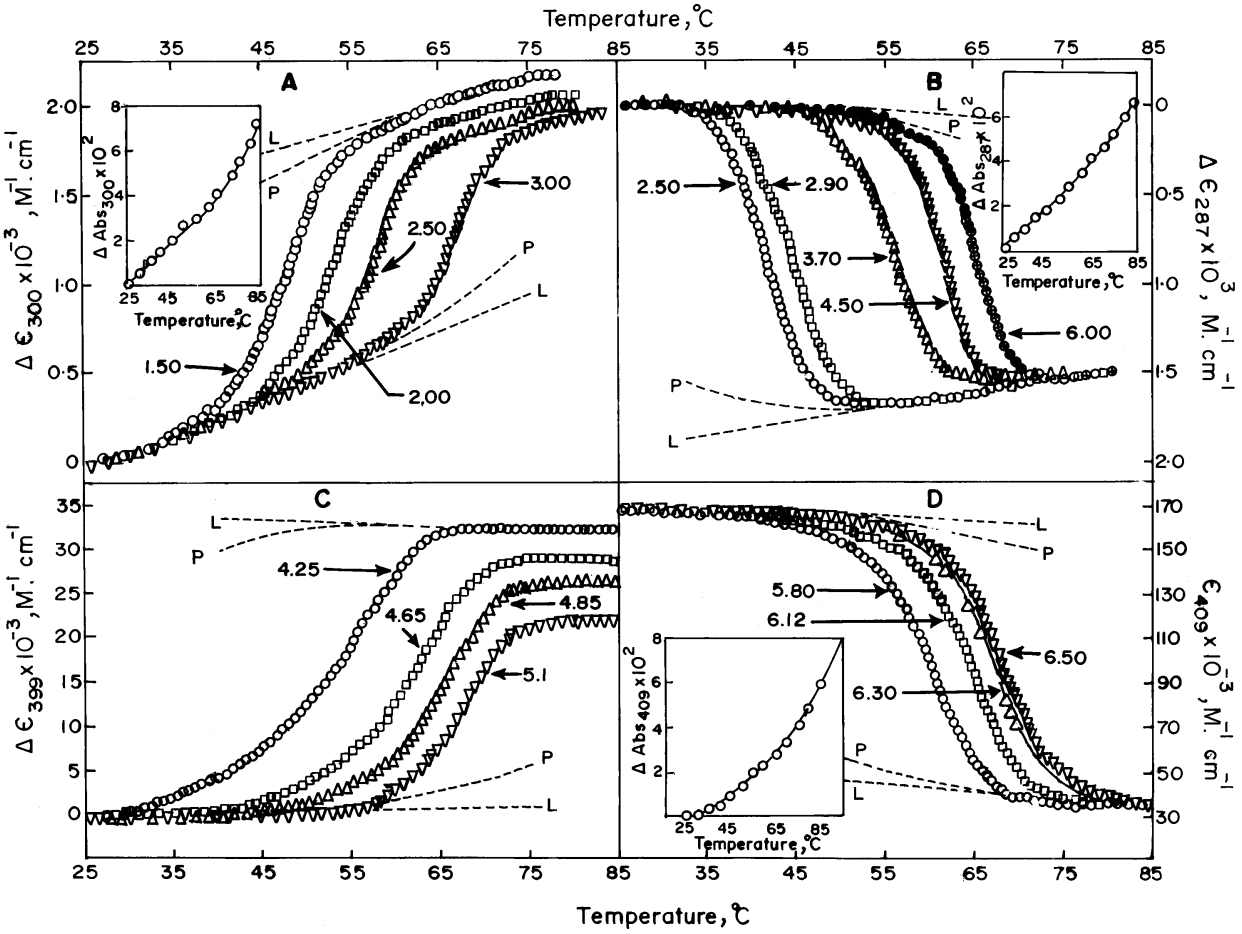

Figure 1 Thermal-denaturation curves of lysozyme (A), RNase A (B), cyt *c* with 0.60 M GdmCl (C) and Mb with 0.60 M GdmCl (D) at different pH values given in the Figure

The dashed lines represent the extrapolated baselines assuming that the temperature-dependence of the optical property is linear (L) and parabolic (P). The insets in (**A**), (**B**) and (**D**) show the difference absorbencies of L-Trp at 300 nm, L-Tyr at 287 nm and haem at 409 nm, respectively. In order to maintain clarity not all data points are shown on the curves.

especially NMR, CD, and optical rotary dispersion 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^0_D spans more than 30 years, we do not seem to have confidence in the procedures for the estimation of protein stability.

The statement made by Privalov et al. [17] is very puzzling. If their view is the case, then it means that the estimate of protein stability from the equilibrium method has no validity. In this paper we address specifically this issue by measuring thermodynamic parameters from the heat-induced conformational-transition curves of four proteins, namely, lysozyme, RNase A, Mb and cyt *c* at different pH values. There are two reasons for choosing these proteins. One is that the analysis of conformational-transition curves of these proteins is simple, for the heat-induced denaturation of each protein is a two-state process [16]. Another reason is that our equilibrium data can be validated against calorimetric data, for DSC measurements have provided all thermodynamic parameters required for a complete description of the heat-induced denaturation of each protein [16,19]. Naturally, estimates of thermodynamic parameters from the conformational-transition curve will depend on the function(s) by which the extrapolations of the pre- and post-transition baselines in the transition region are carried out [6,20]. We have used three different methods to analyse a two-state optical-transition curve of a protein. One is the conventional method, called here the 'linear model', which assumes that the temperature-dependence of the pre- and post-transition baselines is linear. Another method, called here the 'mixed linear/parabolic model' assumes that a parabolic function describes the temperature-dependence of the pre-transition baseline, whereas the functional dependence of the post-transition baseline is linear with temperature [6,20]. A third one, the 'parabolic model', assumes that the dependence of the pre-transition baseline as well as the post-transition baseline on temperature is described by a parabolic function. The latter model is based on our experimental observations that a parabolic function describes the temperature-dependencies of the absorption properties of tyrosine, tryptophan and haem. We report here that (i) different models of analysis of the same set of data of a protein gave different values of ΔH^{van}_m, the van't Hoff enthalpy change at T_m, the midpoint of denaturation. The linear model gave the lowest estimate of ΔH^{van}_m, which is significantly smaller than ΔH^{cal}_m, the value obtained by calorimetric measurements; the parabolic model gave the highest estimate of ΔH^{van}_m, which is in excellent agreement with ΔH^{cal}_m; and the mixed linear/parabolic model

gave an ΔH_m^{van} value between the estimates of ΔH_m^{van} using linear and parabolic models. (ii) For each protein a plot of ΔH_m^{van} measured at different pH values versus corresponding T_m gave a ΔC_p value that was not only independent of the model of analysis of the pre- and post-transition baselines but which was also in excellent agreement with the calorimetric value.

MATERIALS AND METHODS

Commercial lyophilized chromatographically purified horse heart Mb (type III), cyt *c* (type IV), bovine pancreatic RNase A (type XII-A), hen's egg lysozyme (grade I), L-tyrosine, L-tryptophan and haem were purchased from Sigma. Since all proteins gave single band on SDS/PAGE, they were used without further purification. An ultrapure GdmCl sample was purchased from Schwarz/Mann Biotech (Cleveland, OH, U.S.A.). Analytical-grade sodium salt of cacodylic acid and KCl were from Aldrich and Merck (India), respectively. These and other chemicals were analytical-grade reagents.

Cyt *c* and Mb were oxidized first by adding 0.01 % and 0.1 % potassium ferricyanide, respectively. These oxidized samples and RNase A and lysozyme were dialysed at 4 °C against several changes of 0.1 M KCl (pH 7.0). Protein stock solutions were filtered using 0.45-μm Millipore filter paper, and their concentrations determined experimentally using known values of ϵ_λ ($M^{-1} \cdot cm^{-1}$), the molar absorption coefficient at the wavelength λ (nm): $\epsilon_{409} = 106000$ for cyt *c* [21], $\epsilon_{409} = 171000$ for Mb [22], $\epsilon_{277.5} = 9800$ for RNase A [23] and $\epsilon_{280} = 39000$ for lysozyme [24]. All solutions for absorption measurements were prepared in the appropriate buffers containing 0.1 M KCl. Thermal-denaturation studies of proteins were carried out in a JASCO V-560 UV/VIS spectrophotometer with a heating rate of 1 °C/min. About 250–270 data points were collected.

Thermal denaturations of Mb and cyt *c* in the presence of 0.60 M GdmCl at different pH values were followed by observing changes in absorbance at 409 and 399 nm, respectively. The heat-induced denaturation of lysozyme and RNase A was followed by monitoring the changes in absorbance at 300 and 287 nm, respectively. After denaturation, the protein solution was cooled immediately to 25 °C to measure the absorption. The agreement between this absorption and the absorbance of the native protein (unheated sample) at 25 °C was taken as a measure of reversibility of the heat-induced denaturation. Thermal perturbations of absorbance of free L-tyrosine, L-tryptophan and haem were also measured at 287, 300 and 409 nm, respectively.

RESULTS

Figure 1 shows heat-induced denaturation curves of lysozyme, RNase A, cyt *c* and Mb at different pH values. It has been observed that these optical-transition curves are reversible. It should, however, be noted that thermal denaturations of cyt *c* and Mb are reversible only in the presence of 0.60 M GdmCl. Furthermore, calorimetric data suggested that thermal denaturation of lysozyme, RNase A, cyt *c* and Mb is a two-state process [16,19,25]. Following the approach of Santoro and Bolen [26], a non-linear least-squares analysis was used to fit each thermal-denaturation curve, shown in Figure 1, to the relation:

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

where $y(T)$ is the experimentally observed absorption property of the protein at temperature T (K), $y_N(T)$ and $y_D(T)$ are the absorption properties of the native and denatured molecules at T (K), R is the gas constant, ΔH_m is ΔH_m^{van}, the van't Hoff enthalpy change at T_m, the midpoint of the thermal denaturation. Different values of ΔH_m^{van} and T_m of a protein at a fixed pH were obtained from the analysis of the same transition curve according to eqn. (1) using three different models that describe the temperature-dependencies of $y_N(T)$ and $y_D(T)$, namely, the linear model (i.e. $y_N = a_N + b_N T$, and $y_D = a_D + b_D T$), the mixed linear/parabolic model (i.e. $y_N = a_N + b_N T + c_N T^2$, and $y_D = a_D + b_D T$), and the parabolic model (i.e. $y_N = a_N + b_N T + c_N T^2$, and $y_D = a_D + b_D T + c_D T^2$), where a, b and c are temperature-independent coefficients and subscripts N and D refer to the native and denatured protein molecules, respectively. Results of the non-linear least-squares analysis of each transition curve of a protein using linear, mixed linear/parabolic and parabolic models are given in Tables 1, 2 and 3, respectively. These tables also show ΔH_m^{cal}, the values obtained by DSC measurements [27].

Table 1 Thermodynamic parameters of proteins using the linear model [$y_N(T) = a_N + b_N T$ and $y_D(T) = a_D + b_D T$]

ΔH_m^{cal} at a given T_m was estimated with the help of the relation $\Delta H_m^{cal} = \Delta H_D^0 + \Delta C_p T_m$ with ΔH_D^0 ($kJ \cdot mol^{-1}$) and ΔC_p (kJ. $mol^{-1} \cdot K^{-1}$) values of −1670 and 6.35 for lysozyme, −1253 and 5.22 for RNase A, −1434 and 5.18 for cyt *c*, and −3451 and 11.41 for Mb, which were derived from the data of lysozyme, RNase A and Mb given in [27], and cyt *c* in [19]. The estimated error in ΔH_m^{cal} is ±20 $kJ \cdot mol^{-1}$ [34]. Values given in parentheses were obtained using a different procedure [28]. Standard errors are given for each fit, and were similar for each of at least three independent experiments.

pH	T_m (K)	ΔH_m^{van} ($kJ \cdot mol^{-1}$)	ΔH_m^{cal} ($kJ \cdot mol^{-1}$)
Lysozyme			
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
RNase A			
6.00	338.9 ± 0.1 (338.9)	481 ± 8 (494 ± 8)	516
4.50	335.4 ± 0.1 (335.3)	452 ± 8 (460 ± 8)	498
3.70	325.3 ± 0.2 (325.3)	397 ± 8 (389 ± 17)	445
2.90	318.1 ± 0.2 (317.7)	356 ± 8 (364 ± 8)	407
2.50	315.2 ± 0.2 (315.0)	343 ± 4 (343 ± 8)	392
Cyt *c*			
5.11	340.1 ± 0.2 (340.2)	272 ± 4 (280 ± 17)	328
4.85	338.3 ± 0.2 (338.1)	255 ± 8 (255 ± 17)	318
4.65	335.7 ± 0.3 (334.9)	238 ± 8 (230 ± 17)	305
4.25	330.3 ± 0.4 (330.2)	205 ± 4 (201 ± 12)	277
Mb			
6.50	340.4 ± 0.1 (340.6)	339 ± 8 (356 ± 12)	433
6.30	339.8 ± 0.2 (339.7)	326 ± 4 (330 ± 8)	426
6.12	337.9 ± 0.2 (337.9)	314 ± 8 (310 ± 17)	404
5.80	334.6 ± 0.1 (334.4)	280 ± 8 (280 ± 8)	367

The insets of Figure 1 show the temperature-dependencies of free L-tryptophan, L-tyrosine and haem. It is seen in this Figure that the absorption properties vary non-linearly with temperature. It has been observed that the temperature-dependence of each chromophore is described by a parabolic function, $a + bT + cT^2$, where a, b and c are temperature-independent parameters.

Each heat-induced transition curve shown in Figure 1 was also analysed for $\Delta G_D(T)$ values in the range $-5.4\ kJ \cdot mol^{-1} \leqslant \Delta G_D(T) \leqslant 5.4\ kJ \cdot mol^{-1}$ using eqn. (2) (see below). Figure 2 shows the stability curves of lysozyme, RNase A, cyt *c* and Mb at different pH values. Each stability curve was used to determine ΔH_m and T_m using a different procedure [28]. This involves (i) determining ΔS_m, the entropy change at T_m from the slope $(\delta \Delta G_D/\delta T)_p$ at T_m [where p is pressure; if the plot of ΔG_D versus T is non-linear over the range of temperatures at which protein

Table 2 Thermodynamic parameters of proteins using the mixed linear/parabolic model [$y_N(T) = a_N + b_N T + c_N T^2$ and $y_D(T) = a_D + b_D T$]

For details see Table 1.

pH	T_m (K)	ΔH_m^{van} (kJ·mol^{-1})	ΔH_m^{cal} (kJ·mol^{-1})
Lysozyme			
3.00	340.2±0.2	439±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
RNase A			
6.00	339.0±0.1	502±4	516
4.50	335.5±0.3	481±4	498
3.70	325.5±0.2	423±8	446
2.90	318.4±0.2	377±8	409
2.50	315.6±0.2	364±4	394
Cyt *c*			
5.11	340.5±0.2	318±4	330
4.85	338.8±0.3	297±8	321
4.65	335.9±0.2	276±4	306
4.25	330.7±0.3	247±4	279
Mb			
6.50	340.6±0.1	385±8	435
6.30	340.2±0.2	372±4	431
6.12	338.5±0.2	360±4	411
5.80	334.9±0.2	335±4	370

Table 3 Thermodynamic parameters of proteins using the parabolic model [$y_N = a_N + b_N T + c_N T^2$ and $y_D = a_D + b_D T + c_D T^2$]

For details see Table 1.

pH	T_m (K)	ΔH_m^{van} (kJ·mol^{-1})	ΔH_m^{cal} (kJ·mol^{-1})
Lysozyme			
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
RNase A			
6.00	339.1±0.1	515±8	517
4.50	336.0±0.2	498±4	501
3.70	325.3±0.2	444±8	445
2.90	318.3±0.2	389±8	408
2.50	315.5±0.2	372±8	394
Cyt *c*			
5.11	341.5±0.2	339±8	335
4.85	339.6±0.2	318±8	325
4.65	336.6±0.3	305±4	309
4.25	331.1±0.2	268±8	281
Mb			
6.50	341.2±0.1	431±8	442
6.30	340.2±0.2	418±4	431
6.12	338.9±0.2	402±4	415
5.80	335.0±0.2	377±4	371

denatures, ΔS_m is then determined from the limited (ΔG_D, T) data on both sides of T_m that fall on a straight line]; and (ii) ΔH_m^{van} is obtained from the product of ΔS_m and T_m. Results of these analysis are given in parentheses in Table 1.

$$\Delta G_D(T) = \frac{y(T) - (a_N + b_N T)}{(a_D + b_D T) - y(T)} \quad (2)$$

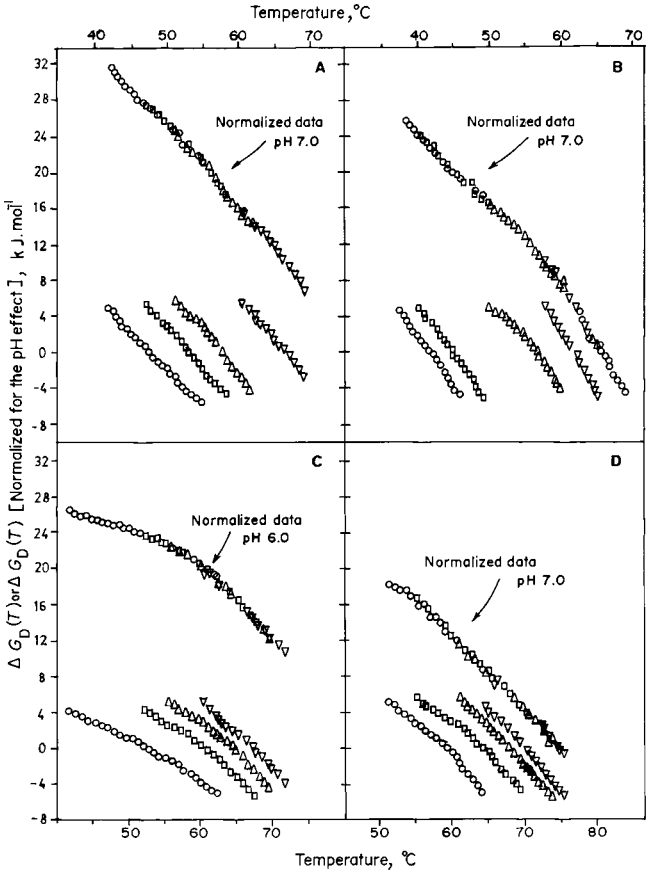

Figure 2 $\Delta G_D(T)$ or $\Delta G_D(T)$ (normalized for the pH effect) versus temperature plots of lysozyme (A), RNase A (B), cyt *c* (C) and Mb (D) at different pH values

At a temperature T (K), the value of $\Delta G_D(T)$ (normalized for the pH effect) $\{ = \Delta G_D(T)$ [observed at a fixed pH] $- F(\text{pH})\}$ was estimated using eqn. (3) with the values of $pK_{1,N} = 1.46$, $pK_{2,N} = pK_{3,N} = 1.66$ and $pK_{1,D}$–$pK_{3,D} = 4.4$ for lysozyme [29]; $pK_{1,N} = 1.5$, $pK_{2,N} = 4.8$, $pK_{3,N} = 5.4$, $pK_{4,N} = 6.2$, $pK_{1,D} = 4.1$, $pK_{2,D} = 4.5$ and $pK_{3,D} = pK_{4,D} = 6.4$ for RNase A [30]; $pK_{1,N} = pK_{2,N} = 4.5$ and $pK_{1,D} = pK_{2,D} = 6.0$ for cyt *c* [31]; and $n = 6$, $pK_{1,N} = 3.8$, $pK_{2,N} = 3.7$, $pK_{3,N} = 6.7$, $pK_{1,D} = 4.0$, $pK_{2,D} = 5.97$ and $pK_{3,D} = 6.5$ for Mb [22]. $\Delta G_D(T)$ data of lysozyme, RNase A and Mb were normalized to pH 7.0, whereas cyt *c* data were normalized to pH 6.0. For each protein, the symbols used in this Figure relate to the pH values that are shown in Figure 1.

All the $\Delta G_D(T)$ values obtained from the analysis of the thermal-denaturation curves of a protein at several pH values using eqn. (2), were adjusted to a common pH with the help of a function F(pH) that gives the pH-dependence of $\Delta G_D(T)$ [9]:

$$F(\text{pH}) = -RT\ln\frac{\prod_{i+1}^{n}(10^{-\text{pH}} + 10^{-\text{p}K_{i,D}})}{\prod_{i+1}^{n}(10^{-\text{pH}} + 10^{-\text{p}K_{i,N}})} \quad (3)$$

where n is the difference in the number of bound protons between D and N states, and $pK_{i,N}$ and $pK_{i,D}$ represent pK values of the ith group in the native and denatured states, respectively. The values of F(pH) were determined using known values of n, $pK_{i,N}$ and $pK_{i,D}$ of lysozyme [29], RNase A [30], cyt *c* [31] and Mb [22]. It is seen in Figure 2 that, for each protein, all normalized $\Delta G_D(T)$ values fall on the same stability curve. It should be noted that the effect of temperature on pH, pK_N and pK_D has been ignored in the estimation of F(pH) at different temperatures.

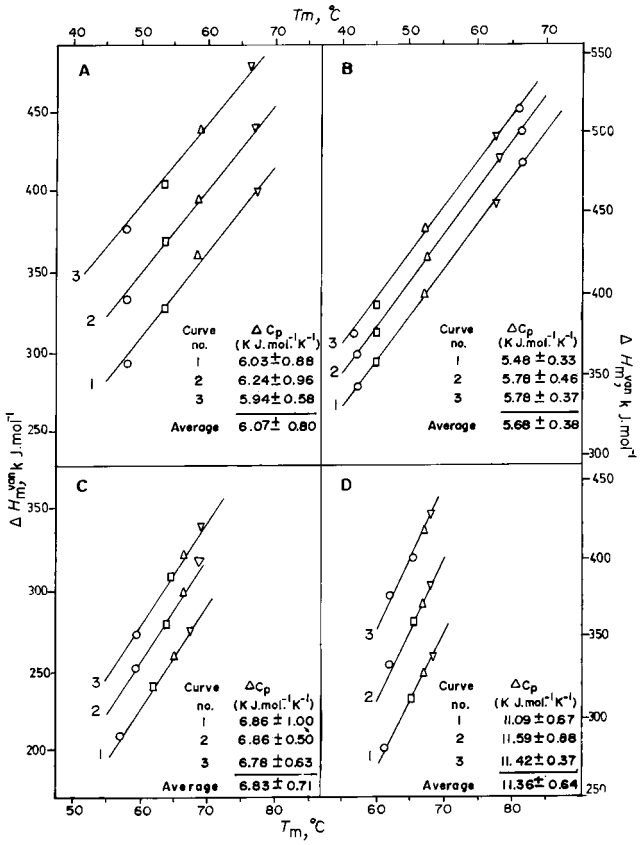

Figure 3 Plots of ΔH_m^{van} of lysozyme (A), RNase A (B), cyt *c* (C) and Mb (D) versus T_m at different pH values

Symbols have the same meaning as in Figure 1. Values of thermodynamic parameters were obtained using the linear (1), mixed linear/parabolic (2), and parabolic (3) models. For each protein, the symbols used in this Figure relate to the pH values that are shown in Figure 1.

Figure 3 shows the plots of ΔH_m^{van} versus T_m of lysozyme, RNase A, cyt *c* and Mb. For each protein curves 1, 2 and 3 represent (ΔH_m^{van}, T_m) data, obtained from the analysis of its transition curves (Figure 1) according to eqn. (1) using linear model (Table 1); the mixed linear/parabolic model (Table 2) and parabolic model (Table 3), respectively. It is seen in this Figure that a linear least-squares fit of these plots of each protein gave the values of ΔC_p, which are, within experimental error, identical to one another. Furthermore, these values of ΔC_p of proteins are in excellent agreement with those obtained by DSC measurements; calorimetric ΔC_p ($kJ \cdot mol^{-1} \cdot K^{-1}$) values are 6.35 ± 0.84 for lysozyme [16,27], 5.22 ± 0.50 for RNase A [16,27], 3.8–7.5 for cyt *c* ([19] and references therein) and 11.41 ± 0.67 for Mb [25,27]. It should be noted that the errors in the determination of van't Hoff (this study) and calorimetric [16,27] ΔH_m have not been considered in the estimation of ΔC_p from the plots of ΔH_m versus T_m.

DISCUSSION

In order to look for a possible source of discrepancy between thermodynamic quantities obtained from the equilibrium method and calorimetry, ΔH_m and ΔC_p of a protein obtained by these two different techniques were compared. In fact, this is possible only when a protein is studied under identical solvent conditions by both techniques. In the cases of cyt *c* and Mb we have, however, determined thermodynamic quantities in the presence of 0.60 M GdmCl (Tables 1–3), whereas calorimetric measurements were performed in the absence of the denaturant (see [19] and references therein, and [25]). Because of the well-documented exothermic interaction of GdmCl with protein, a question then arises: is the comparison of thermodynamic quantities of cyt *c* and Mb obtained from these two different studies valid? We have checked this by correcting the stability curves of both proteins for the effect of 0.60 M denaturant at each pH using a standard procedure that assumes that, at constant pH, ΔG_D can be written as the sum of GdmCl-dependent and temperature-dependent components of Gibbs energy change [9,32]. Each corrected stability curve was then analysed for ΔH_m^{van}. It has been observed that running these two proteins in the presence of 0.60 M GdmCl causes a reduction in ΔH_m^{van} by 4–8 $kJ \cdot mol^{-1}$, which is within the error of the measurements. It is interesting to note that the interaction of GdmCl with proteins has been studied calorimetrically [33]. Using these calorimetric data and eqn. (21) in [33], we estimated that 0.60 M GdmCl caused a reduction in the ΔH_m^{cal} by about 12.5 $kJ \cdot mol^{-1}$. It is noteworthy that this reduction in the thermodynamic quantity in the presence of low denaturant concentration is within the error of calorimetric measurements [34]. Thus a comparison between the equilibrium and calorimetric data for these proteins is valid.

The $\Delta G_D(T)$ value at any temperature T in the transition region of a denaturation curve (Figure 1) may be determined, if values of y_N and y_D are also known at T [see eqn. (2)]. A question that arises is, how does one obtain values of y_N and y_D in the transition region? The practice used in almost all studies is to make linear extrapolations of the pre- and post-transition baselines into the transition zone. Using this approach, $\Delta G_D(T)$ values were determined to construct stability curves of proteins at different pH values (Figure 2). For each protein, the $\Delta G_D(T)$ values measured at different pH values were normalized at a common pH using eqn. (3) with known values of n, $pK_{i,N}$ and $pK_{i,D}$ (see Figure 2). It is seen in Figure 2 that the normalized $\Delta G_D(T)$ values of a protein fall on a smooth curve, indicating that the effect of pH and temperature on the ΔG_D value of a protein is additive [9]. It is interesting to note that Privalov [16], using a different approach, arrived at the same conclusion; that is, the pH-dependent and temperature-dependent components of ΔG_D are additive. This agreement between the conclusions by two different approaches provides evidence for the correctness of our heat-induced denaturation measurements.

There are several methods that have been used to determine ΔH_m^{van} and T_m from a conformational-transition curve induced by heat. The earlier procedure [35–38] involves the estimation of K_D, the equilibrium constant of denaturation, in the range 0.1–10 and fitting the entire data [$\ln K_D$ (or ΔG_D), $1/T$ (or T)] according to the van't Hoff equation (or the Gibbs–Helmoltz equation). In another procedure [26] all the transition data (y, T) are fitted to an equation that gives the dependence of the optical property on temperature {see eqns. (2) and (3) in [39]}. We have proposed recently a simple method for the determination of ΔH_m^{van}, the value of ΔH_m from equilibrium method from the stability curve [28]. All these procedures have one thing in common, namely the assumption that the temperature-dependencies of the pre- and post-transition baselines are linear.

Analysis of each transition curve of a protein according to eqn. (1) assuming linear temperature-dependence of $y_N(T)$ and $y_D(T)$ gave values of ΔH_m^{van} and T_m (Table 1). It is seen in Table 1 that although ΔH_m^{van} values are determined with an accuracy of 2–10 %, which is the same as observed in the DSC measurements [34], they are significantly less than the corresponding ΔH_m^{cal}

values. A lower value of ΔH_m^{van} is, however, expected if the heat-induced denaturation of a protein is not a two-state process [40]. This possibility is ruled out in our case, for the DSC measurements on these proteins have provided strong evidence for a two-state transition [16,19,25]. Another source of discrepancy between ΔH_m^{van} and ΔH_m^{cal} may stem from the assumption used in the derivation of eqn. (1), namely that the effects of ΔC_p on ΔH_D, the enthalpy of denaturation, are negligible over the narrow temperature range of transition [39]. Swint and Robertson fitted the same set of data (y, T) to another relation that assumes the dependence of ΔH_D on temperature {see eqn. (3) in [39]} and observed that this assumption has no significant effect on the estimation of ΔH_m^{van}. In order to see whether the analysis of the denaturation curve according to eqn. (1) is accurate, we have used a different procedure for the determination of ΔH_m^{van} and T_m developed earlier [28]. The values of ΔH_m^{van} and T_m thus obtained are given in parentheses in Table 1, where it is seen that these values are, within fitting errors, identical to those obtained from the analysis of transition data (y, T) using the procedure of Santoro and Bolen [26]. This agreement between the estimates of ΔH_m^{van} and T_m, obtained by two different methods, led us to believe (i) that the treatment of transition data according to eqn. (1) is accurate, and (ii) that the source of discrepancy between ΔH_m^{van} and ΔH_m^{cal} of a protein at any pH is not due to the assumption that the effects of ΔC_p on ΔH_D are negligible over the transition zone.

Naturally, estimates of ΔH_m^{van} will depend considerably on the function(s) by which the extrapolations of the pre- and post-transition baselines into the transition region are carried out [6,20]. In fact, Tiktopulo and Privalov [20] showed that the most symmetrical sigmoidal normalized transition curve, a characteristic of a two-state process, was obtained only when a parabolic function (linear for the first derivative) and a linear function are used for the extrapolations of the pre- and post-transition baselines, respectively, into the transition region of the heat-induced denaturation of RNase A. 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 [20]. Using the same procedure, the optical transition of lysozyme at pH 3.0 was analysed for ΔH_m^{van}, which was found to be in good agreement with the calorimetric value [6]. We have therefore analysed all the transition curves, shown in Figure 1, according to eqn. (1) with the temperature-dependencies of $y_N(T)$ and $y_D(T)$ described by the second-degree polynomial and linear functions, respectively. This analysis gave unique values of ΔH_m^{van} and T_m that are given in Table 2. It is seen in this Table that although the agreement between ΔH_m^{van} and ΔH_m^{cal} of a protein at a given 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 (Table 1), ΔH_m^{cal} is still significantly higher.

Since temperature-dependencies of pre- and post-transition baselines of RNase A at 287 nm, lysozyme at 300 nm and haem proteins (Mb and cyt *c*) in the visible region measure the thermal perturbations of tyrosyl residue [23], tryptophyl residue [41] and haem–protein interaction [42], respectively, we have investigated whether the effect of temperature on the optical properties of these chromophores is non-linear. It has been observed that the change in the optical properties of free tyrosine, tryptophan and haem with temperature is non-linear and is described adequately by a second-degree polynomial equation in temperature (see insets in Figure 1). This observation is taken as a justification for analysing heat-induced denaturation curves according to eqn. (1) with the parabolic model. A comparison of ΔH_m^{van} thus obtained with ΔH_m^{cal} of a protein at a fixed pH suggests that, in all cases, agreement is excellent (Table 3). This agreement provides strong evidence for the validity of analysing thermal-transition curves with the parabolic model.

There are several methods that 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_D as a function of temperature from a van't Hoff analysis of thermal-denaturation curves measured at different pH values or chemical denaturant concentrations [36,43–45]. Once ΔH_D is measured as a function of temperature, ΔC_p is determined using the 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 plot of ΔH_m^{van} versus T_m [16,34]. It should be noted that this method assumes that ΔH_m and ΔC_p do not depend on pH. A third method developed by Pace and Laurent [46] involves (i) measurement of a thermal-transition curve in the native buffer to estimate ΔH_m^{van} and T_m; (ii) measurements of isothermal urea-induced denaturation to estimate $\Delta G_D^0(T)$, the values of ΔG_D in the absence of urea at several temperatures; and (iii) calculation of ΔC_p from the known values of ΔH_m^{van}, T_m and $\Delta G_{D^0}(T)$ using the Gibbs–Helmoltz equation. In another approach according to Swint and Robertson [39], ΔC_p is determined from a non-linear fit of the entire transition data (y, T) to an equation that includes the temperature-dependence of ΔH_D {see eqn. (3) in [39]}.

Assuming that ΔC_p is independent of temperature and pH between 20 and 80 °C [16,39,46–48], we have determined ΔC_p from the slope of the plot of ΔH_m^{van} obtained at different pH values versus corresponding T_m (Figure 3). It is seen in Figure 3 that, for each 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 ($kJ \cdot mol^{-1} \cdot K^{-1}$) are 6.07 ± 0.8 for lysozyme, 5.68 ± 0.38 for RNase A, 6.83 ± 0.71 for cyt *c* and 11.36 ± 0.64 for Mb. These values of ΔC_p are not only in excellent agreement with those obtained from the DSC [16,19,25,27] but also the error involved in the determination of ΔC_p from the conformation-transition curve induced by heat is the same as that observed with a calorimeter (for a critical review, see [34,46]). It should be noted that the errors in the determination of ΔH_m^{cal} have not been considered in the estimation of ΔC_p from the ΔH_m versus T_m plot (Figure 10 in [16]). In order to compare our results with the calorimetric values, we have therefore used the same procedure to determine ΔC_p. In fact, actual errors in ΔC_p from DSC and equilibrium methods will be larger due to the propagation of errors in ΔH_m.

In summary, a comparison of our results with calorimetric data led us to propose the following method for determining thermodynamic parameters from the two-state heat-induced optical-transition curves. (i) Measure thermal-denaturation curves at different pH values, (ii) analyse each optical-transition curve according to eqn. (1) using the parabolic model to determine ΔH_m^{van} and T_m values, and (iii) estimate ΔC_p from the plot of ΔH_m^{van} versus T_m. ΔG_D^0 at any temperature is then estimated from the known values of ΔH_m^{van}, T_m and ΔC_p using the Gibbs–Helmoltz equation.

Finally, we have shown that for a given protein agreement between the calorimetric data and those obtained by absorption measurements of the heat-induced denaturation (this study) is excellent only when the temperature-dependencies of the pre- and post-transition baselines are described by a polynomial function. A question arises: does this apply to other optical probes of structures such as CD and fluorescence? The work on this problem is under investigation in our laboratory. It is noteworthy that the value of ΔH_m^{van} of tryptophan synthase α-subunit from CD is systematically underestimated by 63 $kJ \cdot mol^{-1}$ as compared with ΔH_m^{cal} [49]. Furthermore, as we discussed in the

Introduction, for a given protein, ΔG^0_D from GdmCl-induced denaturation is significantly less than that obtained from heat-induced denaturation. This is indeed a question of great concern because the degree of disordering is essentially different in these two modes of denaturation [39]. This is another problem we are trying to understand.

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