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Thermodynamics of the Trp-cage Miniprotein Unfolding in Urea

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Abstract

The thermodynamic properties of unfolding of the Trp-cage mini protein in the presence of various concentrations of urea have been characterized using temperature-induced unfolding monitored by far-UV circular dichroism spectroscopy. Analysis of the data using a two-state model allowed the calculation of the Gibbs energy of unfolding at 25°C as a function of urea concentration. This in turn was analyzed by the linear extrapolation model that yielded the dependence of Gibbs energy on urea concentration, i.e. the m-value for Trp-cage unfolding. The m-value obtained from the experimental data, as well as the experimental heat capacity change upon unfolding, were correlated with the structural parameters derived from the three dimensional structure of Trp-cage. It is shown that the m-value can be predicted well using a transfer model, while the heat capacity changes are in very good agreement with the empirical models based on model compounds studies. These results provide direct evidence that Trp-cage, despite its small size, is an excellent model for studies of protein unfolding and provide thermodynamic data that can be used to compare with atomistic computer simulations.

Introduction

The design of the Trp-cage mini protein (only 20 amino acid residues) by Andersen et al ¹ provided the protein folding field with a convenient model to examine the thermodynamic and folding properties of a small protein with all the structural attributes of larger globular proteins ^{2–5}, i.e. a well defined secondary and tertiary structure, and a well developed hydrophobic core (Figure 1A). The small size of the protein also made it an attractive model for computer simulations in atomistic details (see e.g. references ^{6–13}). In this paper we provide experimental data on the effects of urea on the thermodynamic properties of the Trp-cage mini protein. These results have two important consequences. One, they provide the experimental data for the effects of urea on the smallest known protein and, thus, will allow us to test empirical predictions for the effects of urea on globular proteins, which were developed based on larger proteins. Second, they provide the thermodynamic information that can be used to calibrate and validate the computational modeling of the effects of urea on protein stability.

Materials and Methods

Sample Preparation

The Trp-cage peptide (amino acid sequence: $\text{2HN-NLYIQWLKDGGPSSGRPPPS-COOH}$) was synthesized using standard Fmoc chemistry (Penn State College of Medicine Macromolecular Core Facility). The peptide was purified as previously described on a C-18 column ² and purity was confirmed with MALDI-TOF. Circular Dichroism (CD)

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experiments were performed in one of two buffers: the first containing 5 mM sodium phosphate and 5 mM sodium citrate, pH 7.0, and the second containing 1 mM sodium phosphate, 1 mM sodium citrate, and 1 mM borate, pH 7.0. For urea denaturation, an 8 M urea stock solution was also made in the above buffers. Both buffers gave similar melting profiles. All peptide samples were dialyzed into MilliQ water and diluted to 10 μ M in the above buffers, containing the following urea concentrations: 0 M, 0.5 M, 0.75 M, 1.0 M, 1.5 M, 2.0 M, 2.5 M, 3.0 M, 3.5M, 4.0 M, 6.0 M. Concentrations of urea were confirmed both before and after CD experiments by comparing measured values with tabulated data from the solution refractive index ¹⁴.

Thermal Unfolding of Trp-cage Monitored Using Far-UV Circular Dichroism (CD)

CD measurements were performed on a Jasco J-715 spectropolarimeter. All measurements were carried out either in a 1 cm rectangular quartz cell with temperature controlled by a six-position Peltier cell changer or in a 1 mm cylindrical cell with the temperature controlled by an external, circulating water bath. Melting profiles were monitored at 222 nm or 225 nm (to reduce the contribution of high concentrations of urea to the CD signal) and from 4 to 100°C, in 1°C increments. The actual temperature inside the cell was measured using an Omega HH24 internal temperature probe. Reversibility of thermal unfolding was confirmed by comparing far-UV CD spectra at 4°C both before and after thermal melting. Measured ellipticity values, Θ , were converted into mean residue ellipticity, $[\Theta]$, using:

$$[\Theta] = \frac{\Theta \cdot MR}{10 \cdot l \cdot c} \quad (1)$$

where MR is the average mass per amino acid, l is the optical length of the cell in centimeters, and c is the concentration of the peptide in mg/ml. All experiments were performed a minimum of two times, with the average values presented.

Calculation of the Accessible Surface Area (ASA)

The accessible surface area was calculated using all 20 Trp-cage NMR structures (PDB code: 1L2Y ¹, using NACCESS V2.1.1 ¹⁵), and the average ASA are reported. The unfolded state was modeled in Swiss PDB Viewer v3.7b2 as an extended beta sheet ($\phi = -120^\circ$, $\psi = 120^\circ$) and also processed using NACCESS. The difference between the accessible surface areas, ΔASA , was calculated using:

$$\Delta ASA = ASA_{Unfolded} - ASA_{Folded} \quad (2)$$

where $ASA_{Unfolded}$ and ASA_{folded} are the surface areas of the unfolded and native states, respectively.

Data Analysis

All CD unfolding data was fitted to a two-state unfolding model, where it is assumed that only the native (N) and unfolded (U) states are populated at any given temperature. For such a two-state model, the temperature dependence of enthalpy, ΔH , and entropy, ΔS , can be defined as:

$$\Delta H(T) = \Delta H(T_m) + (T - T_m) \cdot \Delta C_p \quad (3)$$

and

$$\Delta S(T) = \Delta S(T_m) + \ln(T/T_m) \cdot \Delta C_p \quad (4)$$

where the changes in the heat capacity upon unfolding, ΔC_p , are assumed to be temperature independent. The temperature-dependence of Gibbs free energy can be derived via Gibbs-Helmholtz relationship:

$$\Delta G(T) = [(1 - T/T_m) \cdot \Delta H(T_m)] + (T - T_m) \cdot \Delta C_p - \ln(T/T_m) \cdot T \cdot \Delta C_p \quad (5)$$

Direct DSC analysis of Trp-cage unfolding in aqueous solution (i.e. in the absence of urea) led to the following thermodynamic parameters: $\Delta H(T_m) = 56 \pm 2 \text{ kJ} \cdot \text{mol}^{-1}$, $T_m = 43.9 \pm 0.8 \text{ }^\circ\text{C}$ and $\Delta C_p = 0.3 \pm 0.1 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. During the fit to a two-state model, $\Delta H(T_m)$ and ΔC_p were allowed to vary within the experimental uncertainties.

For urea concentrations greater than 3.5 M, the fraction of the native Trp-cage molecules was less than 50% even at the lowest experimental temperatures (4°C). Consequently, T_m was no longer a valid reference temperature and T_s , the temperature at which entropy is equal to zero, was used¹⁶. For this case, the temperature dependence of ΔH and ΔS can be defined as:

$$\Delta H(T) = \Delta H(T_s) + (T - T_s) \cdot \Delta C_p \quad (6)$$

and

$$\Delta S(T) = \ln(T/T_s) \cdot \Delta C_p \quad (7)$$

where again the changes in the heat capacity upon unfolding, ΔC_p , are assumed to be temperature independent. The temperature-dependence of Gibbs free energy can then be defined as:

$$\Delta G(T) = \Delta H(T_s) + (T - T_s) \cdot \Delta C_p - \ln(T/T_s) \cdot T \cdot \Delta C_p \quad (8)$$

It has been shown that both enthalpy and entropy of unfolding are dependent on urea concentration¹⁷. In addition, the heat capacity of unfolding, ΔC_p , has also been shown to be dependent on urea concentration^{17,18}. To account for these effects, ΔC_p was expressed as the sum of two terms:

$$\Delta C_p^{Urea} = \Delta C_p + \delta C_p \cdot [Urea] \quad (9)$$

where the first term, ΔC_p , refers to the heat capacity of unfolding in the absence of urea and the second term, δC_p , is the increase in the heat capacity of unfolding due to the addition of urea. The value of $\delta C_p = 0.013 \pm 0.006 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ per mole of urea was obtained from the average dependence of the heat capacity on urea concentration for several proteins¹⁷, and was constrained within the experimental uncertainty during the fit.

Using nonlinear regression analysis software (NLREG), the CD unfolding data obtained under low urea concentrations was fit to equations 3, 4, 5 and 9, holding the previously

obtained values for $\Delta H(T_m)$, T_m , and ΔC_p , constant for 0 M urea. The CD data obtained at concentrations of urea of 3.5 M and higher was fit to equations 6, 7, 8, and 9. The CD signal for the native state cannot be determined experimentally and therefore in the fit, the temperature dependence of the CD signal for the native state was assumed to be the same for all melting curves within a corresponding data set. Similarly, the CD signal for the unfolded state was assumed to be the same for all melting curves.

Results and Discussion

Experimental determination of the urea m-value for Trp-cage unfolding

Figure 1B shows the temperature-induced unfolding profiles of Trp-cage for a representative set of urea concentration as monitored using circular dichroism (CD). As expected, increasing concentrations of urea have a destabilizing effect on the Trp-cage mini-protein. The absolute ellipticity values at 4°C decrease with increasing urea, which suggests a decrease in the fraction of the native Trp-cage population. In addition, the transition profiles for higher urea concentrations are shifted towards lower temperatures, suggesting reduced thermal stability. Previously, we have shown that Trp-cage unfolding in the absence of urea closely follows a two-state unfolding model². Therefore, it was assumed that the experimental data in the presence of urea can be equally well described by a two-state process. For 0 M urea, a T_m of 41 ± 2 °C was obtained, which is within the error of previously reported values^{1–3}.

Using the parameters obtained from the fit to a two-state model, the values of the change in Gibbs free energy at 25°C were calculated using equation 5 or 8 (see Table 1). The dependence of $\Delta G(25^\circ\text{C})$ on urea concentration appears to be a linear function of denaturant (see Figure 2). According to the linear extrapolation model^{19,20}, the slope of this dependence corresponds to the m-value and the intercept with the y-axis corresponds to the Gibbs energy of Trp-cage unfolding in 0 M urea:

$$\Delta G(\text{Urea}) = \Delta G(\text{H}_2\text{O}) + m \cdot [\text{Urea}] \quad (10)$$

Linear regression yields an m-value of 1.36 ± 0.08 kJ mol⁻¹ M⁻¹ and $\Delta G(\text{H}_2\text{O}) = 2.8 \pm 0.3$ kJ·mol⁻¹.

Comparison of the m-value for Trp-cage with predictions by empirical methods

It is known that there is a strong correlation between m-values and the total exposed surface area upon unfolding, $\Delta \text{ASA}_{\text{total}}$ ^{21,22}. However, this relationship has not been examined for very small proteins. The Trp-cage mini-protein, consisting of only 20 residues, is therefore a prime candidate for examining this correlation.

According to NACCESS calculations (see Materials and Methods for details), unfolding of Trp-cage results in a change in total accessible surface area, $\Delta \text{ASA}_{\text{total}}$, of 990 ± 50 Å². This value corresponds well with the predicted ASA of 953 Å², using the relationship obtained by Myers et al.²¹:

$$\Delta \text{ASA}_{\text{total}} = -907 + 93 \cdot (\#res) \quad (11)$$

Such a good agreement suggests that the total changes in the accessible surface area of Trp-cage follow a similar trend as those of larger proteins.

According to Myers et al.²¹, there is a linear relationship between ΔASA_{total} and the m-value:

$$m = 1.56 + 0.00046 \cdot \Delta ASA_{total} \quad (12)$$

Using this relationship and the ΔASA_{total} of $990 \pm 50 \text{ \AA}^2$, the predicted m-value is $2.0 \pm 0.2 \text{ kJ mol}^{-1} \text{ M}^{-1}$. It should be noted, however, that equation 12 is strictly empirical; therefore, an m-value of 0 can only be obtained if ΔASA_{total} is negative. If the regression line is forced to pass through the origin, it may be possible to more accurately predict the m-value for proteins with small ΔASA_{total} values, such as Trp-cage. Using the same 45 proteins as Myers et al.²¹, this new correlation can be written as

$$m = 0.00054 \cdot \Delta ASA_{total} \quad (13)$$

Using this equation, the predicted m-value for Trp-cage is $0.54 \pm 0.03 \text{ kJ mol}^{-1} \cdot \text{M}^{-1}$, which is lower than the experimental m-value of $1.36 \pm 0.08 \text{ kJ mol}^{-1} \cdot \text{M}^{-1}$ for Trp-cage. Since the true m-value lies somewhere between the original regression line and one constrained by thermodynamic principles, a better prediction may be possible if the data is fit using a polynomial regression:

$$m = -0.000001 \cdot \Delta ASA_{total}^2 + 0.14 \cdot \Delta ASA_{total} + 180 \quad (14)$$

Using this equation, the predicted m-value is $1.3 \pm 0.03 \text{ kJ mol}^{-1} \cdot \text{M}^{-1}$, which corresponds well to the experimental value. Although this approach is inconsistent with the interpretation of m-value being proportional to the accessible surface area exposed upon unfolding, it is strictly an empirical correlation, similar to equation 12.

In addition to the empirical correlations discussed above, there have been several attempts to predict m-values based on the free energy transfer of the side chains and backbone of a protein. The most notable of these is Tanford's transfer model, which relies on the assumption that the folding free energy is equivalent to the difference in solvation between the native and denatured states²³. More recently, Bolen et. al have suggested that m-values can be accurately predicted using information from the three-dimensional structure²⁴. Assuming the relationship between m-value and transfer energies:

$$m_{calc} = \Delta G_{tr,D}^{1M} - \Delta G_{tr,N}^{1M} = \sum_{i=AA \text{ type}} n_i \Delta g_{tr,i}^{sc} \Delta \alpha_i^{sc} + \Delta g_{tr}^{bb} \sum_{i=AA \text{ type}} n_i \Delta \alpha_i^{bb}, \quad (15)$$

where

$$\Delta \alpha_i^{bb \text{ or } sc} = \left[\sum_{j=1}^n (ASA_{i,j,D} - ASA_{i,j,N}) \right] / n_i ASA_{i, GLY-X-Gly} \quad (16)$$

the calculated m-value for Trp-cage is $1.69 \pm 0.68 \text{ kJ mol}^{-1} \text{ M}^{-1}$. It is worth noting that this calculation involves modeling the solvent accessibility of the unfolded state as the average of two extremes^{25,26}. While more elaborate and rigorous methods have been developed since (see e.g. ref. ²⁷), they are in good agreement with approach used here. It is also worth

noting that this approach is associated with a significant error. Bolen et al. stress that this is the result of the inability to accurately quantify the ASA of the denatured state, and not the fault of the model itself²⁴. Another potential error is that the correction for changes in activity coefficients of model compounds is applied only to the transfer energy of the backbone. Regardless of these shortcomings, the calculated m-value for Trp-cage is in good agreement with that obtained experimentally, suggesting the validity of the transfer model. O'Brien et al^{28,29} reached a similar conclusion based on computer simulations of urea-induced unfolding of lysozyme.

Comparison of the ΔC_p for Trp-cage with predictions by empirical methods

Experimentally determined value of heat capacity of unfolding, ΔC_p , for Trp-cage is positive $0.3 \pm 0.1 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ consistent with the observed positive heat capacity changes for globular protein unfolding². The small absolute value of ΔC_p for Trp-cage is probably the result of smaller size of the Trp-cage miniprotein, and consequently smaller changes of the ASA exposed upon unfolding. A detailed analysis of the factors contributing to the change in heat capacity of protein unfolding suggests that the exposure of non-polar and polar ASA need to be accounted for separately. It has been shown, using the transfer of model compounds, that the exposure of non-polar ASA contributes positively to ΔC_p of unfolding while polar ASA contributes negatively^{21,30,31}. Different absolute values for these contributions per square angstrom of non-polar and polar exposed surfaces were proposed by Meyers et al.²¹, Spolar et al.³¹, Murphy and Freire³⁰, respectively:

$$\Delta C_p = 11.7 \cdot \Delta \text{ASA}_{\text{non-polar}} - 0.838 \cdot \Delta \text{ASA}_{\text{polar}} \quad (17)$$

$$\Delta C_p = 1.34 \cdot \Delta \text{ASA}_{\text{non-polar}} - 0.59 \cdot \Delta \text{ASA}_{\text{polar}} \quad (18)$$

$$\Delta C_p = 1.88 \cdot \Delta \text{ASA}_{\text{non-polar}} - 1.09 \cdot \Delta \text{ASA}_{\text{polar}} \quad (19)$$

Furthermore, the contributions from non-polar and polar surface areas can be subdivided into non-polar aliphatic, non-polar aromatic, polar backbone and polar side chains³²:

$$\Delta C_p = 2.14 \cdot \Delta \text{ASA}_{\text{alp}} + 1.55 \cdot \Delta \text{ASA}_{\text{arm}} - 1.81 \cdot \Delta \text{ASA}_{\text{bb}} - 0.88 \cdot \Delta \text{ASA}_{\text{pol}} \quad (20)$$

Calculations performed using equations 17–20 give ΔC_p values of $0.19 \pm 0.04 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, $0.40 \pm 0.05 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, $0.43 \pm 0.07 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, and $0.22 \pm 0.08 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, respectively. These predictions compare well with the ΔC_p value obtained by DSC and CD, $0.3 \pm 0.1 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ².

Comparison with the computer simulations

One of the attractive features that Trp-cage offers is that its small size allows full-atom computer simulations of unfolding of this protein^{4–11,13,33–36}. For example, Paschek et al¹³ used replica exchange molecular dynamics simulations and obtained a value of ΔC_p of $0.2 \pm 0.05 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ for unfolding to Trp-cage, very similar to the one reported here. The same group obtained an m-value of $0.41 \pm 0.03 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ after performing simulations on Trp-cage in the presence of urea (Angel Garcia, personal communication). This value is in a reasonable correspondence with the experimental value obtained here. Availability of

the thermodynamic parameter for Trp-cage reported here will further stimulate computational modeling efforts.

Concluding remarks

Overall, the Trp-cage mini-protein is well behaved and shares many similarities to larger-sized proteins, during temperature-induced unfolding in both the presence and absence of urea. Globally fitting individual data sets using a two-state model allowed for reliable estimates of $\Delta G(25^\circ\text{C})$ in the presence of different concentrations of urea. Using the linear extrapolation model, the urea m -value for Trp-cage unfolding was determined. The experimental m -value appears to be best predicted by the empirical transfer model²⁴. The experimental ΔC_p can be well predicted by empirical models that account for the changes in the polar and non-polar accessible surface areas^{21,32}. These experimental thermodynamics data will be essential for validation of atomistic simulations of the effects of urea of protein stability by computational methods.

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Figure 1.

A) Ribbon diagram of the Trp-cage miniprotein representing the average of all NMR conformers of 1L2Y, as computed by PyMol³⁷. Several core amino acids are displayed as ball and stick, including Trp6 (light gray) and the amino acids that effectively cage it (all dark gray). B) Thermal unfolding of Trp-cage in various urea concentrations as monitored by changes in ellipticity at 222 nm. The symbols correspond to the experimental melting profiles obtained at different urea concentrations: 0 M as circles, 1 M as triangles, 2 M as squares, and 3 M as diamonds. Solid black lines represent the fit to a two-state model as described in Materials and Methods.

Figure 2.

The plot of $\Delta G(25^\circ\text{C})$ values versus the concentration of urea. Symbols represent the average of multiple experiments at that urea concentration. The solid line represents the fit to a linear extrapolation model (Equation 10), with m-value of $1.36 \pm 0.08 \text{ kJ mol}^{-1} \text{ M}^{-1}$ and a $\Delta G(\text{H}_2\text{O}) = 2.8 \pm 0.3 \text{ kJ} \cdot \text{mol}^{-1}$.

Table 1

Thermodynamic parameters of unfolding of the Trp-cage mini protein in the presence of different concentrations of urea

[Urea] mol	T_m °C	$\Delta H(T_m)$ kJ/mol	$\Delta G(25^\circ\text{C})$ kJ/mol
0	41±2	56±2	2.6±0.3
0.5	39±2	54±4	2.4±0.3
0.75	37±2	47±7	1.8±0.3
1.0	37±2	49±3	1.8±0.3
1.5	31±2	42±8	0.8±0.4
2.0	26±2	41±2	0.0±0.4
2.5	20±2	40±6	-0.7±0.4
3.0	12±3	36±5	-1.6±0.4
3.5	7±4*	29±6	-2.1±0.5
4.0	-2±4*	30±6	-2.1±0.6

* these are T_s and not T_m values. The values of ΔC_p of $0.3 \pm 0.1 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ and δC_p of $0.013 \pm 0.006 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1} \cdot \text{M}^{-1}$ were used as described by the equation (9).