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The heat capacity paradox of ligand binding proteins: reconciling the microscopic and macroscopic world

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Abstract

Differential scanning microcalorimetry (DSC) is a superb method for the analysis of protein energetics. However, the relative simplicity of application has led astray many to assume that a proper analysis of the data was possible without a sound knowledge of the underlying statistical thermodynamic principles. In this study, the question is addressed of how to calculate properly the heat capacity signal of a protein in the presence of high affinity ligands. It is shown that the signal corresponds neither to grand canonic nor to canonic heat capacity. Statistical thermodynamic model calculations result only in the observed macroscopic heat capacity signal, if the protein in the calorimetric cell is assumed to form a grand canonic ensemble (T, p, μ controlled) which is, however, heated under constraints typical for a canonic ensemble (T, p, N controlled). As a consequence, the microscopic statistical thermodynamic heat capacity must be carefully distinguished from the macroscopically observable thermodynamic heat capacity in those cases where proteins unfold in the presence of high affinity ligands. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

In recent years there has been increasing awareness of the fundamental significance of quantitative characterization of protein energetics. Two roots can be held responsible for the growing interest. One is purely scientific. It is connected with the question of the correlation between the environmentally determined microscopic state of the protein and its structure and function. The second reason is highly practical. It stems from the improvement and commercial availability of calorimetric instrumentation. This does not mean that other techniques such as spectroscopy and theoretical calculations were not instrumental in advancing knowledge. It is only to emphasize that direct thermodynamic information can be optimally obtained from direct calorimetric measurements and has therefore relied heavily on that technique. With these developments in mind, the question of the microscopic interpretation of the macroscopic C_p signal was unavoidable. The first answers were given in the early studies by Lumry et al. [1], Freire and Biltonen [2], Privalov [3], and Cooper [4]. These authors observed and postulated that

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denaturation of small proteins can be described frequently by a reversible two-state process. In the following time refinements of the analysis of the C_p curves were presented that permitted the interpretation of more complex equilibrium systems such as proteins in the presence of ligands [5–8] or oligomeric systems [7,9–11].

Based on the observation of a peculiar behavior of the homodimeric protein ROP in DSC studies [12], we decided to have a second look on the energetics of proteins. We were particularly interested in the correlation between microscopic behavior and the macroscopic calorimetric signal [13] as well as in the influence of ligand binding equilibria [14] on the shape of the C_p-curves. From a statistical mechanical point of view, two principal approaches offer themselves for the calculation of heat capacity. (1) The system under investigation is the calorimetric cell, which contains a fixed number of particles and therefore resembles a (T, p, N)-ensemble. This model was called 'stoichiometry model' because the stoichiometry enters the calculations as a physical constraint. (2) Alternatively the protein in the cell could be viewed as a grand canonical ensemble of protein molecules (T, p, μ), that can take up particles, i.e. ligands, from the surrounding reservoir. This model was called the 'proportionality model' because of the conservation of some proportionality properties that are lost in the stoichiometry model [14].

Furthermore, a third model—called the 'classical model'—has been employed for the analysis [5–8,15,16], which has properties that are intermediate between the two models presented by us. It could be shown that all three models provide practically identical numerical values under certain, frequently encountered experimental conditions [14,17].

A prominent problem remained, however, unanswered, despite the apparent numerical convergence of the results at specific ligand concentrations. It can be formulated in the following manner: How must a ligand binding protein be classified from a statistical thermodynamic point of view, or in other words: which of the three models describes the reality properly? In this article we shall treat this fundamental question. We will show the underlying physical processes that govern the behavior of ligand binding proteins at conditions at which the three models will exhibit distinguishable results. For this purpose, we shall calculate first the heat capacity of the protein for the cases of a (T, p, N) and a (T, p, μ) ensemble from the second temperature derivative of the relative partition function. It will be seen that in both cases the resulting calorimetric signals are incompatible with experimental observation. Then the question will be discussed which microscopic reality is reflected in the DSC signal. A simple system of a native protein N that can bind one ligand x and is able to unfold reversibly to the denatured state D, will be treated. Such a scheme will show all important features and can be expanded relatively easily into more elaborate reaction sequences.

2. Calculation of the enthalpy

The enthalpy for the reaction scheme

$$Nx \rightleftharpoons N + x \rightleftharpoons D + x \tag{1}$$

is obtained from the relative partition function Q of the protein [13]

$$Q = \frac{[N]+[Nx]+[D]}{[N]} = 1 + K_x[x] + K_0 \tag{2}$$

by applying the first temperature derivative to Q. $K_x = [Nx]/[N][x]$ and $K_0 = [D]/[N]$ are the equilibrium constants for ligand binding and protein denaturation, respectively. The system under investigation is the protein solution in the calorimetric cell. Obviously, the total concentration $[x]_t$ of ligand is kept constant during the measurement. This corresponds by definition to a (T, p, N)-ensemble. Then the proper temperature derivative is made at constant total ligand concentration $[x]_t$ and constant pressure p

$$\left(\frac{\partial}{\partial 1/RT}\right)_{p,[x]_t} \tag{3}$$

If, however, the system under investigation is defined as consisting of the individual proteins that bind and release particles (the ligands), the system is characterized only by the chemical potential μ in addition to T and p and no longer by a fixed total number of ligands. In this case,

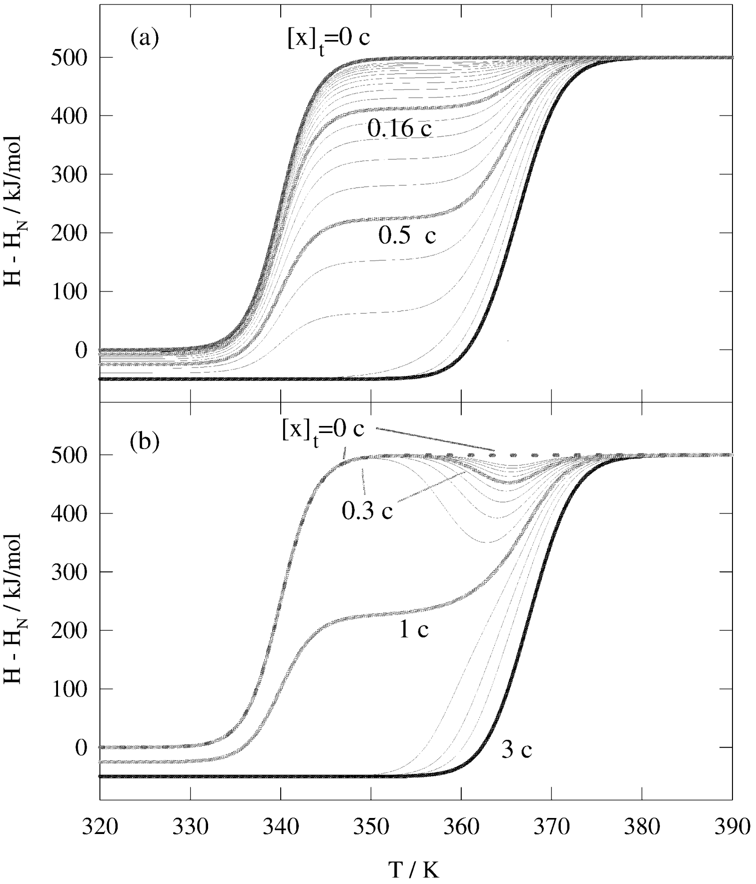

Fig. 1. Grand canonic (a) and canonic (b) enthalpy of a ligand binding protein. The parameters used at $T_{1/2}=340$ K are: $\Delta_N^D H^0=500$ kJ/mol; $\Delta_N^{Nx} H^0=-50$ kJ/mol; pK=11; and $c=10$ μM. For the macrocanonic system Eq. (5) has been used, and Eq. (7) for the canonical system. The ratios of total ligand per protein ($[x]_t/c$) are given at the respective bold curves. The residual curves shown by thin lines refer to $[x]_t/c$ ratios spaced equally by a factor of 1.26.

the derivative has to be calculated at constant free ligand concentration $[x]$:

$$\left(\frac{\partial}{\partial 1/RT}\right)_{p,[x]} \tag{4}$$

The temperature derivative at constant free ligand concentration $[x]$ is quite straightforward. The corresponding enthalpy change ΔH results from the first temperature derivative of the relative partition function Q

$$\begin{aligned}\Delta H=H-H_N&=-\left(\frac{\partial \ln Q}{\partial 1/RT}\right)_{p,[x]}\\&=\Delta_N^{Nx}H^0 f_{Nx}+\Delta_N^D H^0 f_D,\end{aligned} \tag{5}$$

where $f_{Nx}=K_x[x]/Q$ and $f_D=K_0/Q$ are the fractions of the native liganded and denatured state, respectively.

The temperature derivative at constant total ligand concentration $[x]_t$ is much more complicated, since in Eq. (2) for the relative partition function, Q, only the free concentration $[x]$ occurs explicitly. There are two possibilities to get around this problem. On the one hand, Q may be reformulated in terms of $[x]_t$. However, this requires finding the roots of a polynomial of the order $n+1$ for a protein binding n ligands, which is not possible in general. On the other hand, the condi-

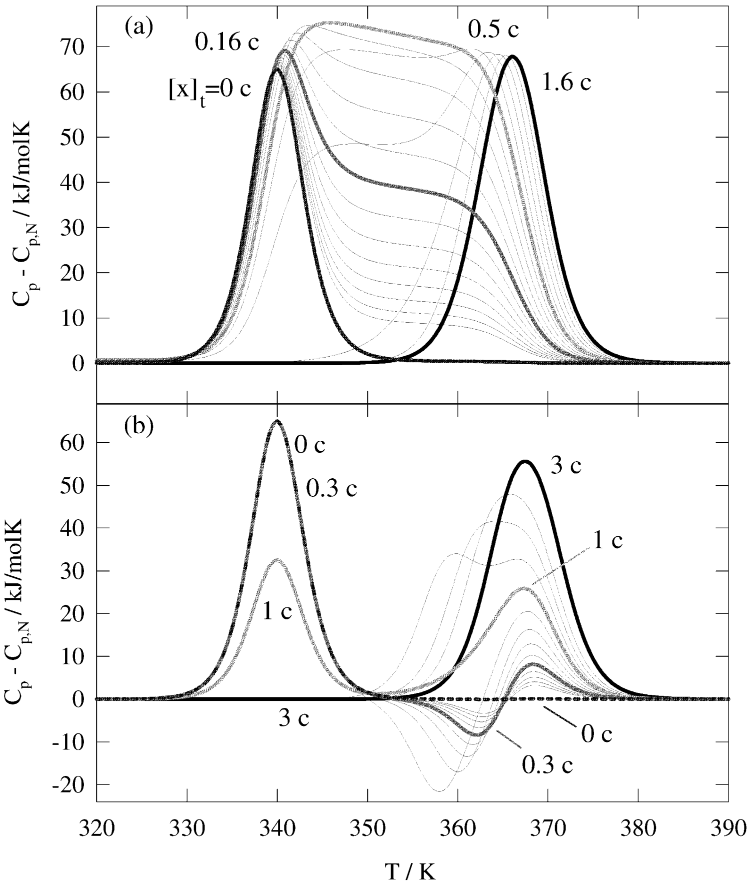

Fig. 2. Heat capacity of a protein binding one ligand calculated for different models. (a) macrocanonic heat capacity Eq. (9), and (b) canonic heat capacity Eq. (10). The parameters are the same as given in Fig. 1. The ratios of total ligand per protein ($[x]_t/c$) are given at the respective bold curves. The residual curves shown by thin lines refer to $[x]_t/c$ ratios spaced equally by a factor of 1.26.

tion of constant total ligand concentration $[x]_t$ can be introduced into the partial derivatives by a base transform [13,14]. By this method, one can obtain an analytical solution for the enthalpy even for an arbitrarily large number of bound ligands. For the reaction scheme given in Eq. (1) the corresponding partial derivative is [14]:

$$\left(\frac{\partial}{\partial 1/RT}\right)_{[x]_t}=\left(\frac{\partial}{\partial 1/RT}\right)_{[x]}-\frac{c}{1+c\left(\dfrac{\partial f_{Nx}}{\partial [x]}\right)_T}\times\left(\frac{\partial f_{Nx}}{\partial 1/RT}\right)_{[x]}\left(\frac{\partial}{\partial [x]}\right)_T \tag{6}$$

c is the molar protein concentration in terms of subunits. The usage of this expression corresponds to introducing the proper physical constraints for a (T, p, N)-ensemble. Application to the partition function Q [Eq. (2)] yields the following expression for the enthalpy change ΔH:

$$\begin{aligned}\Delta H=H-H_N&=RT^2\left(\frac{\partial \ln Q}{\partial T}\right)_{[x]_t}\\&=\Delta_N^D H^0 f_D\frac{1}{1-f_x^2 c/[x]_t}\\&\quad+\Delta_N^{Nx}H^0 f_x\frac{1-f_x c/[x]_t}{1-f_x^2 c/[x]_t}\end{aligned} \tag{7}$$

It was discussed by Rösgen and Hinz [14] that the (T, p, μ) enthalpy given in Eq. (5) and the (T, p, N) enthalpy given in Eq. (7) are usually equivalent. This is, however, no longer the case if the following two conditions (a) and (b) occur simultaneously: (a) protein and ligand concentration are in the same order of magnitude; and (b) the ligand affinity of the protein is exceedingly high $(K_x \geq 1/c)$. If both these conditions do apply, differences become obvious. Therefore, we explore in this study the equations for a protein with high ligand affinity as characterized by a pK value of 11. Such high values are not unusual for some proteinase inhibitor– and DNA–operator interactions.

Fig. 1a shows the (T, p, μ) enthalpy for a protein binding one ligand and denaturing reversibly as obtained from Eq. (5) for different ligand concentrations. The upper curve in this graph refers to zero ligand concentration. The other three curves refer to different $[x]_t/c$ ratios where $[x]_t$ is the total ligand concentration and c is the protein subunit concentration. In the low temperature region the negative enthalpy of ligand-binding (assumed to be -50 kJ/mol) is manifested in a reduction of the starting enthalpy with increasing ligand concentration. Due to the assumed high ligand affinity (p$K = 11$), the protein binds in the transition region practically all accessible ligand, as long as there are free native binding sites available. Therefore, at total ligand concentrations that are lower than the protein concentration, there is a population of unliganded native proteins present, which denature at a lower temperature than the protein–ligand complex. The residual population of native proteins has ligands bound and denatures at a higher temperature.

Fig. 1b shows the corresponding graphs obtained from Eq. (7) for the canonic enthalpy involving the derivative at $[x]_t = \text{const}$. The curves look similar to graphs for the grand canonic enthalpy in Fig. 1a, with one significant exception. As the ratio of total ligand to protein concentration changes from 0 to 1, there occurs a decrease in the enthalpy with increasing temperature close to the end of the transition region as illustrated for $[x]_t/c < 1$.

3. The canonical and the grand canonical heat capacity

The heat capacity difference $C_p - C_{p,N}$ is obtained from the second temperature derivative of the relative partition function:

$$\left(\frac{\partial^2 \ln Q}{\partial\left(1/RT\right)^2}\right) = RT^2(C_p - C_{p,N}) \tag{8}$$

3.1. The grand canonical heat capacity

The grand canonical heat capacity difference is calculated from Eq. (5) by using the temperature derivative shown in Eq. (4). The result is

$$\begin{aligned} C_p = {} & C_{p,N} + \Delta_N^D C_p^0 f_D + \Delta_N^{Nx} C_p^0 f_x \\ & + \frac{(\Delta_N^D H^0)^2}{RT^2} f_D(1 - f_D) \\ & + \frac{(\Delta_N^{Nx} H^0)^2}{RT^2} f_x(1 - f_x) \\ & - 2\frac{\Delta_N^{Nx} H^0 \Delta_N^D H^0}{RT^2} f_x f_D \end{aligned} \tag{9}$$

Fig. 2a illustrates the behavior of the grand canonic heat capacity [Eq. (9)] in the range where protein and ligand concentration are at the same order of magnitude. The peak width is at a maximum, if the total ligand concentration equals half the protein concentration. In this case only half of the native proteins are able to bind ligand, whereas the residual proteins remain unliganded. The ligand-free protein population denatures first. Up to the transition temperature of the protein–ligand complex, 50% of the total amount of proteins are denatured. Therefore, under these conditions the enthalpy distribution has its maximum width, extending over a temperature interval of almost 30 K. This is reflected in the area under the peak for $[x]_t/c = 0.5$ in Fig. 2a, which corresponds to a ΔH_{cal} value larger than 2000 kJ/mol. This value is obviously too high. Indeed it is four times higher than the assumed enthalpy of denaturation of 500 kJ/mol for the native protein or the enthalpy of denaturation of the complex of 550 kJ/mol. Evidently, there is a severe discrepancy between the area under the calculated heat capacity curve that results from the assumption of conditions corresponding to a grand canonic ensemble and the true value of ΔH^0.

Therefore, under the conditions of high ligand affinity, ΔH^0 calculated from the area under a heat capacity–temperature curve is not in agreement with the calorimetrically observed enthalpy change, if the heat capacity is calculated on the basis of a grand canonic ensemble [Eq. (9)]. The second alternative is the calculation of the heat capacity under the assumption of canonical conditions, i.e. $[x]_t = \text{const}$. This is employed in the following chapter.

3.2. The canonical heat capacity

The second derivative with respect to temperature of Eq. (2) under the constraints of constant total ligand concentration [Eq. (3)] results in the following canonic heat capacity [14]

$$
\begin{aligned}
C_p = {} & C_{p,N} + \Delta_N^D C_p^0 \frac{f_D}{1 - f_x^2 c/[x]_t} + \Delta_N^{Nx} C_p^0 f_x \\
& \times \frac{1 - f_x c/[x]_t}{1 - f_x^2 c/[x]_t} + \frac{(\Delta_N^D H^0)^2}{RT^2} \frac{f_D}{\left(1 - f_x^2 c/[x]_t\right)^3} \\
& \times \left[\left(1 - f_x^2 c/[x]_t\right) - f_D\left[1 + f_x^2 c/[x]_t (1 - 2f_x)\right]\right] \\
& + \frac{(\Delta_N^{Nx} H^0)^2}{RT^2} \frac{f_x (1 - f_x)}{\left(1 - f_x^2 c/[x]_t\right)^3} \left(1 - f_x c/[x]_t\right) \\
& \times \left[1 - f_x c/[x]_t (1 - 2f_x)\right] - 2 \frac{\Delta_N^{Nx} H^0 \Delta_N^D H^0}{RT^2} \\
& \times \frac{f_x f_D}{\left(1 - f_x^2 c/[x]_t\right)^3} \left(1 - f_x c/[x]_t\right) \\
& \times \left[1 - f_x c/[x]_t (2 - 2f_x)\right] - 2 \frac{\Delta_N^{Nx} H^0 \Delta_N^D H^0}{RT^2} \\
& \frac{f_x f_D}{\left(1 - f_x^2 c/[x]_t\right)^3} \left(1 - f_x c/[x]_t\right) \left[1 - f_x c/[x]_t (2 - 2f_x)\right]
\end{aligned}
\tag{10}
$$

A graphical representation of Eq. (10) for selected ligand to protein ratios is shown in Fig. 2b. In analogy to the enthalpy decrease with increasing temperature seen in Fig. 1b (at $[x]_t/c < 1$), we find also a negative heat capacity. This behavior is clearly not possible at equilibrium. Therefore, one has to draw the conclusion that also the canonic heat capacity is not an appropriate model for the rationalization of the calorimetric heat capacity signal of ligand binding proteins.

4. The real nature of the temperature dependence of the enthalpy of proteins

So far we have a perturbing result. Straightforward calculation of the heat capacity by applying the second temperature derivative to the relative partition function yields conflicting data: evidently, neither the grand canonic heat capacity (which overestimates the transition enthalpy) nor the canonic heat capacity (which predicts negative heat capacities) is appropriate to rationalize on a microscopic basis, the macroscopically observed heat capacity signal.

Obviously there is an inconsistency between the microscopic behavior expected on the basis of both ensembles and the macroscopically observed signal. To resolve this discrepancy we resort to the operational thermodynamic definition of heat capacity. Heat capacity is the limiting enthalpy change required to increase the temperature of the system by ΔT

$$
\begin{aligned}
C_{p,\text{macroscopic}} &= \lim_{\Delta T \to 0} \frac{H(T + \Delta T) - H(T)}{\Delta T} \\
&\equiv \frac{\mathrm{d}H}{\mathrm{d}T}
\end{aligned}
\tag{11}
$$

This derivative of the enthalpy automatically comprises all applied physical constraints that are operative in the experiment. Particularly it considers the constant total ligand concentration. Therefore, it appears to be an appropriate choice to calculate the enthalpy according to Eq. (5), i.e. under constant free ligand concentration and to apply the second temperature derivative under the constraint of constant total ligand concentration using Eq. (6). When calculating in this manner, the following temperature dependence of C_p is obtained:

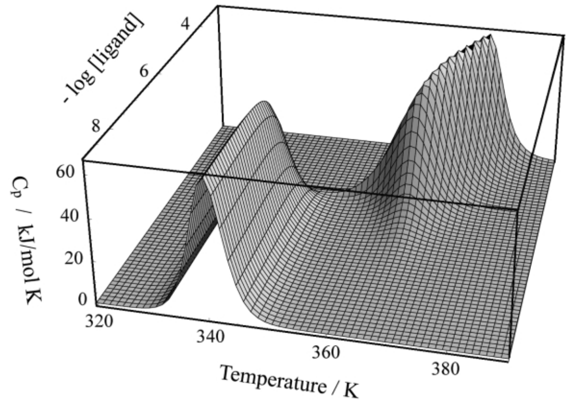

Fig. 3. Heat capacity of a ligand binding protein according to Eq. (12). The parameters are the same as given in Fig. 1. In the vicinity of $\log([x]_t)\approx\log(5\times10^{-6})$ two peaks are obtained.

$$\begin{aligned}C_p={}&C_{p,N}+\Delta_N^D C_p^0 f_D+\Delta_N^{Nx}C_p^0 f_x\\&+\frac{(\Delta_N^D H^0)^2}{RT^2}f_D\frac{\left(1-f_x^2c/[x]_t\right)-f_D}{1-f_x^2c/[x]_t}\\&+\frac{(\Delta_N^{Nx}H^0)^2}{RT^2}f_x(1-f_x)\frac{1-f_xc/[x]_t}{1-f_x^2c/[x]_t}\\&-2\frac{\Delta_N^{Nx}H^0\Delta_N^D H^0}{RT^2}f_xf_D\frac{1-f_xc/[x]_t}{1-f_x^2c/[x]_t}\end{aligned}\qquad(12)$$

The functional dependence of C_p on temperature and total ligand concentration is best visualized graphically. This is done in Fig. 3, which shows a three-dimensional plot of the heat capacity landscape. The most important conclusion is immediately obvious on inspection. In the ligand concentration range where $c\approx[x]_t/2$ and where two native states co-exist—native unliganded and native liganded proteins—two peaks of proper size are predicted by the calculations at positions at which each of the two populations of native proteins is supposed to denature.

If equation Eq. (11) is applied to the canonic enthalpy [Eq. (7)], the result is identical to the equation for the canonic heat capacity [Eq. (10)] with the consequence of negative heat capacities mentioned above.

5. Conclusions

The present study has addressed a very significant question: what is the statistical mechanical ensemble that represents properly the microscopic processes which are observed as heat capacity signals in a differential scanning calorimeter? Two obvious choices are the grand canonic and the canonic ensemble. However, it turned out that neither the grand canonic nor the canonic heat capacity provide a proper description of the C_p data that are obtained in a DSC experiment. The only solution resolving this dilemma resulted from the usage of the operational macroscopic definition of the heat capacity [Eq. (11)]. Based on the grand canonic enthalpy the temperature derivative of the enthalpy must be obtained using canonic constraints, i.e. $[x]_t=\text{const}$. Only in this manner can the proper variation with temperature of the heat capacity be reproduced.

This finding shows that there is an intricate relationship between the statistical mechanic heat capacity of a ligand binding protein and the DSC signal. This is a fundamentally significant insight. However, from a practical point of view under the majority of experimental conditions with lower ligand affinities or excess ligand, both formulas [Eqs. (9) and (12)] will provide similar ΔH^0 results [14]. Therefore, the DSC signal can usually be expected to reflect the statistical mechanic heat capacity. In these cases deconvolution of experimental heat capacity data can be largely simplified without loss of information by using the formula for the grand canonic heat capacity [Eq. (9)], instead of applying Eq. (12) that is based on the temperature derivative of the grand canonical enthalpy under the constraint of constant total ligand concentration.

Acknowledgments

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References

[1] R. Lumry, R. Biltonen, J.F. Brandts, Validity of the 'two-state' hypothesis for conformational transitions of proteins, Biopolymers 4 (1966) 917–944.

[2] E. Freire, R.L. Biltonen, Statistical mechanical deconvolution of thermal transitions in macromolecules, Biopolymers 17 (1978) 463–479.

[3] P.L. Privalov, Stability of proteins, Adv. Prot. Chem. 3 (1979) 167–241.

[4] A. Cooper, Protein fluctuations and the thermodynamic uncertainty principle, Prog. Biophys. Mol. Biol. 44 (1984) 181–214.

[5] C.H. Robert, S.J. Gill, J. Wyman, Quantitative analysis of linkage in macromolecules when one ligand is present in limited total quantity, Biochemistry 27 (1988) 6829–6835.

[6] C.H. Robert, A. Colosimo, S.J. Gill, Allosteric formulation of thermal transitions in macromolecules, including effects of ligand binding and oligomerization, Biopolymers 28 (1989) 1705–1729.

[7] J.F. Brandts, L.-N. Lin, Study of strong to ultratight protein interactions using differential scanning calorimetry, Biochemistry 29 (1990) 6927–6940.

[8] M. Straume, E. Freire, Two-dimensional differential scanning calorimetry: simultaneous resolution of intrinsic protein structural energetics and ligand binding interactions by global linkage analysis, Anal. Biochem. 203 (1992) 259–268.

[9] P.L. Privalov, S.A. Potekhin, Scanning microcalorimetry in studying temperature-induced changes in proteins, Methods Enzymol. 131 (1986) 4–51.

[10] S.-I. Kidokoro, H. Uedaira, A. Wada, Determination of thermodynamic functions from scanning calorimetry data. II. For the system that includes self-dissociation/association processes, Biopolymers 27 (1988) 271–297.

[11] E. Freire, Statistical thermodynamic analysis of the heat capacity function associated with protein folding–unfolding transitions, Comments Mol. Cell. Biophys. 6 (1989) 123–140.

[12] J. Rösgen, B. Hallerbach, H.-J. Hinz, The 'Janus' nature of proteins, Biophys. Chem. 74 (1998) 153–161.

[13] J. Rösgen, H.-J. Hinz, Statistical thermodynamic treatment of conformational transitions of monomeric and oligomeric proteins, Phys. Chem. Chem. Phys. 1 (1999) 2327–2333.

[14] J. Rösgen, H.-J. Hinz, Folding energetics of ligand binding proteins I: theoretical model, J. Mol. Biol. 306 (2001) 809–824.

[15] A. Shrake, P.D. Ross, Ligand-induced biphasic protein denaturation, J. Biol. Chem. 265 (1990) 5055–5059.

[16] A. Shrake, P.D. Ross, Origins and consequences of ligand-induced multiphasic thermal protein denaturation, Biopolymers 32 (1992) 925–940.

[17] A. Rosengarth, J. Rösgen, H.-J. Hinz, Folding energetics of ligand binding proteins II: co-operative binding of Ca^{2+} to annexin I, J. Mol. Biol. 306 (2001) 825–835.