Chemical protein unfolding - A simple cooperative model

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Abstract

Chemical unfolding with guanidineHCl or urea is a common method to study the conformational stability of proteins. The analysis of unfolding isotherms is usually performed with an empirical linear extrapolation method (LEM). The method contradicts however, common expectations. A large positive free energy is assigned to the native protein which is usually considered to be a minimum of the free energy. In addition, the free energy varies linearly, not the quickly with the concentration. Here we present a multistate cooperative model, which addresses specifically the binding of the denaturant to the protein and the cooperativity of the protein unfolding equilibrium. The model is based on a molecular statistical-mechanical partition function of the ensemble but simple solutions for the calculation of the binding isotherm and the associated free energy are presented. The model fits unfolding isotherms of small and large proteins equally well. For a given denaturant, the binding constant depends on temperature and pH, but shows little protein specificity. Chemical unfolding is less cooperative than thermal unfolding. The cooperativity parameter σ is two orders of magnitude larger than that of thermal unfolding. The multistate cooperative model predicts a zero free energy for the native protein, which becomes strongly negative beyond the midpoint concentration of unfolding. The free energy to unfold a cooperative unit corresponds exactly to the diffusive energy of the denaturant concentration gradient necessary for unfolding. The temperature dependence of unfolding isotherms yields the denaturant-induced unfolding entropy and, in turn, the unfolding enthalpy. The multistate cooperative model provides molecular insight, is as simple to apply as the LEM, but avoids the conceptual difficulties of the latter.

Introduction

Chemical denaturation is a process by which the protein conformation is unfolded via addition of denaturants such as guanidineHCl or urea. Chemical denaturation is a common method for determining a protein's conformational stability, relative to its functional properties.[1] Identifying the conditions that maximize the structural stability of a protein is crucial during development of biologics for therapeutic treatments. Several complementary techniques should be applied to provide a systematic analysis of protein stability.[2]

Chemical unfolding of proteins is analysed almost exclusively with a 2-state model.[3, 4] An empirical linear extrapolation method (LEM) is applied. The LEM assigns a large positive Gibbs free energy $\Delta G_0^{H_2O}$ to the native protein, and predicts a linear dependence of the free energy on the concentration of the denaturant. The significance of $\Delta G_0^{H_2O}$ is unclear, because the standard unfolding models assume a free minimum (not a maximum) of the free energy.[5, 6]

The 2-state model is a non-cooperative model. It has no energy parameter for the interaction between neighboring amino acid residues. All amino acid residues unfold simultaneously (all-or-none model).[7] However, the conformational change of all amino acid residues at the same time is physically unrealistic. Instead, "peptides that form helices in solution do not show a simple two-state equilibrium between a fully folded and fully unfolded structure. Instead they form a complex mixture of all helix, all coil or, most frequently central helices with frayed coil ends".[8] A sequential cooperative unfolding of protein domains is therefore physically more realistic.

We have recently proposed a semi-empirical cooperative model that assumes a binding constant K_D for the binding of the denaturant and a cooperativity parameter σ for the cooperative unfolding of the protein.[9] Here we provide a modification of this model, based on a statistical-mechanical partition function leading to simple expressions for the chemical unfolding isotherm and the associated free energy.

Published chemical unfolding isotherms obtained with spectroscopic techniques and with calorimetry are analyzed. GuanidineHCl, urea and sodium dodecyl sulfate (SDS) were used as denaturants. The protein size ranged from 30 to ~1600 amino acid residues, including a monoclonal antibody. The present model yields excellent simulations of all unfolding isotherms. The native protein is the reference state with zero free energy. The free energy becomes negative upon unfolding and decreases with the logarithm of the denaturant concentration. The temperature dependence of the free energy provides the unfolding entropy and, in turn, the unfolding enthalpy. The latter agrees with calorimetric measurements. The binding constants K_D is defined essentially by the type of denaturant and varies little with the nature of the protein. The cooperativity parameter for chemical unfolding is about two orders of magnitude larger than that for thermal unfolding.[10] The multistate cooperative model is firmly grounded on statistical mechanical thermodynamics. With the *multistate cooperative approximation,* we provide a simple expression for chemical denaturation analysis which is easy to apply.

Materials and Methods

Chemical unfolding experiments with guanidine HCl, urea, and sodium dodecyl sulfate SDS), performed with different spectroscopic and calorimetric techniques, are selected from the available literature. The focus is on the chemical unfolding of lysozyme with guanidineHCl, urea, and SDS. Altogether 25 published chemical denaturation isotherms of proteins of different structure and size have been investigated.

Chemical unfolding models

Chemical denaturants such as guanidineHCl or urea are commonly used to study protein stability. They change the polarity of the environment, bind to backbone and amino acid residues and thus change the native protein conformation (N) into the unfolded conformation

(U). Different molecular mechanism of denaturation have been proposed.[4, 11] An indirect mechanism postulates changes in the water structure and of the hydrophobic effect. The alternative view is the direct interaction of the chemical denaturant with the protein. Strong support for the latter mechanism comes from isothermal titration calorimetry (ITC) of guanidineHCl and urea with various proteins.[12] X-ray studies also demonstrate a close interaction between guanidineHCl and the protein backbone of lysozyme.[13]

Multistate cooperative unfolding model

The multistate cooperative model considers the individual amino acid residues of the protein. The amino acid residues in the native or in the unstructured confirmation are designated as "n" and "u". The initial step of this model is the binding of a denaturant D to an amino acid "n" in a structured protein segment, inducing a conformational transition to an unstructured state "u":

$$n + D \rightleftarrows u \qquad (1)$$

This chemical equilibrium is described by a simple equation:

$$K_D(T) = \frac{c_u}{c_n c_D} \qquad (2)$$

The concentrations c_n and c_u are the concentrations of the amino acid residues participating in unfolding. The binding constant $K_D(T)$ is a function of temperature only. Unfolding is a dynamic equilibrium between many different protein conformations.

The statistical interpretation of a chemical equilibrium requires a grand canonical partition function. To make the connection to the textbook literature we consider a system of only two types of particles. "In a grand canonical ensemble, the number of type A particles, N_A, and type B particles, N_B, are both variable. Let μ_A and μ_B be the respective chemical potentials of the two components. The grand partition function is

$$\mathbb{Z}(\mu_A, \mu_B, V, T) = \sum_{N_A, N_B, i} e^{\beta\left(N_A\mu_A + N_B\mu_B - E_{N_A N_B I}\right)} \tag{3}$$

(reference[14], chapter 13.9). Equation 2 defines a 3 component system measuring the ensemble size in concentration units c_i (mol/L). The chemical potential is given by

$$\sum_i \mu_i = \sum_i (\mu_i^0 + RT \ln c_i) = \Delta G(c_D) \tag{4}$$

$$\Delta G(c_D) = -RT(\ln K_D + \ln c_D + \ln c_u - \ln c_n) \tag{5}$$

In chemical unfolding experiments the protein concentration is typically ~ 10 μM, whereas the concentration of the denaturant is 1 - 8 M. The chemical potential of the equilibrium is dominated by the denaturant concentration, leading to the following approximation.

$$\Delta G(c_D) = \sum_i \mu_i \cong -RT \ln(K_D c_D) \tag{6}$$

The conditional probability of a residue "n" in a structured protein segment is defined as 1, the conditional probability of the unfolded residue "u" is

$$q(c_D) = e^{-\Delta G/RT} = K_D c_D \tag{7}$$

The Boltzmann factor $q(c_D)$ is inserted into the Zimm-Bragg partition function $Z(c_D)$[14].

$$Z(c_D) = \begin{pmatrix} 1 & 0 \end{pmatrix} \begin{pmatrix} 1 & \sigma q(c_D) \\ 1 & q(c_D) \end{pmatrix}^{\nu} \begin{pmatrix} 1 \\ 1 \end{pmatrix} \approx \begin{pmatrix} 1 & 0 \end{pmatrix} \begin{pmatrix} 1 & \sigma K_D c_D \\ 1 & K_D c_D \end{pmatrix}^{\nu} \begin{pmatrix} 1 \\ 1 \end{pmatrix} \tag{8}$$

ν denotes the number of amino acid residues participating in the unfolding reaction. The cooperativity parameter σ determines the steepness of the unfolding transition. A small σ leads to a sharp transition. The cooperativity parameter in chemical unfolding is typically $\sigma \sim 10^{-2} - 10^{-3}$ and is 10 to hundred times larger than that of thermal unfolding. The fraction of unfolded protein is:

$$\Theta_U(c_D) = \frac{q(c_D)}{\nu} \frac{d(\ln Z(c_D))}{dc_D} \left(\frac{dq(c_D)}{dc_D} \right)^{-1} \tag{9}$$

For a non-cooperative ensemble with σ = 1 the partition function eq. 8 reduces to $Z(q) = (1+q)^{\nu}$. The fraction of unfolded protein becomes independent of ν and is $\Theta_U(q) = \frac{\partial \ln Z(q)}{\partial \ln q} = \frac{q}{1+q}$, which is identical to the Langmuir adsorption isotherm.

Figure 1 shows the unfolding isotherm $\Theta_U(c_D)$ (eq. 9) for different cooperativity parameters σ. The binding constant is $K_D = 0.25\ M^{-1}$, which is typical for guanidineHCl binding to most proteins. The binding constant is too small to induce complete protein unfolding for a non-cooperative ensemble (σ = 1) as demonstrated by the Langmuir isotherm in figure 1A. A dramatic change in the binding isotherm is induced by including even small cooperative interactions (red to green lines in fig. 1A).

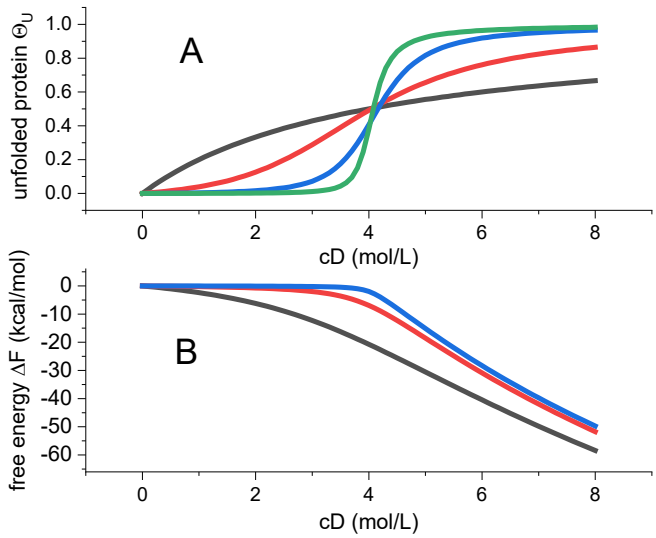

Figure 1. Multistate cooperative unfolding model. Variation of the cooperativity parameter σ. (A) Extent of unfolding $\Theta_U(c_D)$ (eq. 9). Black: σ = 1. Red: σ = 10^{-1}. Blue: σ = 10^{-2}. Green: σ = 10^{-3} (B) Free energy as a function of cooperativity parameter σ and denaturant concentration (eq. 10). Binding constant K_D = 0.25. M^{-1}. Midpoint concentration c_0 = 4.0 M. Number of amino acid residues participating in transition ν =129. Temperature T=298K. The simulation parameters K_D, c_0, ν, T and σ = 10^{-3} correspond to the unfolding of lysozyme in guanidine HCl solution.

The extent of unfolding (eq. 9) is the result of the partition function $Z(c_D)$. Likewise, the free energy is also related to the partition function according to standard statistical thermodynamics.[15] The free energy change of unfolding is

$$\Delta F(c_D) = -RT_0 \ln\left(Z\left(c_D\right)\right) \tag{10}$$

$\Delta F(c_D)$ upon addition of guanidineHCl is displayed in figure 1B. The native protein is the reference state with zero free energy. Upon addition of denaturant the free energy decreases. In the case of no cooperativity ($\sigma = 1$), the free energy decreases already at low concentrations of denaturant. For a cooperative ensemble, the free energy remains close to zero up to the midpoint of unfolding c_0 and decreases rapidly beyond this concentration. Compared to non-cooperative denaturation the free energy change for a co-operative system is distinctly smaller.

The temperature dependence of the free energy is

$$\frac{\partial \Delta F}{\partial T} = -\Delta S \tag{11}$$

The unfolding enthalpy can then be calculated as

$$\Delta H = \Delta F + T\Delta S \tag{12}$$

A simple multistate cooperative approximation

For the biochemical practitioner the above formalism may act as a deterrent. Fortunately, the matrix equation 7 can be replaced by a simple linear expression, which can easily be calculated. The cooperativity parameter in chemical unfolding experiments is always $\sigma \geq 10^{-3}$ and the largest eigenvalue λ_0 of the above matrix is a sufficient approximation, resulting in a simpler partition function [14]

$$Z_\lambda(c_D) = \left(\lambda_0(c_D)\right)^\nu \tag{13}$$

with

$$\lambda_0(c_D) = \frac{1 + K_D c_D + \left[\left(1 - K_D c_D\right)^2 + 4\sigma K_D c_D\right]^{\frac{1}{2}}}{2} \tag{14}$$

The fraction of unfolded protein is given by

$$\Theta_{U\lambda}\left(c_D\right) \cong \frac{1}{2}\left(1 + \frac{K_D c_D - 1 + 2\sigma}{\left[\left(1 - K_D c_D\right)^2 + 4\sigma K_D c_D\right]^{\frac{1}{2}}}\right) \tag{15}$$

At the midpoint of unfolding with $c_D = c_0$ and $\Theta(c_0) = 1/2$ follows

$$K_D c_0 = 1 \qquad \text{or} \qquad K_D = 1/c_0 \tag{16}$$

In the multistate cooperative model, the binding constant of the denaturant is simply the reciprocal of the midpoint concentration of unfolding. Equation 15 can also be written as

$$\Theta_{U\lambda}\left(c_D\right) \cong \frac{1}{2}\left(1 + \frac{c_D / c_0 - 1 + 2\sigma}{\left[\left(1 - c_D / c_0\right)^2 + 4\sigma c_D / c_0\right]^{\frac{1}{2}}}\right) \tag{17}$$

Equations 15 and 17 are equivalent to a cooperative sorption isotherm, which is based on a statistical-mechanical partition function. They are In line with many other sorption isotherms.[16]

The only fit parameter in the simulation of chemical unfolding isotherms is therefore the cooperativity parameter σ.

The free energy of the system is

$$\Delta F_\lambda(c_D) = -\nu R T_0 \ln \lambda_0(c_D) \tag{18}$$

For denaturant concentrations $c_D \gg c_0$, the following approximation is valid

$$\Delta F(c_D) \approx -\nu R T_0 \ln(K_D c_D) \qquad \text{for} \qquad c_D \gg c_0 \tag{19}$$

Chemical equilibrium two-state unfolding

The common model to describe chemical unfolding isotherms is a non-cooperative two-state model, which has dominated the field for the last 30 - 40 years. Only two types of protein conformation are assumed in solution, the native protein (N) and the unfolded protein (U). No intermediate structures and no specific interaction between denaturant D and protein are considered. The equilibrium $N \rightleftarrows U$ is simply

$$K_{NU}(c_D) = \frac{[U]}{[N]} \tag{20}$$

The equilibrium constant $K(c_D)$ varies with the concentration of denaturant. To calculate $K_{NU}(c_D)$ the model makes an unconventional assumption about the free energy.

$$\Delta G_{NU}(c_D) = \Delta G_0^{H_2O} - mc_D \tag{21}$$

Eq. 21 is difficult to understand in thermodynamic terms for two reasons. First, a large positive free energy $\Delta G_0^{H_2O}$ is assigned to the stable native protein. Second, the free energy is a *linear*, not as usual, a logarithmic function of the concentration c_D. The so-called Linear Extrapolation Method allows the calculation of spectroscopic unfolding transitions. The fraction of unfolded protein $\Theta_U(c_D)$ is

$$\Theta_U(c_D) = K_{NU}(c_D)/(1+K_{NU}(c_D)) = e^{\frac{-\Delta G_{NU}(c_D)}{RT_0}} / (1+e^{\frac{-\Delta G_{NU}(c_D)}{RT_0}}) \tag{22}$$

The disadvantage of this model is its conceptual simplicity of just two protein conformations and the rather questionable Linear Extrapolation Method. It is generally believed that the native protein sits in a free energy minimum. In contrast, the LEM predicts a large positive free energy for a stable native protein.

By definition the midpoint concentration c_0 is the concentration where native and unfolded protein occur at equal concentrations and the free energy is consequently a zero in the LEM. It thus follows that

$$m = G_0^{H_2O} / c_0 \qquad (23)$$

m is not an independent variable, which is however often ignored in the analysis of spectroscopic data.

Results

Chemical unfolding of lysozyme with guanidineHCl at 30 °C

Figure 2 displays the chemical unfolding of lysozyme in guanidine HCl at 30 °C. The experimental data in figure 1A are taken from reference.[17] The midpoint concentration of unfolding is c_0 = 3.8 M. Chemical unfolding takes place in the concentration range of. $2.45M \le c_D \le 5.45M$

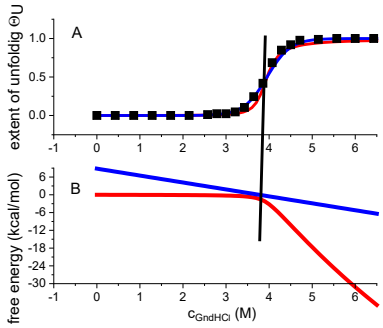

Figure 2. Unfolding of lysozyme in guanidineHCl solution at 30 °C. Red lines: multistate co-operative model. Blue lines: chemical two-state model (Linear Extrapolation Method). (A) Experimental data (■) taken from reference.[17] Midpoint concentration c_0 = 3.95 M. Simulation parameters of multistate cooperative mode: $K_D = 1/c_0 = 0.26\ M^{-1}$, $\sigma = 1x10^{-3}$ LEM parameters: $\Delta G_0^{H_2O}$ =8.837 kcal/mol, $m = \Delta G_0^{H_2O} / c_0$ =2.34 kcalL/mol^2. The vertical line denotes the midpoint of unfolding. (B) Temperature profiles of the free energy.

The solid lines in figure 2A are the simulations of the experimental data with the two models using the parameters listed in the legend to figure 2. Both methods simulate the

unfolding transitions equally well. Both models predict exactly 50% unfolding at the midpoint concentration c_0, that is, $\Theta_U(c_0) = ½$. However, the free energies at c_0 are quite different.

Figure 2B shows the variation of the free energy with the concentration of guanidine HCl. The chemical equilibrium two-state model predicts a large positive free energy $\Delta G_0^{H_2O} = 8.93$ kcal/mol to the native protein. The free energy decreases linearly with the concentration of denaturant. At the midpoint concentration c_0 the free energy is exactly $\Delta G(c_0) = 0$. .

The multistate cooperative model starts with zero free energy change ΔF of the native protein. The free energy change becomes immediately negative upon addition of guanidineHCl and is $\Delta F(c_0) = -2.0$ kcal/mol at the midpoint. At concentrations $c_D > c_0$, $\Delta F(c_D)$ decreases rapidly, approximately according to $\Delta F(c_D) \sim \ln(c_D)$ (eq. 18).

The unfolding reaction of lysozyme at 30 °C takes place between 2.9 M and 5.3 M guanidine HCl, corresponding to the fraction of unfolding $0.01 \leq \Theta_U(c_D) \leq 0.95$. The change in free energy is thus calculated as $\Delta F = -21.7$ kcal/mol for the multistate cooperative model and as $\Delta G = -11.8$ kcal/mol for the chemical two-state model. The spectroscopic unfolding isotherms can be simulated equally well with both models and allow no decision regarding the validity of the two models.

Lysozyme unfolding in urea

Urea is less common in chemical denaturation studies than guanidine HCl. The reason for this becomes obvious by inspection of figure 3. Urea has a distinctly lower affinity to proteins. The urea midpoint concentration for lysozyme is 6.7 M, compared to 4.1 M for guanidine HCl. The urea binding constant $K_D = 0.15\ M^{-1}$ is only about half of that of guanidineHCl with 0.245 M^{-1}.

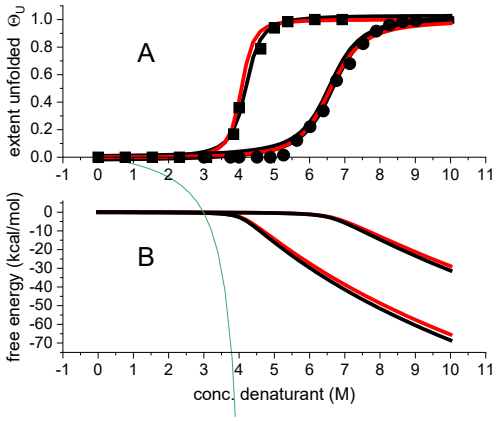

Figure 3. Chemical unfolding of lysozyme with guanidineHCL (■) and urea (●) at 25 °C and pH 7. Data taken from reference.[18] The midpoint concentrations c_0 = 4.1 M for guanidine HCl cooperativity parameter is $\sigma = 2x10^{-3}$ for both denaturants. The figure also compares the exact and approximate solutions for binding isotherm and free energy. (A) Extent of unfolding. Red line: matrix solution eq. 9. Black line: simplified isotherm eq. 17. (B) Free energy of unfolding. Red line: eq. 10. Black line: eq.18.

The denaturation with urea requires a higher concentration compared to guanidine HCl, and leads to a broader transition over a concentration range of $\Delta c \approx 5$ M (guanidine HCl $\Delta c \approx 3$ M). The cooperativity parameter is nevertheless identical for both denaturants. The broadening of the transition region in urea solution is caused by the low urea binding constant. Accordingly, the free energy profile of urea is also shifted towards higher concentrations compared to guanidine HCl (figure 3B). The free energy change for unfolding of $0.01 \leq \Theta_U \leq 0.95$ is virtually identical for the two denaturants.

Figure 3 also includes a comparison of the exact solutions for the extent of unfolding and the free energy (eqs. 9 & 10) with the approximations (eqs 17 & 18). The adsorption isotherm eq. 17 and the free energy approximation eq. 18 show an almost complete overlap with the exact matrix solutions. This holds true for all proteins mentioned in this work. A large number

of lysozyme denaturation studies have been published. Table 1 provides a selection of the studies as analysed with eqs. 9 and 10.

Table 1

Temperature-dependence of chemical unfolding of lysozyme

Lysozyme unfolding depends on pH and temperature. A decrease in pH or an increase in temperature shift the unfolding transition towards lower c_0 values. This is illustrated in figure 4 for chemical denaturation with guanidineHCl at three different temperatures.

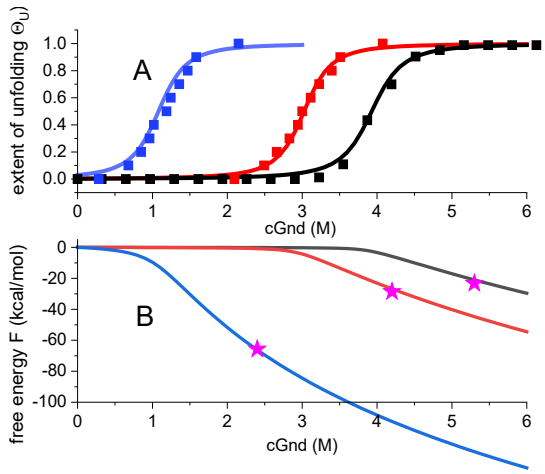

Figure 4. Temperature dependence of lysozyme unfolding in guanidineHCl solution. Data points taken from figure 2 of reference[19]. Solid lines are the simulations with the multistate cooperative model. Blue: 60 °C, K_D = 0.9 M^{-1}, σ = 3x10^{-2}. Red: 40 °C, K_D = 0.34 M^{-1}, σ =2.5x10^{-3}. Black: 10 °C, K_D = 0.26 M^{-1}, σ = 1x10^{-3}. (A) Extent of unfolding $\Theta_U(c_D)$. Simulations with eq. 9. (B) Free energy $F(c_D)$, calculated with eq. 10. (★) Data points keynote the free energy at 95% unfolding, calculated with eq. 19.

Figure 4A displays unfolding isotherms obtained with UV- and CD-spectroscopy.[19] The solid lines are the simulations with the multistate cooperative model. The binding constants K_D are exactly the reciprocal of the midpoint concentrations c_0. The binding constants increase with

temperature and the transition regions broaden. The cooperative interactions are reduced, leading to a larger cooperativity parameter σ. A temperature increase from 10 °C to 60 °C increases the cooperativity parameter by a factor of 10.

Figure 4B displays the predicted concentration dependence of the free energy. The free energy is zero for the native protein and decreases rapidly beyond the midpoint of unfolding c_0. The solid lines are calculated with the exact solution eq. 9. The three ★-marked data points were calculated with eq. 19, corresponding to 95% unfolding. The comparison with the exact solution, demonstrate that the simple equation 19 is an excellent approximation for the free energy if $c_D \gg c_0$.

We have analysed published denaturation experiments of polypeptides and proteins of different size and structure. The fit of the experimental data with the multistate cooperative model were all of comparable quality as shown for lysozyme in figures. 2-4. The data are summarised in table 2.

Table 2

Chemical unfolding with sodium dodecyl sulfate (SDS)

Sodium dodecyl sulfate (SDS) is a much stronger denaturant then guanidine HCl or urea as shown in figure 5 (data taken from reference[20]). Unfolding was measured with calorimetry. At 25 °C the midpoint concentration of unfolding is only 4.35 mM, resulting in a large binding constant of $K_D = 230\ M^{-1}$. The cooperativity of SDS-induced unfolding is low with $\sigma = 7x10^{-2}$.

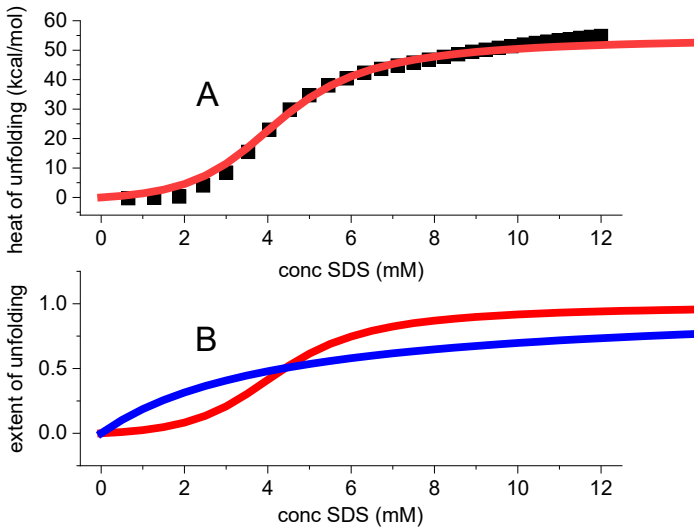

Figure 5. Calorimetric titration of 68 μM lysozyme with sodium dodecyl sulfate (SDS) solution at 25 °C. (A) Experimental data taken from reference,[20] table 1. The published data were normalized to 1 mole lysozyme. Each data point is a separate measurement. Red line: multistate cooperative model. K_D *= 230* M^{-1}*,* $\sigma = 7x\ 10^{-2}$*, enthalpy of unfolding* ΔH_0 *= 55 kcal/mol. (B) unfolding isotherm. Red line: multistate cooperative model, calculated with the same parameters as listed in panel A. Blue line:* K_D *as in panel A, but* $\sigma = 1$ *(non-cooperative Langmuir isotherm).*

The enthalpy of unfolding, ΔH_0, is the most important parameter in thermal unfolding studies. Spectroscopic measurements of chemical unfolding isotherms cannot provide this thermodynamic property. It is obtained, however, by a direct calorimetric measurement as shown in figure 5. Lysozyme is titrated with low concentrations of sodium dodecyl sulfate (SDS) and the heat of reaction is measured in a calorimeter. Each data point in figure 5 corresponds to an independent measurement.[20] The total unfolding enthalpy is ΔH_n = 55 kcal/mol. This is in agreement with the result of an early isothermal enthalpimetric titration of lysozyme with guanidineHCl at pH 2.5 and 40 °C yielding 56 kcal/mol.[21]

Discussion

Characteristics of the multistate cooperative model

The multistate cooperative model is based on a well-defined statistical-mechanical partition function that determines the extent of unfolding and the thermodynamic properties of the system. The partition function contains two molecular parameters, the binding constant of the denaturant, K_D and the protein cooperativity σ. The binding constant describes the affinity of the denaturant to the cooperative unit (e.g. amino acid residue) of the protein. The binding constant K_D is the reciprocal of the midpoint concentration c_0, i.e. $K_D = 1/c_0$. The cooperativity parameter determines the steepness of the unfolding transition as illustrated in figure 1A. The model takes into account the number ν of amino acid residues participating in the unfolding reaction and makes the following predictions. The free energy of the $n \rightarrow u$ transition is identical to the free energy provided by the concentration gradient ΔG_{diff} needed to induce complete unfolding. The free energy of the native protein is the reference state with zero free energy. Upon unfolding the free energy change is negative, but still close to zero and decreases strongly when the midpoint of unfolding is reached.

The exact model may be demanding because of its matrix formulas, however, a simple approximation can be given (eqs 17 & 18). If one is interested only in the energy between the native protein and its unfolded structure at concentration c_{end}, equation 19 yields a simple answer.

Temperature dependence of unfolding

The temperature dependence of the binding constant K_D and the cooperativity parameter σ together with that of the free energy is displayed in figure 6 for lysozyme (cf. Table 1). The simulation of the unfolding isotherms yields to excellent fits in each case. Nevertheless, the scatter of the data in figure 6 is considerable as the isotherms are obtained by different authors

under different experimental conditions. Two temperature regions can be discerned. Between 10 °C and 35 °C the effect of temperature is small, above 35 °C all properties change rapidly.

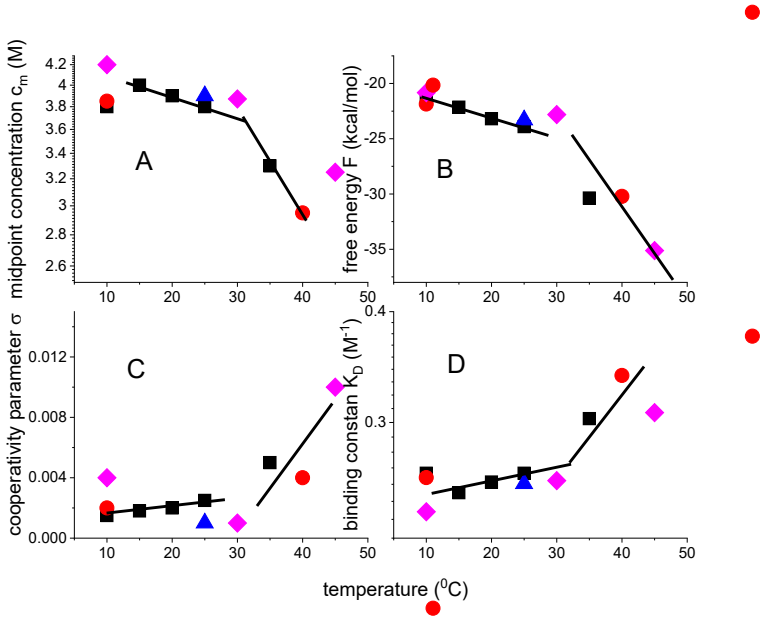

Figure 6. Lysozyme unfolding experiments in guanidineHCl at different temperatures and by different authors. (■)[22], (●)[19], (◆)[17, 23], (▲)[24]. *The solid lines are introduced to guide the eye.*

The midpoint concentration and the free energy decrease with temperature, the binding constant and the cooperativity parameter increase (that is, cooperativity decreases). At higher temperature the protein stability and the cooperativity of unfolding increase. The cooperativity parameter of chemical unfolding is about two orders of magnitude larger than that of terminal unfolding.[10, 25-27] Chemical unfolding is thus less cooperative than thermal unfolding.

Free energy of chemical unfolding

For the last six decades, the general understanding in the protein folding field has been that proteins fold into their native conformations driven by decrease in Gibbs free energy (negative ΔG).[6] The unique secondary and tertiary structure of a protein is, thermodynamically,

the most stable conformation and the thermodynamic funnel hypothesis has become the leading model of protein folding.

The multistate cooperative model is fully consistent with this hypothesis. The native protein is the reference state with zero free energy and unfolding requires energy. In returning reversibly to the native state, the unfolded protein loses its free energy. The concentration profiles of the free energy shown in figures 1-5 thus correspond to one leg of an *inverted* funnel with the native protein at the bottom.

The multistate cooperative model is further supported by considering the temperature dependence of the chemical unfolding isotherm. The temperature dependence of the free energy leads to the unfolding entropy $-\Delta S = \frac{\partial \Delta F}{\partial T}$ and, in turn, to the enthalpy $\Delta H = \Delta F + T\Delta S$. Figure 7A displays data obtained from a consistent set of experiments for the temperature range 10 °C to 25 °C (figure 1 in reference[22]).

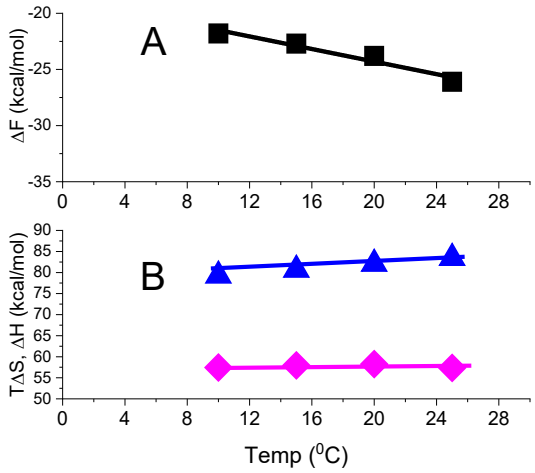

Figure 7. Chemical unfolding of lysozyme in guanidineHCl solution at different temperatures. (A) Free energy ΔF(T) calculated with the multistate cooperative model. Evaluation of unfolding isotherms of reference.[22] Straight line: linear regression analysis with slope -ΔS = -0.28 ± 0.046 kcal/molK (R^2=0.947). (B) (◆) Enthalpy ΔH, (▲) Entropy TΔS.

From the negative slope of the straight line in figure 7A the entropy is calculated as ΔS = 0.280±0.047 kcal/molK. Figure 7B displays the calculated entropy $T\Delta S$ and the enthalpy ΔH. The average enthalpy is $\Delta H_0 = 58 \pm 1$ kcal/mol. An early isothermal titration study of lysozyme with guanidineHCl, at pH 2.5 and 40 °C yielded ΔH = 56 kcal/mol.[28]. The present result is also in agreement with the SDS isothermal calorimetry shown in figure 5, resulting in an enthalpy of 55 kcal/mol at 25 °C.

Concentration gradient $\Delta c = c_{end} - c_{ini}$ *and the free energy of the* $n \rightarrow u$ *transition*

The multistate cooperative model predicts a one-to-one relationship between the free energy of the concentration gradient $\Delta c = c_{end} - c_{ini}$ and the energy required to induce the $n \rightarrow u$ transition. Unfolding takes place in the concentration interval $c_{ini} \leq c_D \leq c_{end}$. The concentration gradient $\Delta c = c_{end} - c_{ini}$ constitutes a diffusive or osmotic free energy

$$\Delta G_{Diff} = -RT_0 \ln \frac{c_{end}}{c_{ini}} \tag{24}$$

The free energy at ambient temperature is typically ΔG_{Diff} ~ -0.4 to -1.0 kcal/mol.

In the multistate cooperative model, the binding of denaturant D is associated with the with the probability $q(c_D) = K_D c_D$. The free energy of unfolding per amino acid residue, Δg_{nu} is

$$\Delta g_{nu} = -RT_0 \ln \frac{q(c_{ini})}{q(c_{end})} = RT_0 \ln \frac{c_{en}}{c_{ini}} \tag{25}$$

The predicted unfolding free energy per amino acid residue is thus identical to the concentration gradient (with opposite sign), that is, $\Delta g_{nu} + \Delta G_{diff} = 0$. The multistate cooperative model is thus consistent with the thermodynamic expectation for a reversible equilibrium.

Guanidine HCl and urea binding constants

The multistate cooperative model considers explicitly the binding of the denaturant D and predicts a reciprocal relation between binding constant K_D and the midpoint concentration c_0, that is $K_D = \frac{1}{c_0}$ (equation 16). The guanidineHCl binding constants deduced from the lysozyme isotherms are $0.2\ M^{-1} \leq K_D \leq 0.9\ M^{-1}$ in the temperature range 10 – 60 °C (table 1). Urea binds with a lower affinity of $K_D = 0.15\ M^{-1}$ (at 25 °C).

Isothermal titration calorimetry was used to measure guanidineHCl and urea binding to different proteins.[12] The data were analyzed by assuming a set of independent non-cooperative binding sites.[12] The binding constants deduced for lysozyme were K_D ~0.4 – 0.8 M^{-1} for guanidine HCl and 0.06 $M^{-1}$for urea. This is in broad agreement with the results of the multistate cooperative model.

The binding constants of guanidineHCl and urea are small and the binding of a single guanidineHCl molecule is not sufficient to induce the $n \rightarrow u$ transition of an amino acid residue. Only the cooperative binding of many denaturants induces protein unfolding.

The adsorption isotherm (equation 15) is applicable also to denaturation with organic solvents. Lysozyme denaturation isotherms have been reported for ethanol and DMSO.[29]. The midpoint concentrations and corresponding binding constants are 5 M and 0.25 $M^{-1}$for ethanol and 7 M and 0.17 M^{-1} for DMSO. In contrast, anionic sodium dodecyl sulfate (SDS) has a high affinity for cationic lysozyme[20]. Based on figure 1 of reference[20] the midpoint concentration is ~4.3 mM and the binding constant K_D ~230 M^{-1}.

For a larger set of different proteins, the average binding constant for guanidineHCl is $K_D = 0.55 \pm 0.25\ M^{-1}$ and for urea $K_D = 0.22 \pm 005\ M^{-1}$. The cooperativity parameter is $\sigma \sim (1.5 \pm 0.9) \times 10^{-2}$ for both denaturants (cf. Table 2

Free energy of the $N \rightleftarrows U$ equilibrium

The conventional two-state and the multistate cooperative model provide quantitative expressions for the total free energy of the $N \rightarrow U$ transition. The two-state model uses an empirical relationship (equation 21) with no thermodynamic foundation. The free energy is simply a linear function of the detergent concentration. To fit the data, a large positive free energy must be assigned to the native protein.

The multistate cooperative model provides a simple adsorption isotherm to analyse chemical unfolding (equation 15). The only unknown parameter is the cooperativity σ. The free energy follows from the partition function (equations 10 or 18). The free energy of the native protein is zero and the concentration profile is shown in figure 1. Addition of denaturant initially leads to only a small negative free energy. The free energy becomes distinctly more negative at concentrations near and above the midpoint concentration c_0. For large denaturant concentrations $c_D \gg c_0$. a simple approximation of $\Delta F(c_D) \approx -RT_0 N \ln(K_D c_D)$ (equation 19) is valid. Numerical values of the free energy change $\Delta F = \Delta F(c_{end}) - \Delta F(c_{ini})$, calculated with equation 10, are listed in table 1. The extent of unfolding in these calculations is $0.01 \leq \Theta_U \leq 0.95$. It should also be noted that the shape of the free energy in chemical unfolding (figure 1) is identical to the free energy temperature profile in temperature-induced unfolding.[10] However, the free energy change ΔF in chemical unfolding is about three fold higher more negative than the free energy of thermal unfolding.[10] This must be traced back to the binding of the denaturants.

Cooperativity in chemical and thermal unfolding

Chemical σ parameters are plotted as a function of the midpoint temperature in figure 3. Cooperativity is $\sigma \simeq 3 \times 10^{-3}$ for $c_0 \geq 3$ M and increases exponentially at lower concentrations. The increase in temperature facilitates chemical unfolding by decreasing the cooperativity of

the protein. Chemical unfolding occurs at constant temperature and at constant K_D. The concentration of the denaturant is the only parameter that determines the chemical equilibrium. The Gibbs free energy to start a new folded sequence within an unfolded region is given by $\Delta G_\sigma = -RT \ln \sigma$.

Conclusions

It is obvious that the Linear Extrapolation Method is a poor model to describe chemical unfolding. The two fit parameters have no well-defined thermodynamic or molecular basis. It is therefore attractive to modify an existing multistate cooperative model for the thermal protein unfolding to address chemical unfolding. The new two-parameter model, includes the binding constant of the denaturant and the cooperativity of protein unfolding. It is simple and easy to apply as the binding constant is the reciprocal of the midpoint concentration of unfolding. Simple equations can be given for the binding isotherm and the free energy. The free energy profile of chemical unfolding parallels the experimental results for thermal unfolding. The free energy of the native protein is zero and becomes distinctly negative upon unfolding, only after reaching the midpoint concentration. The new model is satisfying as it provides an excellent description of chemical denaturation isotherms and for the first time allows comparing cooperative, chemical and thermal unfolding.

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Tables

Table 1. Chemical unfolding of lysozyme with guanidineHCl and urea at different temperatures

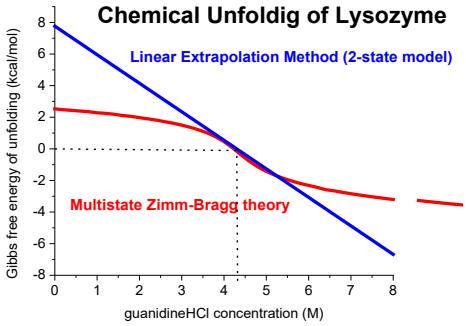
Temp	c_{mid}	K_D	σ	c_{ini}	c_{end}	ΔF	g_{den}	ΔG_{H_2O}	m
°C	M			M	M	kcal/mol	kcal/mol	kcal/mol	kcal L/mol^2
10[22]	3.8	0.263	1.50E-03	2.7	5.3	-21.8	-0.379	9.55	2.512
15[22]	4.0	0.25	1.80E-03	2.7	5.6	-22.7	-0.417	8.598	2.150
20[22]	3.9	0.257	2.00E-03	2.6	5.5	-23.8	-0.436	8.598	2.210
25[22]	3.8	0.263	2.50E-03	2.4	5.5	-26.1	-0.491	9.554	2.513
35[22]	3.3	0.303	5.00E-03	1.75	5.1	-32.6	-0.654	8.598	2.605
10[19]	3.8	0.26	1.00E-03	2.9	5.25	-20.1	-0.334	9.55	2.483
40[19]	2.9	0.34	2.50E-03	1.9	4.1	-26.41	-0.493	8.36	2.842
60[19]	1.1	0.9	3.00E-02	0.25	2.4	-65.5	-1.5	4.299	3.869
25[24]	3.9	0.256	1.00E-03	2.8	5.15	-21.2	-0.361	10.03	2.568
10[23]	4.1	0.243	2.00E-03	2.7	5.8	-22.55	-0.43	8.837	2.147391
30[17]	3.9	0.258	1.00E-03	2.9	5.3	-21.7	-0.363	8.837	2.236
45[17]	3.2	0.31	1.00E-02	1.3	5.3	-39.3	-0.888	4.777	2.236

Table 2. Chemical unfolding of peptides and proteins of different size and structure in guanidine Cl and urea

	N_{aa}	pH	Denat.	K_D	c_{mid}	s	c_{ini}	c_{end}	Δc	g_{den}	ΔF	n	Lit.		
							M	M	M	kcal/mol	kcal/mol				
BBL	41	7	GndHCl	0.33	3.03	3.00E-02	0.2	7.5	7.3	-2.14	-20.9	0.238204	[30]	fig 2b95 nm	
ubiquitin WT	76		GndHCl	0.34	2.94	7.00E-03	1.4	5	3.6	-0.753	-22.1	0.386175	[31]	fig1	
ubiquitin L67S	76		GndHCl	0.72	1.39	1.30E-02	0.5	2.7	2.2	-0.998	-28.4	0.374433	[31] mut76	fig 1	
ubiquitin	76	2	GnddHCl	0.395	2.53	8.00E-03	1.2	4.5	3.3	-0.782	-24.1	0.405505	[32] pH 2	fig 4	
ubiquitin	76	5.5	GndHCl	0.295	3.39	1.20E-02	1.3	6.3	5	-0.934	-26.4	0.371915	[32] pH fig 4		
Apo lipoprotein A1	120	8	GndHCl	1	1.00	2.00E-02	0.3	2	1.7	-1.12	-49.7	0.369792	[33]	fig 1	
Apo lipoprotein A1	122	7.4	GndHCl	0.952	1.05	5.00E-03	0.6	1.7	1.1	-0.61	-32.8	0.440742	[34]	fig.7	
Apo lipoprotein A1	122	7.4	GndHCl	0.95	1.05	3.00E-02	0.3	2.3	2	-1.21	-55.9	0.378675	[35]		
lysozyme	129	7	GndHCl	0.245	4.08	2.00E-03	2.8	5.8	3	-0.431	-24.6	0.442454	[18]	fig 1	
lysozyme	129	2	GndHCl	0.42	2.38	2.00E-02	0.8	4.6	3.8	-1.03	-49.6	0.373297	[36]	fig 2A	
lysozyme	129	2.5	GndHCl	0.48	2.08	5.00E-03	1.1	3.2	2.1	-0.632	-31.1	0.381464	[36]	fig 1	
PMS-Ct	145	4	GndHCl	0.52	1.92	1.00E-03	1.1	2.5	1.4	-0.486	-22.7	0.322123	[37]	fig 4	
PMS-Ct	241	4	GndHCl	0.513	1.95	4.00E-03	1.1	2.7	1.6	-0.531	-45.3	0.353986	[37]	fig 4	
average				0.550769	2.22	0.012077			2.930769	-0.90	-33.3538	0.37			
STDV				0.255951	0.929147	0.009611			1.659186	0.432221	11.82557	0.049515			

Ac-tyr-(ala-glu-ala-ala-lys-ala)$_5$-phe-NH_2	32	7	urea	0.25	4.00	8.00E-02							[5]	fig 1	
Ac-tyr-(ala-glu-ala-ala-lys-ala)$_8$-phe-NH_2	50	7.0, 273 K	urea	0.2	5.00	1.00E-02	2.2	9.8	7.6	-0.884	-18.1	0.409502	[38]	fig. 1 n=50, 0 ^{0}C	
HPr proteun	85	7	urea	0.215	4.65E+00	8.00E-03	2	8	6	-0.793	-26.2	0.388695	[39]	fig 1b, 288K	
cytochrome c	106	5	urea	0.2	5.00E+00	4.00E-03	2.9	7.7	4.8	-0.578	-25.1	0.41			
cytochrome c	106	7	urea	0.155	6.46E+00	4.00E-03	3.6	9.5	5.9	-0.574	-22.5	0.369798	[2]	Fig 5	
P22 1-domaiin	123	6	urea	0.3	3.33	9.00E-03	1.5	5.6	4.1	-0.753	-35.18	0.379836	[40]	fig 2	
lysozyme	129	2	urea	0.303	3.30	2.00E-02	2.2	6.4	4.2	-0.632	-47.9	0.587528	[36]	fig 2B	
lysozyme	129	5.5	urea	0.15	6.67	2.00E-03	4.5	9.3	4.8	-0.43	-23.3	0.420047	[18]	fig 1	
anti-EF-R mab	1600	7	urea	0.18	5.56	2.00E-02	2	8.3	6.3	-0.85	-407	0.299265	[2]	Fig 2	
average				0.22	4.80	1.74E-02			5.46	-0.69	-75.66	0.41			
STDV				0.05	1.15	0.022955			1.12	0.15	125.54	0.08			
lysozyme	129		SDS	230.00	0.00	7.00E-02	0.001	0.012	0.011	-1.47	-77.7	0.409745	[20]	table 1, 298K	ig 1

TOC Image


Chemical Unfoldig of Lysozyme
Linear Extrapolation Method (2-state model)
Multistate Zimm-Bragg theory
Gibbs free energy of unfolding (kcal/mol)
guanidineHCl concentration (M)
8
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-2
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