Thermochimica Acta, 227 (1993) 185–195
Elsevier Science Publishers B.V., Amsterdam

The deconvolution of multi-state transition DSC curves of biological macromolecules: bovine serum albumin and bovine seminal ribonuclease [1]

G. Barone *, P. Del Vecchio, D. Fessas, C. Giancola and G. Graziano

Department of Chemistry, University "Federico II", via Mezzocannone 4, I-80134 Naples, Italy

(Received 15 March 1993; accepted 20 April 1993)

Abstract

The denaturation of the ordered, specific structures of biological macromolecules is a highly cooperative process which many researchers have likened to the melting or solution of a crystal. However, with increasing numbers of studied systems using different experimental approaches, it has become clear that only some small globular proteins undergo a two-state transition. Differential scanning microcalorimetry, giving direct thermodynamic information, has proved to be very useful in clarifying the details of the unfolding processes. For the same reasons, it can provide a reliable experimental basis for developing more complex models. In this paper, two proteins are considered: bovine seminal ribonuclease, which consists of two identical sub-units, covalently bonded; and bovine serum albumin, a protein formed by three domains, two of which are probably strongly interacting. Two possible kinds of mechanisms have been tested: the independent transition process and the sequential denaturation. Both approaches give satisfactory results and the apparent contradiction is discussed on the basis of the particularly compact structure of these macromolecules.

INTRODUCTION

Differential scanning microcalorimetry has proved to be a useful and powerful approach for studying the conformational transitions of biological macromolecules because it can measure directly the enthalpy associated with the process. The enthalpy, as a thermodynamic potential, contains all the information on the states to which the system can belong within the investigated temperature range [1, 2]. Some of the small globular proteins show calorimetric scanning curves which are well represented by the two-state transition model. In these cases, the thermodynamic analysis

* Corresponding author.

[1] Presented at the 14th National Conference on Calorimetry and Thermal Analysis, Udine, Italy, 13–17 December 1992.

0040-6031/93/$06.00 © 1993 – Elsevier Science Publishers B.V. All rights reserved

results are straightforward and have wide general applicability [3, 4]. An increasing number of experimental studies, however, have shown that many biological macromolecules [5–8] give calorimetric curves which differ significantly from those expected for a simple two-state process.

To analyse these DSC curves in a physically significant manner and to obtain the desired thermodynamic parameters concerning the stability of protein native forms, it is necessary to accomplish the so-called "deconvolution" of the thermal profiles. This means trying to extract information on all the single transitions which constitute the overall denaturation process. Basically, two models have been developed for the analysis of complex calorimetric curves: the independent transition model, which considers the total process as the sum of a set of two-state, mutually independent transitions [9–11]; and the sequential transition model, which describes the process as a sequential passage of the whole tertiary structure from the native form through a certain number of intermediate states to the denatured state [12, 13].

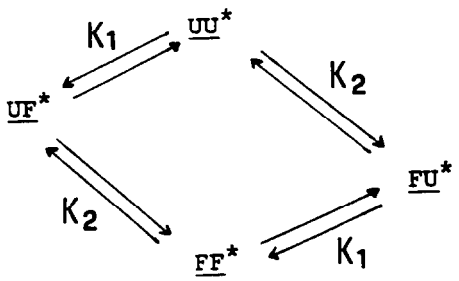
The aim of this paper is to review the two models and to apply them to two experimental cases. Both models give a good fit and the values of the thermodynamic parameters are in agreement with each other. This will be briefly discussed.

INDEPENDENT TRANSITION MODEL

We have chosen to study a hypothetical protein whose structure can be considered as consisting of two cooperative and independent sub-units, each of which has only two accessible thermodynamic states. The denaturation process is described in Scheme 1 and by the equations

$$K_1 = \exp[-\Delta^{U^*}_{F^*} G^{\ominus}/RT] \quad (1)$$

$$K_2 = \exp[-\Delta^{U}_{F} G^{\ominus}/RT] \quad (2)$$



Scheme 1.

For the overall macromolecule, four thermodynamic macroscopic states are then accessible, which can be labeled FF* (completely folded conformation), UF* and FU* (partially unfolded conformations), and

UU* (completely unfolded conformation), where F and U refer to one of the two cooperative sub-units and F* and U* to the other. If the two sub-units are really independent, the two pairs of transitions are equal, as indicated in the scheme: the transition FF* $\rightleftharpoons$ UF* has the same equilibrium constant as FU* $\rightleftharpoons$ UU*, namely K_2.

The canonical partition function for this system is the product of the two-state partition functions valid for each independent cooperative sub-unit, assuming the fully folded conformation as the reference state, i.e. a statistical weight equal to one [14]

$$Q(T)^{\text{Indep}} = (1 + K_1)(1 + K_2) = 1 + K_1 + K_2 + K_1K_2 \tag{3}$$

The partition function is clearly factorized into two terms and it is useful to take advantage of this peculiar feature. Indeed, it is very simple to obtain an analytical expression of the heat capacity function in excess with respect to the native state, $\langle \Delta C_p(T) \rangle$, starting from the partition function of a system with only two accessible states, native and denatured, N and D

$$\begin{aligned} Q(T) &= 1 + K(T) = 1 + \exp[-\Delta_N^D G^\ominus / RT] \\ &= 1 + \exp[(-\Delta_N^D H^\ominus / R)(1/T - 1/T_d)] \end{aligned} \tag{4}$$

In the last term, we have written the temperature dependence of the equilibrium constant K in terms of the parameters characterizing a DSC curve: $\Delta_N^D H^\ominus$, the standard enthalpy change associated with the transition, corresponding to the area of the peak, and T_d, the half transition temperature, usually corresponding to the maximum of the peak. From the fundamental statistical thermodynamic relation connecting the excess average enthalpy (referred to the zero state), $\langle \Delta H(T) \rangle$, to the partition function of the system [15]

$$\langle \Delta H(T) \rangle = RT^2[\mathrm{d} \ln Q(T)/\mathrm{d}T] \tag{5}$$

it follows in the present case that

$$\langle \Delta H(T) \rangle = \Delta_N^D H^\ominus [K(T)/Q(T)] \tag{6}$$

Finally, it is possible to obtain the excess heat capacity $\langle \Delta C_p(T) \rangle$ as the derivative of the excess enthalpy function

$$\begin{aligned} \langle \Delta C_p(T) \rangle &= [\mathrm{d}\langle \Delta H(T) \rangle / \mathrm{d}T] \\ &= \Delta_N^D C_p f_D + \Delta_N^D H^\ominus [\mathrm{d}f_D / \mathrm{d}T] \end{aligned} \tag{7}$$

where f_D represents the population fraction of the denatured state, according to its statistical thermodynamic definition, and $\Delta_N^D C_p$ is the experimentally determined net heat capacity change associated with the transition from native to denatured state. This relation is an analytical expression that allows the simulation of two-state transition curves as a dependence on three parameters: T_d, $\Delta_N^D H^\ominus$ and $\Delta_N^D C_p$, which is composed

of two terms normally defined as the heat capacity change *between* the states, $\Delta_N^D H^{\ominus}[df_D/dT]$, and the intrinsic heat capacity change *in* the states, $\Delta_N^D C_p f_D$. The latter is not so important for the energetics of the process and also complicates the deconvolution procedure. For these reasons its contribution has been eliminated from the experimental curves through an iterative algorithm [16]. The other term represents the latent heat associated with the transition from state N to state D.

If the protein consists of two independent sub-units and assuming that each unfolds by a two-state mechanism independently from the behaviour of the rest of the macromolecule, the overall calorimetric curve will be simply the sum of the component sub-unit transitions. A non-linear regression algorithm will then be sufficient to fit the experimental curve to the sum of a defined number of curves representative of the two-state transitions [17]. This kind of deconvolution allows the determination of the change in enthalpy associated with the unfolding of single sub-units ΔH_i, and the corresponding temperature of the maximum for each component transition $T_{d,i}$. It is also possible, useful and very important to determine the temperature dependence of the population fractions of the four accessible states for the hypothetical selected protein

$$f_{FF^*} = 1/Q(T)^{Indep}$$

$$f_{FU^*} = K_1(T)/Q(T)^{Indep}$$

$$f_{UF^*} = K_2(T)/Q(T)^{Indep}$$

$$f_{UU^*} = K_1(T)K_2(T)/Q(T)^{Indep}$$

SEQUENTIAL TRANSITION MODEL

In this model, we consider a protein denaturation process with an intermediate thermodynamic state

$$N \underset{}{\overset{K_1}{\rightleftharpoons}} I \overset{K_2}{\rightleftharpoons} D$$

Taking the native form as reference state, the canonical partition function of this system can be written

$$Q(T)^{Seq} = 1 + K_1 + K_1K_2$$
$$= 1 + \exp[-\Delta_N^I G^{\ominus}/RT] + \exp[-\Delta_N^D G^{\ominus}/RT] \quad (8)$$

The deconvolution procedure for DSC curves according to this model was developed in a series of important papers by Freire and Biltonen [12, 18–21]. These authors showed that the conformational partition

function of the macromolecule in solution can be determined directly from experimental calorimetric data. Indeed, the excess heat capacity function $\langle \Delta C_p(T) \rangle$ can be readily obtained from DSC measurements, making a linear regression on the predenaturation range and extrapolating this straight line over the total range of interest. In fact, it is reasonable to assume that the heat capacity of the native state is a linear function of temperature. In this manner, the measured apparent heat capacity is converted to the excess heat capacity with respect to the native state, which is the most convenient reference state for studying these conformational transitions. By a double integration, with respect to temperature, of the excess heat capacity function $\langle \Delta C_p(T) \rangle$, it is possible to obtain the canonical partition function $Q(T)^{\mathrm{Seq}}$ of the system under investigation. From this function $Q(T)^{\mathrm{Seq}}$, using a fundamental algorithm elaborated by Freire and Biltonen, the sequential deconvolution of the whole complex calorimetric curve can be accomplished. Even in this case it is possible to determine the enthalpy change associated with each component transition ΔH_i, and the corresponding temperature of half transformation $T_{d,i}$. But it must be noted that they do not correspond to the unfolding of independent domains of the macromolecule. The temperature dependence of the population fractions of three thermodynamic states (to which the hypothetical selected protein can belong) are determined by the relations

$$f_N = 1/Q(T)^{\mathrm{Seq}}$$

$$f_I = K_1(T)/Q(T)^{\mathrm{Seq}}$$

$$f_D = K_1(T)K_2(T)/Q(T)^{\mathrm{Seq}}$$

The fundamental difference between the two models is the number of thermodynamically accessible states which they generate: two independent one-step transitions give 2^2 states; in general, N independent one-step transitions give 2^N states; and two sequential transitions give 3 states; in general, N sequential transitions give $N + 1$ thermodynamic states.

From the above analysis it seems incredible that the two different descriptions can represent an experimental complex DSC curve with the same accuracy. However we have found that both models describe quite well the experimental data of the two systems, bovine serum albumin at pH 8.0 and bovine seminal ribonuclease at pH 5.0.

MATERIALS AND METHODS

The bovine serum albumine (BSA) used was a commercial Sigma product, sample A 7511 from Fraction V (defatted to less than 0.005%, according to Chen [22]). The monomeric form of BSA was obtained by passing about 200 mg of this defatted protein down a 1-m-long Sephadex G 75 column, equilibrated with 0.15 M NaCl and 0.01 M phosphate buffer at

pH 7. The protein was dialysed exhaustively in the cold against distilled and deionized water, and finally lyophilized for storage. The molecular weight of BSA was taken as 66300.

The bovine seminal ribonuclease (RNAase BS), obtained from bull seminal plasma, was kindly provided by Professors G. D'Alessio and R. Piccoli and was used without further treatment. The molecular weight of RNAse BS was taken as 27400 [23]. The concentrations of the solutions for calorimetric measurements were determined spectrophotometrically, using the following molar extinction coefficients, $\varepsilon_{279} = 44200\,M^{-1}\,cm^{-1}$ for BSA and $\varepsilon_{278} = 12740\,M^{-1}\,cm^{-1}$ for RNase BS. Calorimetric measurements were carried out on a second-generation Setaram Micro-DSC apparatus, expressly designed for studies in dilute solutions of biological macromolecules. It was interfaced with a data translation A/D board for the automatic accumulation of experimental data on an IBM-compatible PC. All the analysis and data treatments were accomplished with software programs developed in our laboratory: THESEUS for the calibration, buffer–buffer subtraction, definition of correct baseline and the thermodynamic parameters (ΔH_{exp}, T_{max}, ΔC_p) determination for each calorimetric curve [16]; DEDALUS for the deconvolution procedure according to the independent transition model [17]; and MINOS for the deconvolution procedure following the sequential transition model [17]. A scan rate of $0.5\,K\,min^{-1}$ was chosen for the present study.

RESULTS AND DISCUSSION

One of the greatest advantages of DSC is that it is possible to obtain simultaneously the thermodynamic enthalpy for the process and the analytical enthalpy calculated from the van't Hoff relation, on the basis of the measured degree of progress of the reaction induced by temperature. In the case of a two-state transition, the calorimetric and analytical enthalpy values must coincide [1, 3, 12]. For both the proteins, BSA and RNAase BS, the DSC curves are not well represented by a two-state transition because the ratio $\Delta H_{exp}/\Delta H_{v.H.}$ is unequivocally greater than one. This is not unexpected if one considers that a one-step unfolding mechanism is an indication that a unique cooperative unit corresponds to the whole macromolecule, as occurs for some investigated small globular proteins. An increase in the polypeptide chain length leads, in general, to a more complex organization of the tertiary structure. Each domain often behaves as a distinct cooperative unit. Indeed, it is well known from X-ray diffraction determinations that RNAase BS is a dimeric protein consisting of two identical sub-units (each 80% identical to pancreatic RNAase A), covalently linked to each other by two disulphide bridges from the neighbouring cysteines 31 and 32 [24, 25]. However, it is firmly established from hydrodynamic and spectroscopic studies that the tertiary structure of

BSA is arranged in three domains [26]. Moreover, in a very recent crystallographic study, it has been shown that for human serum albumin HSA, homologous to BSA, the determined structure corresponds to an arrangement in which there is a strong association between two of the domains [27].

We have studied extensively the thermal unfolding process of monomeric and oligomeric BSA on changing the pH of the solutions and other conditions, and the results will be published elsewhere [28]. For the present purposes, we have chosen calorimetric measurements at pH 8.0 and phosphate buffer, because in these conditions the exothermic phenomenon, attributed to aggregation of denatured albumin molecules, is well separated from the endothermic conformational transition. It must be noted that the unfolding process of BSA is irreversible, probably due to aggregation of denatured molecules or to side-reactions at high temperatures. The irreversibility could invalidate the deconvolution analyses presented above, all based on classical and statistical equilibrium thermodynamics. Sturtevant and coworkers, however, in a series of papers concerning this point [29–33], have shown that the usual thermodynamic treatment is still applicable in these cases. For this reason, we proceeded to analyse the DSC curves with the two models. However, no calculations are possible for the temperature dependence of the Gibbs standard energy function $\Delta G^{\ominus}(T)$, which is correctly defined and evaluated only for reversible processes.

For RNAase BS, we have chosen the calorimetric curves at pH 5.0 and acetate buffer, where the denaturation process is reversible and the thermodynamic analysis strictly correct. In both cases, the two deconvolution procedures showed the presence of two independent transitions or two sequential transitions, respectively. The determined values for the thermodynamic parameters ΔH_i and $T_{d,i}$, characterizing each transition, are reported in Table 1 for BSA and Table 2 for RNAase BS. The value of ΔH_{exp} for BSA is in good agreement with that obtained at pH 7.0 for HSA in a recent calorimetric investigation of its thermal denaturation [34]. From non-calorimetric studies, it has been argued that the unfolding process of BSA is a multi-state transition [35, 36]. For RNAase BS, the determined value of ΔH_{exp} is less than double that obtained for RNAase A in the same experimental conditions [37]. This may be due to a minor loss of structural organization in the denaturation process of dimeric protein with respect to the monomeric analogue (the coincidence of most of the structural parameters suggests this). There are some indications that the different thermal stability of the two (at least) domains of RNAase BS does not concern the two monomer units, but rather the central framework, connected by the pair of disulphide bridges, and the rest of the molecule.

The deconvolutions of the thermal profiles according to the two models are shown in Figs. 1–3 for monomeric BSA and in Fig. 4 for RNAase BS: Fig. 1, the deconvolution according to the independent two-state transition

TABLE 1

Thermodynamic parameters from the analysis of the experimental calorimetric curves of monomer BSA at pH 8.0, 0.01 M phosphate buffer, for the two models [a]

	Independent transition	Sequential transition
ΔH_{exp}/kJ mol^{-1}	1190	1190
ΔH_1/kJ mol^{-1}	325	350
ΔH_2/kJ mol^{-1}	880	840
$(\Delta H_1 + \Delta H_2)$/kJ mol^{-1}	1205	1190
$T_{d,1}$/°C	64.0	64.0
$T_{d,2}$/°C	68.7	68.7
Chi-square	854	912

[a] These results were obtained on a single calorimetric measurement which was selected for the deconvolution analyses from a set of four experiments in good agreement with each other.

model obtained with the DEDALUS program of a DSC curve of BSA; Fig. 2, the superposition of the curve obtained from the sequential deconvolution accomplished with the MINOS program and the experimental curve of BSA (it is worth noting that in this case it is impossible to determine the single-component transitions as they are mutually dependent because the assumed mechanism is sequential); Figs. 3(a) and 4(a), the population fractions as functions of temperature for the four states accessible to both proteins, according to the independent transition model; Figs. 3(b) and 4(b), the population fractions as functions of temperature for the three states accessible to both proteins, following the sequential transition model.

Although very different in their theoretical bases, both models represent the experimental calorimetric curves very well. The reasons or causes can

TABLE 2

Thermodynamic parameters from the analysis of the experimental calorimetric curves of RNAase BS at pH 5.0, 0.01 M acetate buffer, for the two models [a]

	Independent transition	Sequential transition
ΔH_{exp}/kJ mol^{-1}	863	863
ΔH_1/kJ mol^{-1}	402	398
ΔH_2/kJ mol^{-1}	466	463
$(\Delta H_1 + \Delta H_2)$/kJ mol^{-1}	868	861
$T_{d,1}$/°C	58.4	58.4
$T_{d,2}$/°C	62.3	62.3
Chi-square	1102	1193

[a] These results were obtained on a single calorimetric measurement which was selected for the deconvolution analyses from a set of four experiments in good agreement with each other.

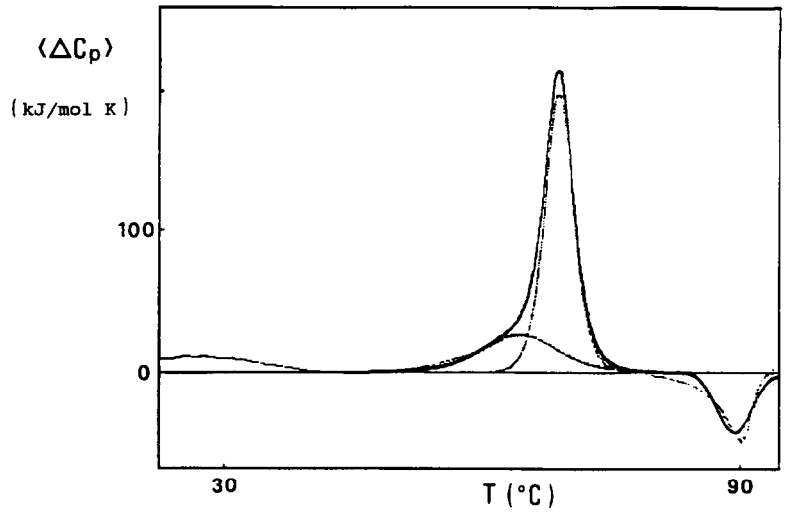


Fig. 1. Deconvolution analysis according to the independent transition model of an experimental DSC curve for BSA at pH 8.0, 0.01 M phosphate buffer and 0.15 M NaCl. At high temperatures, an exothermic peak due to the aggregation of denatured albumin molecules is also present.

be understood by considering the temperature dependence of the population fractions. It can be noted that the number of significantly populated states in the temperature range in which the denaturation process occurs, is practically identical for both models.

For the proteins examined, the fourth state, UF*, established on the basis of the independent two-state transition model, never has a physically

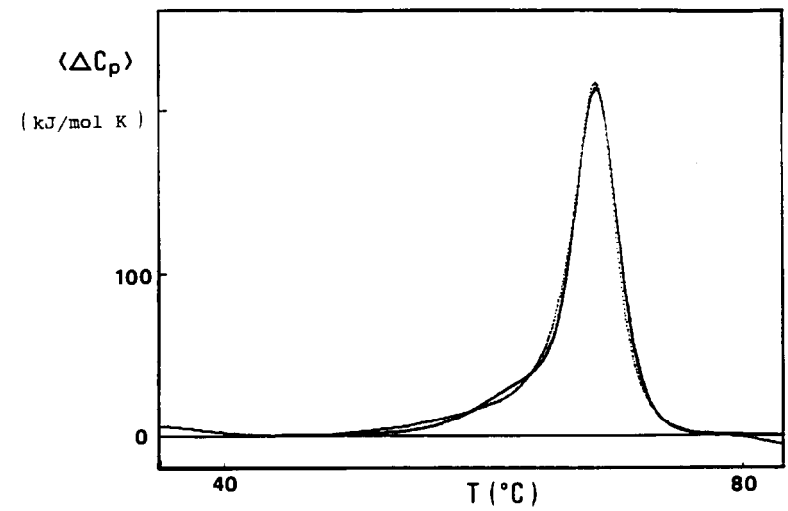


Fig. 2. Experimental and simulated DSC curves for BSA at pH 8.0, 0.01 M phosphate buffer and 0.15 M NaCl. The simulation is based on the sequential transition model.

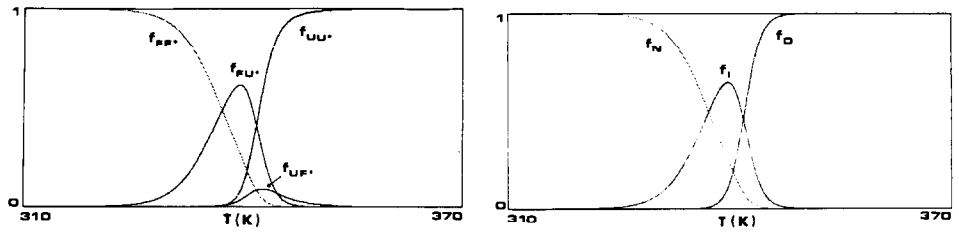


Fig. 3. (a) Population fractions as a function of temperature of the states accessible to BSA at pH 8.0, calculated using the independent transition model. (b) Population fractions as a function of temperature of the states accessible to BSA at pH 8.0, calculated using the sequential transition model.

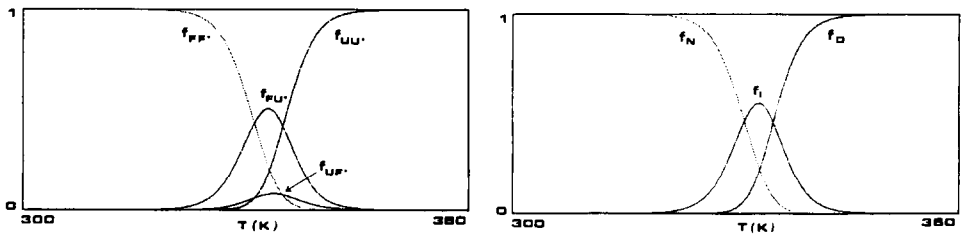


Fig. 4. (a) Population fractions as a function of temperature of the states accessible to RNAase BS at pH 5.0, 0.1 M acetate buffer, calculated using the independent transition model. (b) Population fractions as a function of temperature of the states accessible to RNAase BS at pH 5.0, 0.1 M acetate buffer, calculated using the sequential transition model.

significant population fraction, i.e. it is always less than 0.1. In other words, the physical observable heat capacity is unable to show unequivocally the effects due to the small amount of protein molecules present in this state. Thus, looking to the respective schemes of the two models, it is possible to conclude that the results are very similar because only one of the two allowed ways of accomplishing the unfolding process for the independent transition model is practically and physically relevant. Considering the well-confirmed domain structure of proteins, it seems that one of the two domains is less stable and unfolds at lower temperatures than the other. Thus the denaturation process, although it occurs in distinct regions of the macromolecule (because there are distinct cooperative sub-units or domains), approaches a sequential mechanism, i.e. a step-by-step mechanism. The connection between the two representations relies on the number of thermodynamic states which are sufficiently populated to contribute appreciably to the energetics of the denaturation process [21, 38].

ACKNOWLEDGEMENTS

This work was financed by the Italian National Research Council (C.N.R. Rome) Target Program on "Chimica Fine" and by the Ministry of University and Scientific and Technological Research.

REFERENCES

1 P.L. Privalov, Adv. Protein Chem., 33 (1979) 179.
2 P.L.Privalov, and S.A. Potekhin, Methods Enzymol., 131 (1986) 4.
3 P.L. Privalov and N.N. Khechinasvili, J. Mol. Biol., 86 (1974) 665.
4 W.J. Becktel and J.A. Schellman, Biopolymers, 26 (1987) 1859.
5 P.L. Privalov and V.V. Filimonov, J. Mol. Biol., 122 (1978) 447.
6 H. Klump, Ber. Bunsenges. Phys. Chem., 91 (1987) 206.
7 V.V. Novokhatny, S.A. Kudinov and P.L. Privalov, J. Mol. Biol., 179 (1984) 215.
8 J.W. Shriver and U. Kamath, Biochemistry, 29 (1990) 2556.
9 P.L. Privalov, Adv. Protein Chem., 35 (1982) 1.
10 S.V. Matveev, V.V. Filimonov and P.L. Privalov, Mol. Biol. (USSR), 16 (1983) 172.
11 L.H. Chang, S.J. Li, T.L. Ricca and A.G. Marshall, Anal.Chem., 56 (1984) 1502.
12 E. Freire and R.L. Biltonen, Biopolymers, 17 (1978) 463.
13 V.V. Filimonov, S.A. Potekhin, S.V. Matveyev and P.L. Privalov, Mol. Biol. (USSR), 16 (1982) 551.
14 S.J. Gill, B. Richey, G. Bishop and J. Wyman, Biophys. Chem., 21 (1985) 1.
15 T.L. Hill, An Introduction to Statistical Thermodynamics, Dover, New York, 1986.
16 G. Barone, P. Del Vecchio, D. Fessas, C. Giancola and G. Graziano, J. Therm. Anal., 38 (1992) 2779.
17 G. Barone, P. Del Vecchio, D. Fessas, C. Giancola and G. Graziano, in N. Russo, J. Anastassopoulou and G. Barone (Eds.), Chemistry and Properties of Biomolecular Systems, Kluwer, Dordrecht, 1993, in press.
18 E. Freire and R.L. Biltonen, Biopolymers, 17 (1978) 481.
19 E. Freire and R.L. Biltonen, Biopolymers, 17 (1978) 497.
20 E. Freire and R.L. Biltonen, Biopolymers, 17 (1978) 1257.
21 R.L. Biltonen and E. Freire, Crit. Rev. Biochem., 5 (1978) 85.
22 R.F. Chen, J. Biol. Chem., 25 (1967) 173.
23 G. D'Alessio, A. Floridi, R. De Prisco, A. Pignaro and E. Leone, Eur. J. Biochem., 26 (1972) 153.
24 L. Mazzarella, C.A. Mattia, S. Capasso and G. Di Lorenzo, Gazz. Chim. Ital., 117 (1987) 91.
25 S. Capasso, F. Giordano, C.A. Mattia, L. Mazzarella and A. Zagari, Biopolymers, 22 (1983) 327.
26 T. Peters, Adv. Protein Chem., 23 (1985) 161.
27 D.C. Carter, X.-M. He, S.H. Munson, P.T. Twigg, K.M. Gernert, M.B. Broom and T.Y. Miller, Science, 244 (1989) 1195.
28 G. Barone, S. Capasso, P. Del Vecchio, D. Fessas, C. Giancola, G. Graziano and P. Tramonti, to be published.
29 J.M. Sturtevant, Ann. Rev. Phys. Chem., 38 (1987) 463.
30 S.P. Manly, K.S. Matthews and J.M. Sturtevant, Biochemistry, 24 (1985) 3842.
31 V. Edge, N.M. Allewell and J.M. Sturtevant, Biochemistry, 24 (1985) 5899.
32 C.Q. Hu and J.M. Sturtevant, Biochemistry, 26 (1987) 178.
33 V. Edge, N.M. Allewell and J.M. Sturtevant, Biochemistry, 27 (1988) 8081.
34 P.D. Ross and A. Shrake, J. Biol. Chem., 263 (1988) 11196.
35 T.E. Creighton, J. Mol. Biol., 129 (1972) 235.
36 A. Wada, Y. Soito and M. Ohogushi, Biopolymers, 22 (1983) 197.
37 G. Barone, P. Del Vecchio, D. Fessas, C. Giancola, P. Pucci, A. Riccio and M. Ruoppolo, J. Therm. Anal., 38 (1992) 2791.
38 S.J. Gill, C.H. Robert and J. Wyman, M.N. Jones (Ed.), in Biochemical Thermodynamics, Elsevier, Amsterdam, 2nd edn., 1988.