

# **Molecular Understanding of Calorimetric Protein Unfolding Experiments**

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## ABSTRACT

Protein unfolding is a dynamic cooperative equilibrium between short lived protein conformations. The Zimm-Bragg theory is an ideal algorithm to handle cooperative processes. Here, we extend the analytical capabilities of the Zimm-Bragg theory in two directions. First, we combine the Zimm-Bragg partition function  $Z(T)$  with statistical-mechanical thermodynamics, explaining the thermodynamic system properties enthalpy, entropy and free energy with molecular parameters only. Second, the molecular enthalpy  $h_0$  to unfold a single amino acid residue is made temperature-dependent. The addition of a heat capacity term  $c_v$  allows predicting not only heat denaturation, but also cold denaturation. Moreover, it predicts the heat capacity increase  $\Delta C_p^0$  in protein unfolding. The theory is successfully applied to differential scanning calorimetry experiments of proteins of different size and structure, that is, gpW62 (62aa), ubiquitin (74aa), lysozyme (129aa), metmyoglobin (153aa) and mAb monoclonal antibody (1290aa). Particular attention was given to the free energy, which can easily be obtained from the heat capacity  $C_p(T)$ . The DSC experiments reveal a zero free energy for the native protein with an immediate decrease to negative free energies upon cold and heat denaturation. This trapezoidal shape is precisely reproduced by the Zimm-Bragg theory, whereas the so far applied non-cooperative 2-state model predicts a parabolic shape with a positive free energy maximum of the native protein. We demonstrate that the molecular parameters of the Zimm-Bragg theory have a well-defined physical meaning. In addition to predicting protein stability, independent of protein size, they yield estimates of unfolding kinetics and can be connected to molecular dynamics calculations.

## Key words

Protein-cooperativity, differential-scanning-calorimetry, cold-denaturation, free-energy-trapeze, 2-state-model

## INTRODUCTION

Protein stability is an important issue in the development of pharmaceutical biologics. Thermodynamic aspects of protein folding have acquired significant practical importance because they provide the theoretical framework for rational protein design and protein modifications.[1] On the experimental level, differential scanning calorimetry (DSC) is the method of choice for thermodynamic studies of protein folding/unfolding equilibria.[2, 3] Analysis of DSC experiments with simple thermodynamic models has been key for developing our understanding of protein stability.[4] So far, the reversible denaturation reaction has been analyzed with a non-cooperative 2-state model.[5] However, its application is limited to small proteins with about 50 to 150 amino acids. Moreover, the free energy predicted by the 2-state model disagrees with the experimental finding obtained with differential scanning calorimetry.

The protein folding/unfolding transition is a dynamic equilibrium with many short-lived intermediates.[6] A multistate cooperative algorithm is therefore a physically more realistic alternative. We have shown with a variety of proteins that the cooperative multistate Zimm-Bragg theory is such a potential alternative. The theory yields a quantitated measure of cooperativity, is not limited in protein size and provides excellent simulations of protein unfolding thermodynamics.[7-14] Here we propose a significantly improved model where the Zimm-Bragg theory is combined with statistical-mechanical thermodynamics. First, we show that the macroscopic system parameters of unfolding, that is, enthalpy, entropy and free energy, can be traced back to simple molecular parameters of well-defined physical meaning. Second, the enthalpy  $h_0$  needed to unfold a single amino acid residue is supplemented with a heat capacity  $c_v$ . A second unfolding transition at low temperature (cold denaturation) occurs and the heat capacity increase  $\Delta C_p^0$  upon protein unfolding is also explained. The new model is validated by analyzing DSC measurements of different proteins, that is, lysozyme[10], the classic example of protein unfolding,

gpW62[15], an ultrafast folding protein, mAb, a large monoclonal antibody[13], metmyoglobin[16], a protein exhibiting cold denaturation, and ubiquitin, an  $\alpha$ -helical protein.[17] In the long-standing history of protein DSC measurements the heat capacity function  $C_p(T)$  was mainly used to evaluate the enthalpy, whereas entropy and free energy were ignored, although both functions are easily derived from  $C_p(T)$ . Here we systematically evaluate entropy and free energy from  $C_p(T)$  and analyze them with the multistate Zimm-Bragg theory. We show that the DSC observed temperature dependence of the free energy displays a trapezoidal shape with a zero free energy of the native state. This unique thermodynamic signature is precisely predicted by the Zimm-Bragg theory, but disagrees with the parabolic shape predicted by the 2-state model with a physically unrealistic positive free energy of the native state. Based on the molecular fit parameters at 20-90 °C the entropies of structurally different proteins were calculated at a denaturation temperature of 225 °C and were identical with the predictions of the Dymameomics Entropy Dictionary.[18]

## **THEORY**

### *Zimm-Bragg theory extended to cold denaturation*

Protein unfolding is a dynamic process in which individual amino acid residues flip from their native (n) to their unfolded (u) state. Rapid equilibria between many short-lived intermediates can be expected. An early example of cooperative unfolding is the  $\alpha$ -helix-to-random coil transition of synthetic peptides described with the Zimm-Bragg theory.[19-21] The cooperative folding theory distinguishes between a growth process with an equilibrium constant  $q(T)$  and a nucleation step with an equilibrium constant  $\sigma q(T)$  where  $\sigma$  is the cooperativity or nucleation parameter. Growth is defined as the addition of a new helical segment to an already existing  $\alpha$ -helix. Nucleation is the formation of a new helical segment within an unstructured region. The steepness

of the transition is determined by the cooperativity parameter  $\sigma$ , which is typically in the range of  $\sigma \sim 10^{-3}$  to  $10^{-7}$ . The cooperativity parameter of a non-cooperative system is  $\sigma = 1$ .

The central element of the Zimm-Bragg theory is the partition function  $Z(T)$  that collects all the energetic states of the folding process

$$Z(T) = (1 \quad 0) \begin{pmatrix} 1 & \sigma q(T) \\ 1 & q(T) \end{pmatrix}^v \begin{pmatrix} 1 \\ 1 \end{pmatrix} \quad (1)$$

$v$  denotes the number of amino acid residues involved in unfolding. The equilibrium constant  $q(T)$  is given by

$$q(T) = e^{\frac{-h(T)}{R} \left( \frac{1}{T} - \frac{1}{T_\infty} \right)} \quad (2)$$

$h(T)$  is the enthalpy needed to induce the  $n \rightarrow u$  conformational change. Up until now, this parameter was assumed to be temperature-independent with  $h_0 \sim 0.9 - 1.3$  kcal/mol.[20, 22-25]

Here we introduce a temperature-dependent unfolding enthalpy

$$h(T) = h_0 + c_v(T - T_0) \quad (3)$$

$T_0$  is the midpoint temperature of heat-induced unfolding. The heat capacity  $c_v$  refers to the unfolding of a single amino acid residue. The reference temperature  $T_\infty$  in equation (2) determines the position of the heat capacity maximum on the temperature axis. It is identical with  $T_0$  if the number of amino acid residues  $v$  is much larger than  $\sigma^{-1/2}$ . The steepness of the unfolding transition is determined by both  $\sigma$  and  $v$ . The smaller the cooperativity parameter  $\sigma$ , the steeper is the unfolding transition. Conversely, the shorter the chain length  $v$ , the broader is the unfolding transition. Short chains have a broad transition.[20]

The fraction of unfolded protein  $\Theta_U(T)$  is

$$\Theta_U(T) = \frac{q(T)}{v} \frac{d(\ln Z(T))}{dT} \left( \frac{dq(T)}{dT} \right)^{-1} \quad (4)$$

For a non-cooperative system with  $\sigma = 1$  the multistate Zimm-Bragg theory reduces to a 2-state model.[14]

### *Cold denaturation*

The heat capacity term  $c_v$  has two consequences. First,  $c_v$  produces the heat capacity increase  $\Delta C_p^0$  of the unfolded protein. Second,  $c_v$  leads to an additional transition at low temperature (cold denaturation). The exponent in the equilibrium constant  $q(T)$  (eq. (2)) has zeros at  $T_0$  and at  $h(T) = 0$ . The latter relation leads to

$$T_c = T_0 - \frac{h_0}{c_v} \quad (5)$$

$T_0$  is the midpoint of heat denaturation,  $T_c$  that of cold denaturation. The temperature difference between heat and cold denaturation is  $\Delta T = h_0/c_v$ .

### *Partition function and statistical-mechanical thermodynamics*

We show that the thermodynamics of protein unfolding and, consequently, the DSC experiments can be simulated without macroscopic fit parameters. According to statistical mechanical thermodynamics the partition function  $Z(T)$  is the sum of all conformational energies and is sufficient to determine the thermodynamic system parameters, that is, the inner energy  $E(T)$ , the entropy  $S(T)$ , and the Helmholtz free energy  $F(T)$ . [26, 27] The relevant equations are as follows

$$F(T) = -RT \ln Z(T) \quad (6)$$

$$E(T) = RT^2 \frac{d \ln Z(T)}{dT} \quad (7)$$

$$S(T) = \frac{E(T) - F(T)}{T} \quad (8)$$

$$C_v(T) = \frac{dE(T)}{dT} \quad (9)$$

### *Differential scanning calorimetry (DSC)*

Differential scanning calorimetry (DSC) is the method of choice to determine the thermodynamic properties of protein unfolding. DSC measures the temperature course of the heat capacity  $C_p(T)$  that includes not only the conformational enthalpy proper, but also the increase in heat capacity  $\Delta C_p^0$  between native and denatured protein.[28, 29] Stepwise integration of  $C_p(T)$  provides unfolding enthalpy, entropy, and Gibbs free energy:

$$H(T_i) = \sum_1^i \left[ \frac{C_p(T_i) + C_p(T_{i+1})}{2} \right] [T_{i+1} - T_i] \quad (10)$$

$$S(T_i) = \sum_1^i \frac{C_p(T_{i+1}) + C_p(T_i)}{2T_i} [T_{i+1} - T_i] \quad (11)$$

$$G(T_i) = H(T_i) - T_i S(T_i) \quad (12)$$

"It is clear that in considering the energetic characteristics of protein unfolding one has to take into account all energy which is accumulated upon heating and not only the very substantial heat effect associated with gross conformational transitions, that is, all the excess heat effects must be integrated".[30] The DSC-measured thermodynamic parameters characterizing the total unfolding transition are denoted  $\Delta H_{cal}$ ,  $\Delta S_{cal}$  and  $\Delta G_{cal}$ .

DSC measurements are made at constant pressure. The volume changes in protein unfolding are very small and the following relations are valid without loss of accuracy. Heat capacity  $C_p(T) \cong C_v(T)$ , enthalpy  $H(T) \cong$  inner energy  $E(T)$ , entropy  $S_p(T) \cong S_v(T)$ , Gibbs free energy  $G(T) \cong$  Helmholtz free energy  $F(T)$ .

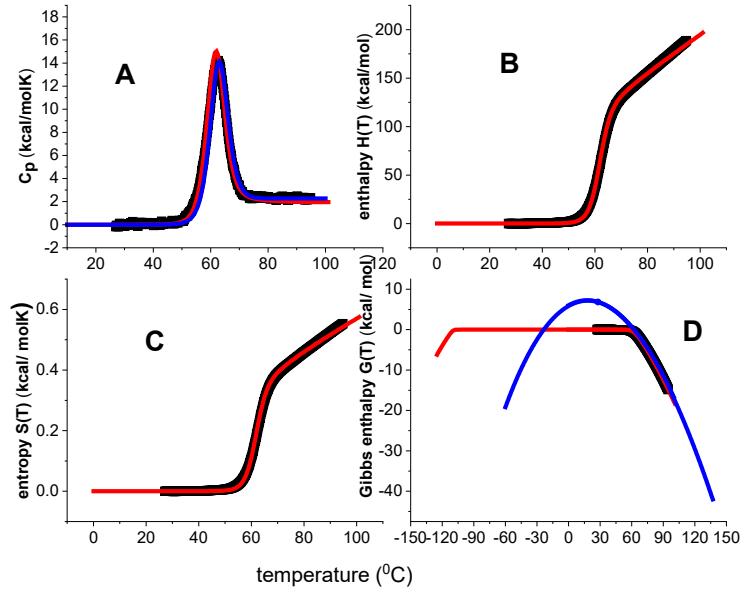
## Results

Our earlier simulations of DSC experiments with the Zimm-Bragg theory required molecular as well as macroscopic fit parameters. The extent of unfolding  $\Theta_U(T)$  was calculated with molecular parameters ( $h_0$ ,  $\sigma$ ) and was then multiplied with macroscopic parameters, that is, the unfolding enthalpy  $\Delta H_0$  and the heat capacity increase  $\Delta C_p^0$ . Here we eliminate  $\Delta H_0$  and  $\Delta C_p^0$  by combining the partition function  $Z(T)$  with statistical-mechanical thermodynamics (eqs. (6) - (9)). All macroscopic thermodynamic properties are now predicted exclusively with molecular parameters ( $h_0$ ,  $c_v$ ,  $\sigma$ ,  $v$ ). Of particular interest is the free energy of unfolding  $G(T) \cong F(T)$ . Proposed as a parabolic function in the theory of the 2-state model, the DSC-accessible free energy  $G_{\text{cal}}(T)$  (eq. (12)) appears to be completely ignored in applications of the 2-state model. In the following we compare proteins of different size and structure, which were carefully studied with DSC. The analysis of the  $C_p(T)$  thermograms provides the caloric, model-independent results for enthalpy  $H_{\text{cal}}(T)$ , entropy  $S_{\text{cal}}(T)$ , and free energy  $G_{\text{cal}}(T)$ . The measured thermodynamic properties are then compared to the predictions by the cooperative Zimm-Bragg theory and the 2-state model.

### *Lysozyme unfolding. DSC and molecular multistate partition function*

Lysozyme is a 129-residue protein composed of ~25%  $\alpha$ -helix, ~40%  $\beta$ -structure and ~35% random coil in solution at room temperature.[10] Upon unfolding, the  $\alpha$ -helix is almost completely lost and the random coil content increases to ~60%. Thermal unfolding is completely reversible and lysozyme is the classical example to demonstrate 2-state unfolding.[2, 31, 32] Figure 1A shows the  $C_p(T)$  thermograms of lysozyme unfolding with a resolution of 0.17°C [10] Unfolding takes place in





1. Figure 1. Differential scanning calorimetry of 50  $\mu\text{M}$  lysozyme in 20% glycine buffer, pH 2.5. Black data points: DSC thermograms obtained with a heating rate of  $1\text{ }^\circ\text{Cmin}^{-1}$  and a step size of  $0.17\text{ }^\circ\text{C}$ . Red lines: simulations with the Zimm-Bragg theory with  $h_0 = 1.0\text{ kcal/mol}$ ,  $c_v = 6\text{ cal/molK}$ ,  $\sigma = 1.0 \times 10^{-6}$ ,  $\nu = 129$ . (A) Heat capacity  $C_p(T)$ . (B) Unfolding enthalpy  $H(T)$ . (C) Unfolding entropy  $S(T)$ . (D) Free energy of unfolding  $G(T)$ . Blue line:  $\Delta G(T)$  (eq.(13)) predicted by the 2-state model calculated with a conformational enthalpy  $\Delta H_0 = 107\text{ kcal/mol}$  and a heat capacity increase  $\Delta C_p^0 = 2.269\text{ kcal/mol}$ . Data taken from reference.[10]

the temperature range of  $45\text{ }^\circ\text{C} \leq T \leq 73\text{ }^\circ\text{C}$  (midpoint temperature  $T_0 = 61.7\text{ }^\circ\text{C}$ ). The unfolding enthalpy is  $\Delta H_{\text{cal}} = 147\text{ kcal/mol}$  (eq. (10)) and the entropy  $\Delta S_{\text{cal}} = 0.437\text{ kcal/molK}$  (eq. (11)) The enthalpy/entropy ratio  $\Delta H_{\text{cal}}/\Delta S_{\text{cal}} = 335.5\text{ K} = 62.5\text{ }^\circ\text{C}$  agrees with the midpoint temperature  $T_0$ . The molar heat capacity of unfolded lysozyme increases by  $\Delta C_p^0 = 2.269\text{ kcal/molK}$ , in agreement with literature data.[28, 29, 32]

The high cooperativity of lysozyme unfolding (cooperativity parameter  $\sigma = 1.0 \times 10^{-6}$ ) leads to a sharp unfolding transition, which is well approximated by a 2-state equilibrium. 2-state model and Zimm-Bragg theory both provide excellent simulations of the heat capacity  $C_p(T)$  (fig. 1A).

Differences between the two models exist, however, in predicting the temperature dependence of the free energy. The 2-state model defines the free energy as

$$\Delta G(T) = \Delta H_0 \left(1 - \frac{T}{T_0}\right) + \Delta C_p^0 (T - T_0) - T \Delta C_p^0 \ln\left(\frac{T}{T_0}\right) \quad (13)$$

yielding the parabolic shape in figure 1D (blue line, same parameters as in Figure 1A). The free energy has a positive maximum of 7.18 kcal/mol for the native protein at 20 °C and becomes zero at  $T_0 = 62^\circ\text{C}$ , where lysozyme is 50% unfolded. However, this is not what is seen in the DSC experiment (fig. 1D, black data points). The free energy of the native protein is zero and becomes immediately negative upon unfolding. Between 27°C and  $T_0$  the DSC experiment reports a negative free energy change  $\Delta G_{\text{cal}} = -0.7$  kcal/mol, which increases rapidly beyond  $T_0$  to  $\Delta G_{\text{cal}} = -6.06$  kcal/mol at 73 °C (90% unfolding). The Zimm-Bragg theory (eq. (7)) reproduces precisely the DSC result (fig. 1D, red line). Extending this simulation to low temperatures yields cold denaturation at  $T_{\text{cold}} = -103$  °C. The Zimm-Bragg theory thus predicts a trapezoidal shape of the free energy and the flat, near zero region extends over almost 140 °C. An experimental prove for the for the free energy trapeze is given in figure 4.

### *gpW62, an ultrafast folding protein*

gpW62 is a 62-residue  $\alpha+\beta$  protein that belongs to a group of ultrafast folding proteins.[4, 15] gpW62 folding was investigated with a variety of techniques, including DSC. The heat capacity  $C_p(T)$  (Figure 2A, data taken from reference[15]) is evaluated here according to eqs. (10) - (12). The midpoint temperature is  $T_0 = 67$  °C. The unfolding enthalpy is  $\Delta H_{\text{cal}} = 91.7$  kcal/mol.,

measured between 37 °C and 102 °C. This is a large enthalpy for a 62-residue protein. The free energy change between native and unfolded gpW62 is  $\Delta G_{\text{cal}} = -8.11$  kcal/mol. The free energy per residue  $g_{\text{cal}} = -131$  cal/mol is almost 3 times larger than that of lysozyme with  $g_{\text{cal}} = -47$  cal/mol.

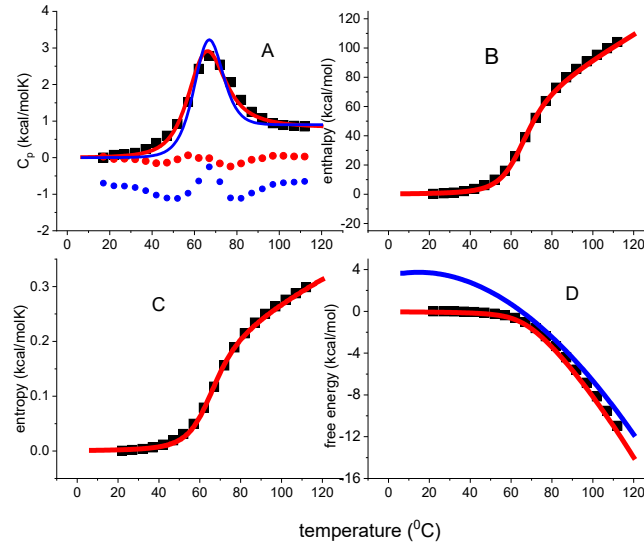


Figure 2. gpW62 DSC unfolding. (A) Heat capacity  $C_p(T)$ . Black data points taken from reference.[15] Data points in panels B-D calculated from data points in panel A with equations (10)-(12). Red solid lines: simulations with the Zimm-Bragg theory with  $h_0 = 1.26$  kcal/mol,  $c_v = 5$  cal/molK,  $\sigma = 5.0 \times 10^{-4}$ ,  $v = 62$  residues. Blue solid lines: 2-state model with  $\Delta H_0 = 49$  kcal/mol and  $\Delta C_p^0 = 0.9$  kcal/molK. The dotted lines in panel A are the differences between the experimental DSC data and the simulations. For better visibility the blue dotted line is shifted by -1 kcal/molK. (B) Unfolding enthalpy  $H(T)$ . (C) Unfolding entropy  $S(T)$ . (D) Free energy of unfolding  $G(T)$ . Data taken from reference[15].

Several aspects of the gpW62 folding equilibrium are unusual. The cooperativity is low with  $\sigma = 5 \times 10^{-4}$  and the unfolding extends over a broad temperature range of  $\Delta T \sim 65$  °C. This may be

compared to lysozyme with  $\sigma = 1 \times 10^{-6}$  and  $\Delta T \sim 30$  °C. As the transition is broad, the Zimm-Bragg theory provides a much better fit than the 2-state model (Figure 2A). The low cooperativity could facilitate fast folding by a low nucleation free energy (see Discussion). Fast folding of gpW62 could also be promoted by the large free energy change of the unfolding reaction. According to the thermodynamics of irreversible processes, the chemical reaction rate is proportional to the affinity, i.e. the free energy, of the reaction.[33-35] The ultra-fast folding of gpW62 could thus arise from the combination of a low nucleation energy and a large unfolding affinity.

#### *Monoclonal antibody mAb*

The 2-state model works in a limited size range of about 100-200 kDa. No such size limitation exists for the Zimm-Bragg theory. This is demonstrated for the recombinant monoclonal IgG1 antibody mAb with a molecular weight of 143 kDa (~1280 amino acids).[13] The antibody is formed of two identical heavy chains of ~450 residues each and two identical light chains of ~200 residues. The heavy and light chains fold into domains of ~110 aa residues. The secondary structure of mAb is composed of 7-11%  $\alpha$ -helix and 40-45%  $\beta$ -sheet.[36]

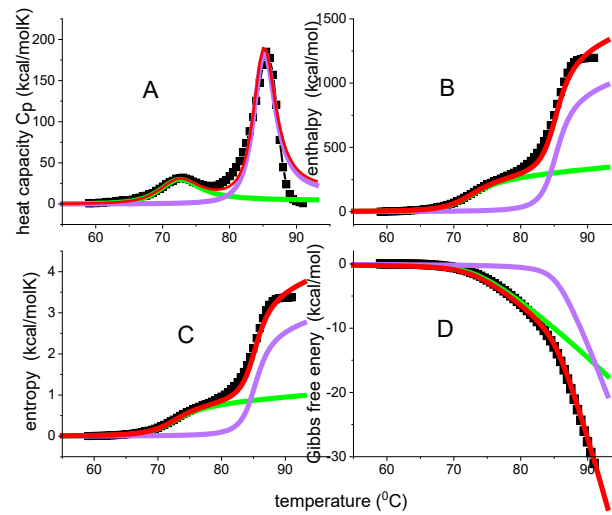


Figure 3. Thermal unfolding of monoclonal antibody mAb at pH 6.2. (■) DSC experiment. Solid lines are simulations with the Zimm-Bragg theory: green= pre-transition; violet= main transition; red= sum of pre- and main transition. (A) Molar heat capacity. (B) Unfolding enthalpy. (C) Unfolding entropy. (D) Free energy.  $h_0 = 1.1$  kcal/mol,  $c_v = 7.0$  cal/molK. Pre-transition:  $T_0 = 73$  °C,  $v_{pre} = 263$ ,  $\sigma = 5 \times 10^{-5}$ . Main transition:  $T_0 = 85.4$  °C,  $v_{main} = 880$ ,  $\sigma = 2 \times 10^{-5}$ . Data taken from reference.[13]

The DSC experiment (Figure 3A) reveals a pre-transition at 73 °C and a main transition at 85 °C. The unfolding enthalpy of the pre-transition is  $\Delta H_{cal} = 290$  kcal/mol involving  $v_{pre} = \Delta H_{cal}/h_0 = 263$  amino acid residues. The main transition has an enthalpy of  $\Delta H_{cal} \simeq 1000$  kcal/mol with  $v_{main} \simeq 880$  amino acid residues. Taken together, pre- and main transition account for ~90% of all amino acid residues. A molecular interpretation of pre- and main transition based on the mAb structure has been given.[13] The pre-transition results from the unfolding of 2  $C_{H2}$  domains, whereas the main transition represents the unfolding of 8 domains of the Fab fragment and 2 domains of the Fc fragment. In Figure 3 the pre-transition (green) and main transition (violet) are superimpose (red). The pre-transition is slightly less cooperative with  $\sigma = 5 \times 10^{-5}$  than the main

transition with  $\sigma = 2 \times 10^{-5}$ . The same molecular parameters  $h_0 = 1.1$  kcal/mol and  $c_v = 7.0$  cal/molK were used in all simulations. The theory predicts the heat capacity increase upon unfolding as  $\Delta C_p^0 = 6.34$  kcal/molK for the pre-transition and  $\Delta C_p^0 = 17.19$  kcal/mol-K for the main transition, consistent with the number of amino acids involved. The DSC-measured temperature profile of the free energy follows the same pattern as observed for lysozyme and gpW62. The free energy of the native mAb is zero up to about 65 °C followed by a biphasic decrease. As shown in Figure 3D the contributions of the pre-transition (green) and the main transition (violet) are well separated. The multistate partition function  $Z(T)$  precisely predicts this biphasic behavior. The free energy per residue is  $g_{cal} = -30 \pm 1$  cal/mol for both pre- and main transition and thus clearly smaller than those of lysozyme (-47 cal/mol) and gpW62 (-136 cal/mol). Neither the pre-transition nor the main transition can be simulated with the 2-state model.

### *Heat and cold denaturation of metmyoglobin*

A protein that is stable at ambient temperature can be unfolded by heating or, less common, by cooling. The original Zimm-Bragg theory was modified here to include cold denaturation. Cold denaturation of most proteins occurs at subzero temperatures. Rather drastic conditions are needed to shift cold denaturation above 0 °C (e.g. addition of denaturants, low or high pH). DSC experiments reporting cold denaturation or at least partial cold denaturation are available for metmyoglobin[16], staphylococcus nuclease[37],  $\beta$ -lactoglobulin[38], streptomyces subtilisin inhibitor[39] or thioredoxin.[40]

Metmyoglobin (153 residues) consists of 8  $\alpha$ -helical regions connected by loops.[41] DSC at pH 4.1 (Figure 4A ) displays heat denaturation at  $T_0 = 69$  °C and cold denaturation starting at 3 °C (data from Figure 13, reference [16]). Heat denaturation of the partially destabilised protein

is characterized by  $\Delta H_{\text{cal}} = 146$  kcal/mol,  $\Delta S_{\text{cal}} = 0.431$  kcal/mol and  $\Delta G_{\text{cal}} = -6.4$  kcal/mol. The ratio  $\Delta H_{\text{cal}}/\Delta S_{\text{cal}} = 339$  K = 66 °C is consistent with the  $C_p(T)$  maximum. Cold denaturation is predicted at  $T_{\text{cold}} = -4.5$  °C and at -3°C by the 2-state model. The Zimm-Bragg theory (fit parameters in table 1) provides a clearly better fit to  $C_p(T)$  than the 2-state model (fit parameters  $\Delta H_0 = 112$  kcal/mol,  $\Delta C_p^0 = 2.9$  kcal/molK).

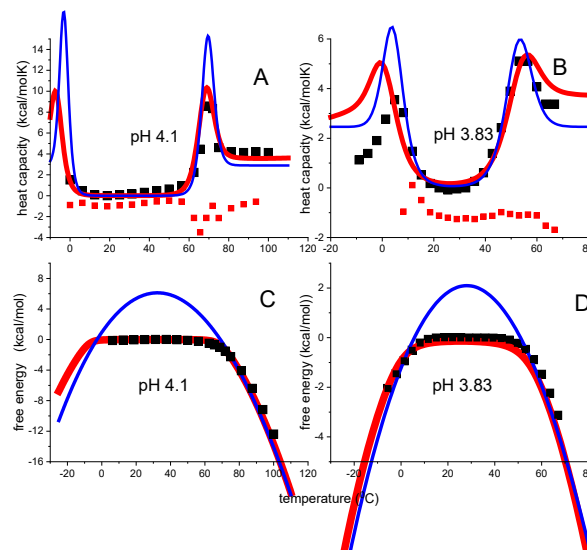


Figure 4. Unfolding of metmyoglobin at acid pH. (■) Experimental data taken from reference[16]. Red lines: simulations with the cooperative Zimm-Bragg theory (fit parameters listed in table 1). (■) Differences between DSC data and the Zimm-Bragg simulation (shifted in 4B by -1 kcal/molK for better visibility). Blue lines: 2-state model. (A)  $C_p(T)$  at pH 4.1 (Figure 13 in reference[16]). (B)  $C_p(T)$  at pH 3.83 (Figure 12 in reference[16]). (C, D) Free energies calculated using the heat capacity data shown in panels 4A and 4B.

At pH 3.83 metmyoglobin is even more destabilized and DSC reports two unfolding transitions with  $C_p(T)$ -maxima at  $T_{\text{cold}} = 8$  °C and  $T_0 = 56.5$  °C (Figure 4B). The enthalpy of heat

unfolding is  $\Delta H_{\text{cal}} = 96$  kcal/mol and the entropy  $S_{\text{cal}} = 0.291$ . The ratio  $\Delta H_{\text{cal}}/\Delta S_{\text{cal}} = 329.9$  K = 56.9 °C agrees with  $T_0$ . Cold denaturation is not the mirror image of heat denaturation as unfolding enthalpy and entropy are much smaller with  $\Delta H_{\text{cold}} \sim 53.8$  kcal/mol and  $\Delta S_{\text{cold}} \sim 0.193$  kcal/molK, respectively. The ratio  $\Delta H_{\text{cold}}/\Delta S_{\text{cold}} = 274$ K = 1°C is different from  $T_{\text{cold}} = 8$  °C. The low-temperature transition could be distorted in the DSC experiment. Again the Zimm-Bragg theory (fit parameters table 1) provides a better simulation than the 2-state model (fit parameters  $\Delta H_0 = 58$  kcal/mol,  $\Delta C_p^0 = 2.46$  kcal/molK). . The free energy profile is displayed in Figure 4D. DSC yields a zero free energy for the native protein and negative free energies for heat and cold denaturation. This trapezoidal temperature profile is correctly predicted by the Zimm-Bragg theory. The specifics in the simulation of cold denaturation are a large heat capacity  $c_v$  and a small unfolding enthalpy  $h_0$  (cf. Table 1). In contrast, the 2-state model predicts a parabolic profile and assigns a large positive free energy to the native molecule.

Metmyoglobin at pH 3.83 was analyzed previously by a hierarchical algorithm defining a partition function in terms of multiple levels of interacting folding units.[42] The model reproduces an idealized, symmetrical shape of the heat capacity  $C_p(T)$  and a parabolic free energy function.

### Table 1

Table 1 summarizes the DSC results and the Zimm-Bragg fit parameters of all proteins discussed. The table includes additional measurements of metmyoglobin at pH 10 and 12 and of ubiquitin[17] for which no simulations are shown.



## Discussion

Proteins in solution do not show a simple, 2-state equilibrium between a fully folded and a fully unfolded conformation. Depending on temperature, they form a complex mixture of many short-lived intermediates. Here we present a new model, which predicts the important thermodynamic functions enthalpy, entropy and free energy on the basis of molecular parameters only. The performance of the model is demonstrated by comparison with DSC experiments. The Zimm-Bragg theory provides excellent simulations of the temperature course of all thermodynamic functions reported by DSC. With this model we obtain insights into the cooperativity and dynamics of protein folding.

### *Protein stability*

The most relevant parameter of protein stability is the midpoint temperature of unfolding  $T_0$ . Protein unfolding can be approximated by a first-order phase transition, and  $T_0$  is determined by  $T_0 = \Delta H_{\text{cal}} / \Delta S_{\text{cal}}$ .  $\Delta H_{\text{cal}}$  and  $\Delta S_{\text{cal}}$  are the DSC-measured unfolding enthalpy and entropy, respectively. Minor changes in  $\Delta H_{\text{cal}}$  or  $\Delta S_{\text{cal}}$  produce distinctive shifts in  $T_0$ . The ultrafast folder gpW62 (62 aa) and ubiquitin (74aa) are short proteins with almost identical unfolding enthalpies of 91.7 kcal/mol and 89.7 kcal/mol, respectively. Nevertheless, their transition temperatures are  $\sim 20$  °C apart with gpW62 at 67 °C and ubiquitin at 89.5 °C. The difference is caused by the larger gpW62 entropy  $\Delta S_{\text{cal}} = 0.269$  kcal/molK compared to  $\Delta S_{\text{cal}} = 0.25$  kcal/molK of ubiquitin. The difference becomes even more obvious on a per residue basis with  $s_{\text{cal}} = \Delta S_{\text{cal}} / v = 4.34$  kcal/mol for gpW62 and  $s_{\text{cal}} = 3.41$  kcal/mol for ubiquitin. Likewise, very small differences in enthalpy and entropy cause the 12 °C difference in the midpoint temperatures of the two mAb domains. *A priori* predictions of  $T_0$  therefore require highly precise MD calculations of unfolding enthalpy and entropy.

In a true first-order phase transition (e.g. melting of ice) the heat  $\Delta H_{\text{cal}}$  is absorbed at the constant temperature  $T_0$  and the heat capacity is a sharp peak (singularity). In contrast,  $\Delta H_{\text{cal}}$  in protein unfolding is absorbed over 20-60°C and the heat capacity  $C_p(T)$  is a broad peak. In particular, and not generally recognized, the relation  $\Delta H_{\text{cal}} = T_0\Delta S_{\text{cal}}$  is limited to the overall reaction, but not applicable to the measured heat  $H(T_0)$  and entropy  $S(T_0)$ . Considering lysozyme as an example, the DSC-measured heat absorbed up to  $T_0$  is  $H(T_0) = 63.4\text{kcal/mol}$  (eq. 10) and is less than half of the total heat of 147.2 kcal/mol). The corresponding entropy is  $S(T_0) = 0.191\text{ kcal/molK}$  (eq. 11). As  $H(T_0) < T_0S(T_0)$  this results in a negative free energy  $G(T_0) = -0.677\text{ kcal/mol}$  (eq. 12), more realistic for a~50% unfolded protein than the zero free energy predicted by the 2-state model. Analogous results are obtained for all proteins discussed here.

The two hallmarks of the 2-state model, that is, the positive free energy of the native protein and the zero free energy of the 1:1 folded/unfolded mixture, are thus not confirmed by DSC. Instead, the free energy shows a trapezoidal shape that is precisely predicted by the new Zimm-Bragg folding model

#### *Molecular unfolding enthalpy $h(T)$*

The Zimm-Bragg parameter  $h_0$  is an average value of all types of interactions, independent of specific conformations ( $\alpha$ -helix,  $\beta$ -sheet, ionic interactions, etc.).  $h_0$  is close to the calorimetric average  $h_{\text{cal}} = \Delta H_{\text{cal}}/v$ . Metmyoglobin is an  $\alpha$ -helical protein and the average enthalpy of the native protein (pH 10) is  $h_{\text{cal}} = 178/153 = 1.16\text{ kcal/mol}$  while  $h_0 = 1.08\text{ kcal/mol}$  (Figure 3 of ref[16], parameters listed in table 1, simulation not shown). For the ~50%  $\alpha$ -helical apolipoprotein A1 the DSC result is  $h_{\text{cal}} = 1.08\pm 0.07\text{ kcal/mol}$  and the Zimm-Bragg parameter  $h_0 = 1.1\text{ kcal/mol}$ . [9] Lysozyme, a globular protein with mainly  $\beta$ -sheet structure, yields  $h_{\text{cal}} = 1.14\text{kcal/mol}$  and  $h_0 =$

0.99 kcal/mol (pH 2.5).[10] A comparison of a larger set of proteins has led to the conclusion that the Zimm-Bragg parameter is  $h_0 = 1.1 \pm 0.2$  kcal/mol.[10]

The enthalpy  $h_0$  is usually associated with the opening of an  $\alpha$ -helix hydrogen bond.[20, 22-24] However, MD calculations have led to the conclusion that “hydrogen bond formation contributes little to helix stability [...] The major driving force for helix is associated with interactions, including van der Waals interactions, in the close packed helix conformation and the hydrophobic effect”.[43] This is supported by experimental results obtained with short alanine-based peptides, where hydrophobic interactions play the dominant role in stabilizing isolated  $\alpha$ -helices.[25]

### *Cold denaturation*

Cold denaturation has been proposed as a tool to measure protein stability.[44] The heat capacity  $c_v$  is a new parameter in the Zimm-Bragg theory leading to a second unfolding transition at low temperature. The temperature difference between heat and cold denaturation is given by  $\Delta T = h_0/c_v$  (eq. (5)). Cold denaturation near ambient temperature thus requires a small  $h_0$  and a large  $c_v$ . This is confirmed by metmyoglobin at pH 4.1 and 3.83, where  $h_0$  is distinctly reduced to  $h_0 = \sim 0.6$  kcal/mol where  $c_v = 10-15$  cal/molK, that is, twice as large as that of the native protein (cf. table 1). Proteins with  $h_0/c_v \geq 100$  K display cold denaturation at very low sub-zero temperatures.

### *Molecular unfolding entropy.*

Molecular dynamics (MD) calculations consider all possible degrees of dihedral freedom of each amino acid residue in sampling the conformational space. In contrast, the Zimm-Bragg theory is an algorithm that differentiates only between folded and non-folded amino acid residues, independent of the specific conformation. However, the theory makes predictions, solidly based on experimental data, which can be compared to MD calculations. The temperature course of the

entropy is a good example. The entropy  $S(T)$  can be calculated with the Zimm-Bragg theory according to eq.(8). The excellent agreement with the experimental data is displayed in Figures 1-3. In Figure 5 we have repeated these calculations, including also ubiquitin, for the much larger temperature range of 298K-498K as this is the temperature interval of the “Dynameomics Entropy Dictionary”. [18]

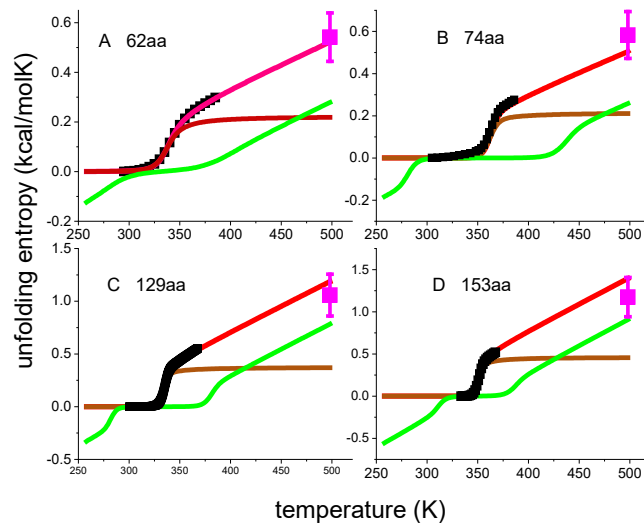


Figure 5. Unfolding entropy. (■) Experimental data.. (■) Dynameomics Entropy Dictionary [18]. (A) gpW62. [15] .(B) Ubiquitin. [17] (Figure 1 in. [17]) (C) Lysozyme [10] (D) Metmyoglobin. [16] (Figure 3 in ref. [16], pH 10). Red lines: Zimm-Bragg total entropy. Brown lines: conformational entropy proper. Green lines: contribution of the heat capacity term  $c_v$ , Zimm-Bragg parameters listed in table 1.

By averaging some 800 MD calculations the Dynameomics Entropy Dictionary provides the unfolding entropies of all amino acids when heated from the native state (298K) to the fully denatured state (498K). Using table 2 in reference [18], we calculated the MD unfolding entropies at 498K for the specific amino acid sequences of the 4 proteins in Figure 5. The results are shown in Figure 5 by the magenta data points at 498K with the error bars also taken from reference. [18]

In parallel, the Zimm-Bragg simulations were extended to 498K with the same parameters as deduced from  $C_p(T)$  at low temperature. This extrapolation is in excellent agreement with the Dymameomics Entropy Dictionary and supports the relevance of the molecular parameters of the Zimm-Bragg theory in protein unfolding.

The entropy  $S(T)$ (red) can be divided into the conformational entropy proper (brown line,  $h_0 = \text{const.}$ ,  $c_v = 0$ ) and the contribution of the heat capacity term (green,  $h_0 = 0$ ,  $c_v = \text{const.}$ ).  $h_0$  determines  $S(T)$  up to the end of the conformational transition where  $\Theta_U \sim 0.85-0.9$ . However, it takes another temperature increase of more than 100 °C to reach complete denaturation.

The average entropy is  $s_{\text{cal}} = \Delta S_{\text{cal}}/v = 3.25 \pm 0.25$  cal/molK per residue (table 1) and involves at least 3 single bonds. The entropy per single bond is  $\sim 1.1$  cal/molK and can be compared with other phase transitions. The solid-fluid transition of long chain paraffins yields a much larger entropy of 1.8-2.7 cal/molK per C-C bond. The gel-to liquid crystal transition of phospholipid bilayers leads 1.25 - 1.6 cal/molK per C-C bond (table 2.7, p. 47 in reference[45]). As judged by the small entropy of 1.1 cal/molK the unfolded proteins are still characterized by a restricted motion of their molecular constituents. For metmyoglobin it was noted that “the unfolded state retains some residual ellipticity, which may be caused by the fluctuating  $\alpha$ -helical conformation of the unfolded polypeptide chain”. [16] Likewise, the combination of FRET, NMR and SAX techniques has revealed residual structure in denatured ubiquitin. [46]

### *Protein cooperativity*

The folding/unfolding transition of proteins is a cooperative process. The Zimm-Bragg theory provides a quantitated measure of cooperativity. In fact, the cooperativity parameter  $\sigma$  plays an important role in the energy and kinetics of the folding process as it determines the free energy to start a new fold within an unfolded region (nucleation). [47] The corresponding free energy is

$$\Delta G_{\sigma} = -RT_0 \ln(\sigma) \quad (14)$$

A large  $\sigma$  corresponds to low cooperativity and to a small nucleation energy  $\Delta G_{\sigma}$ . gpW62 has a cooperativity parameter  $\sigma = 5 \times 10^{-4}$  and hence a nucleation energy of  $\Delta G_{\sigma} = 5.13$  kcal/mol. In contrast, lysozyme unfolding is highly cooperative with  $\sigma = 1 \times 10^{-6}$  and  $\Delta G_{\sigma} = 9.21$  kcal/mol. The low gpW62 nucleation energy makes gpW62 folding easier than that of lysozyme. If  $\Delta G_{\sigma}$  is assumed to be correlated with the kinetic activation energy, then gpW62 folding should be  $\sim 500$  times faster than that of lysozyme. In infra-red T-jump experiments of gpW62 the return to equilibrium followed a single exponential with a relaxation time of  $\tau = 15.7$   $\mu$ s at 57 °C.[15] In contrast, lysozyme was found to fold in a two-step mechanism with a slow nucleation ( $\tau = 14$  ms) followed by a fast growth step ( $\tau = 300$   $\mu$ s) at room temperature.[48]

### *Free energy of unfolding*

The free energy of unfolding  $\Delta G_{\text{cal}}$  scales with the size of the protein and a per residue free energy  $g_{\text{cal}} = \Delta G_{\text{cal}}/v$  is better suited for comparative purposes. According to the Zimm-Bragg theory  $g_{\text{cal}}$  can be approximated by

$$g_{\text{cal}} \simeq h(T_{\text{end}})(1 - T_{\text{end}}/T_0) = -(h_0 + c_v \Delta T)(\Delta T/T_0). \quad (15)$$

$T_{\text{end}}$  denotes the temperature at the end of the conformational transition and  $\Delta T = T_{\text{end}} - T_0$  is the half-width of the transition. The approximation eq. (15) agrees within 2% with the DSC measurement for the proteins listed in table 1. The free energy per amino acid residue varies between  $g_{\text{cal}} = -131$  cal/mol for gpW62 to  $g_{\text{cal}} = -33$  cal/mol for mAb. In parallel, the width of the transition decreases from 65 °C for gpW62 to 20 °C for mAb (large domain). An approximately linear relationship between  $g_{\text{cal}}$  and  $\Delta T$  is predicted by eq. (15) and is confirmed by DSC, that is, a

large  $g_{cal}$  correlates with a broad transition. Considering the three parameters  $T_0$ ,  $g_{cal}$ , and  $\Delta T$ , gpW62 is the least stable protein discussed here.

## Conclusion

The protein folding/unfolding transition is a highly cooperative process which cannot be adequately simulated by the non-cooperative 2-state model. A multistate cooperative model is provided by the Zimm-Bragg theory. Here we combined the partition function of the Zimm-Bragg theory with statistical-mechanical thermodynamics. The model predicts the DSC-measured enthalpy, entropy, and free energy of protein unfolding with molecular parameters, which have well-defined physical meanings. We analyzed the DSC thermograms of proteins of different chain length and structure. We show that the temperature profile of the free energy is characterized by a trapezoidal shape. The new model is in excellent agreement with this experimental finding. In contrast to the 2-state model that postulates a parabolic shape. The present model reveals whether a protein is a fast or a slow folder and predicts heat as well as cold denaturation. The results of the new model can be compared to molecular dynamics calculations. Using the molecular parameters derived from DSC the entropy at complete unfolding at 498K was calculated for four different proteins. The results are in excellent agreement with the predictions of the Dynameomics Entropy Dictionary. A detailed theoretical discussion of the free energy in thermal and chemical protein unfolding is available.[49]

**Table 1**

*Thermal unfolding. Differential scanning calorimetry and parameters of the Zimm-Bragg theory*

Protein		DSC	DSC	DSC	DSC	DSC	ZB	Zimm-Bragg theory	ZB	ZB
	$v$	$\Delta H_{cal}$	$h_{cal}=H_{cal}/v$	$S_{cal}=S_{cal}/v$	$g_{cal}=G_{cal}/v$	$\Delta C_p^{0,i)}$	$H_{con}^{k)}$	$h_0$	$c_v$	$\sigma$
<i>native protein → unfolded protein</i>		kcal/mol	kcal/mol	cal/molK	kcal/mol	kcal/molK	kcal/mol	kcal/mol	cal/molK	
gpW62 <sup>a)</sup>	62	91.7	1.48	4.34	-0.131	0.9	66.2	1.26	5	$5 \times 10^{-4}$
ubiquitin <sup>b)</sup>	74	89.7	1.21	3.41	-0.067	1.04	68.1	1.06	4.3	$1 \times 10^{-6}$
lysozyme <sup>c)</sup>	129	147.2	1.14	3.39	-0.047	2.28	113.3	0.99	6	$1 \times 10^{-6}$
metmyoglobin <sup>d)</sup>	153	178.1	1.16	3.32	-0.039	2.89	142	1.08	7	$7 \times 10^{-6}$
mAb pre <sup>e)</sup>	263	290.6	1.10	3.11	-0.031	4.7	244	1.1	7	$5 \times 10^{-5}$
mAb main <sup>e)</sup>	880	1020	1.16	3.23	-0.033	17	900	1.1	7	$2 \times 10^{-5}$
<i>destabilized protein → unfolded protein</i>										
metmyoglobin pH 12 <sup>f)</sup>	145	144.7	1.00	3.04	-0.048	2.54	93.7	0.75	7	$1 \times 10^{-5}$
metmyoglobin pH 4.1 <sup>g)</sup>	145	141.0	0.97	2.90	-0.044	3.58	70.5	0.6	10	$2 \times 10^{-6}$
metmyoglobin pH 3.84 <sup>h)</sup>	100	96.0	0.96	2.91	-0.031	3.74	43.8	0.6	15	$7 \times 10^{-5}$
			Average 1.13					Average = 1.05		
			STDV 0.16					STDV = 0.21		



- a) reference [15], fig. 4A (this work: figs. 2 & 5 A); b) reference[17], Figure 1 (this work: Figure 5B)
- c)reference[10], Figure8 (this work: figs. 1 & 5C); d) reference[16], Figure3b, pH 10.7 (this work: Figure 5D);
- e) reference[13] (this work: Figure 3); f) reference[16], Figure 3b (no simulation shown); g) reference[16] Figure 13 (this work: figs. 4A&4C); h) reference[16] figure12 (this work: figs. 4B&4D); i). DSC-measured increase in molar heat capacity upon protein unfolding;
- k) conformational unfolding enthalpy proper, calculated with the Zimm-Bragg theory by setting  $c_v = 0$ .

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