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Three Easy Pieces

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ABSTRACT

Background: Differential scanning calorimetry is a powerful method that provides a complete thermodynamic characterization of the stability of a protein as a function of temperature. There are, however, circumstances that preclude a complete analysis of DSC data. The most common ones are irreversible denaturation transitions or transitions that take place at temperatures that are beyond the temperature limit of the instrument. Even for a protein that undergoes reversible thermal denaturation, the extrapolation of the thermodynamic data to lower temperatures, usually 25 °C, may become unreliable due to difficulties in the determination of ΔC_p .

Methods: The combination of differential scanning calorimetry and isothermal chemical denaturation allows reliable thermodynamic analysis of protein stability under less than ideal conditions.

Results and Conclusions: This paper demonstrates how DSC can be used in combination with chemical denaturation to address three different scenarios: 1) Estimation of an accurate ΔC_p value for a reversible denaturation using as a test system the envelope HIV-1 glycoprotein gp120; 2) Determination of the Gibbs energy of stability in the region in which thermal denaturation is irreversible using HEW lysozyme at different pH values; and, 3) Determination of Gibbs energy of stability for a thermostable protein, thermolysin.

Keywords: DSC, ICD, chemical denaturation, HIV-1 gp120, HEW lysozyme, thermolysin

1. INTRODUCTION

The conformational stability of a protein is dictated by its Gibbs energy, ΔG° , which is a function of temperature as well as other physical and chemical conditions. Two techniques allow measurement of ΔG° : 1) Differential scanning Calorimetry (DSC); and, 2) Chemical Denaturation measured at a single temperature (ICD). Differential scanning calorimetry measures the temperature dependence of the heat capacity function of the protein (1-3). Subsequent analysis of the heat capacity function provides a complete thermodynamic description of a transition, the changes in enthalpy, entropy and heat capacity, which allows definition of ΔG° at any temperature (3, 4).

There are situations, however, in which the analysis of the heat capacity function becomes extremely difficult or impossible to implement. In this paper we will consider three different conditions and how a combination of DSC and chemical denaturation data can address those difficult situations and provide reliable estimates of ΔG° at different temperatures. The first case to be considered is the situation in which ΔC_p determination is unreliable due to an inaccurate determination of the initial and final baselines (i.e. the heat capacity of the native and denatured states). The second case corresponds to the situation in which the denaturation transition is irreversible. In many cases reversibility is observed at low pH but the transition becomes irreversible at neutral pH, which which usually is the pH of interest (1, 5, 6). The third case corresponds to the situation

in which the protein is extremely stable and denaturation occurs above the temperature range of the instrument.

2. MATERIALS AND METHODS

2.1 Reagents

Lyophilized thermolysin from *Bacillus thermoproteolyticus* (EC 3.4.24.27) and hen egg white lysozyme (EC 3.2.1.17) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used without any further purification. Thermolysin was dissolved in 10 mM hepes, 100 mM NaCl and 10 mM CaCl₂, pH 7.5. Lysozyme was prepared in PBS, pH 7.5. The HIV-1 envelope glycoprotein gp120 from the YU2 strain was expressed in human 293F cells and purified by affinity chromatography using the monoclonal antibody 17b as described in detail elsewhere (7). gp120 was finally dialyzed against PBS, pH 7.5. Ultrapure urea was from J.T. Baker (Center Valley, PA) and molecular grade GdnHCl was from Promega Corporation (Madison, WI, USA). PBS was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other buffer components were from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Differential Scanning Calorimetry

Thermal denaturation experiments were performed using a VP-DSC microcalorimeter from MicroCal/Malvern Instruments LLC (Northampton, MA, USA). The protein samples were dialyzed into buffer, concentrated to ~ 1 mg/mL and thoroughly degassed before loading of the calorimetric cell (~0.5 mL). The reference cell was filled with dialysis buffer. The cells were pressurized at 25 psi and all scans were carried out at a rate of 1 °C/min. The experiments with gp120

and lysozyme were carried out in PBS, pH 7.5. The experiment with thermolysin was carried out in 10 mM hepes, 100 mM NaCl and 10 mM CaCl_2 , pH 7.5.

2.3 Chemical Denaturation Experiments

All denaturation experiments were carried out using Unchained Labs Hunk Automated Protein Denaturation System with fluorescence detector (Unchained Labs, Norton, MA). The excitation wavelength was 280 nm and scans of the emission intensity were recorded between 300 and 500 nm. For each denaturation experiment, protein, buffer and denaturant (urea or GdnHCl) were dispensed into 36 wells with a linear increase in denaturant concentration. The experiments with gp120 were carried out using 0 – 5 M urea in PBS, pH 7.5. The tryptophan emission maximum occurs at 344 nm for the native state and changes to 356 nm upon denaturation of gp120, which allows for easy monitoring of the transition. The experiments with lysozyme were performed at pH 4.0, 5.0, 6.0, 7.0, and 7.5 using a gradient of 0 – 6.4 M GdnHCl. Denaturation experiments with lysozyme at pH 7.5 were also performed using a gradient of 0 – 5.5 M GdnHCl in the presence of 1, 2, and 3 M urea. The buffers used were 10 mM sodium phosphate (pH 6.0 – 7.5), 10 mM sodium acetate (pH 5.0), and 10 mM sodium formate (pH 4.0). All buffers contained 150 mM NaCl. Because of the very high stability of thermolysin, denaturation gradients of 0 – 6.4 M GdnHCl were run in the presence of 2.5, 3, and 3.5 M urea. The buffer used for all experiments with thermolysin was 10 mM hepes, pH 7.5 with 100 mM NaCl and 10 mM CaCl_2 . The protein concentration in the wells was 30 $\mu\text{g/mL}$ in all

experiments. To test the reversibility of chemical denaturation, protein was denatured in high concentration denaturant followed by dilution of the denaturant and incubation for different amount of times in order to assess the kinetics of renaturation. The experimental protocol provided with the instrument allowed for the measurement of full renaturation curves as a function of the incubation time. The protocol was implemented as follows: HEW lysozyme was prepared in a denaturing solution of 5 M GdnHCl plus 2 M urea and diluted 12.5 times into a 36 point linear gradient between 0.4 and 5.9 M GdnHCl and constant 2 M urea. Thermolysin was prepared in a denaturing solution of 6 M GdnHCl plus 3 M urea and diluted 12.5 times into a 36 point linear gradient between 0.48 and 6.88 M GdnHCl gradient and constant 3 M urea. Renaturation of lysozyme was complete within two hours. Renaturation of thermolysin was slower and was measured 11, 16, 24 and 48 hours after dilution from high denaturant concentrations. All measurements including incubation of samples were carried out at 25 °C. Chemical denaturation as a technique to measure protein stability has been around for many years. Reference (8) provide protocols to implement the technique manually.

3. RESULTS

3.1 Case 1: Thermodynamic Analysis when ΔC_p Determination is

Unreliable. The thermal denaturation of the HIV-1 envelope glycoprotein gp120 from the YU2 strain is reversible at neutral pH. gp120 is a glycosylated protein with a molecular weight of more than 100 kDa of which more than 50% corresponds to carbohydrate residues. Previous studies have shown that unliganded gp120 undergoes reversible thermal denaturation in PBS at pH 7.5 (9). **Figure 1** shows the heat capacity function of gp120 in PBS at pH 7.5. The DSC trace is characterized by a native state baseline with a pronounced positive slope whereas the denatured baseline is almost devoid of a temperature dependence. This situation has been observed with different proteins (10-12) and precludes a meaningful thermodynamic analysis since a simple extrapolation as shown in the figure will produce a divergent ΔC_p value at low temperatures and consequently unrealistic ΔH° , ΔS° and ΔG° values at low temperatures. In situations like this, only the values for T_m (60.8 ± 0.1 °C) and ΔH° at T_m (130 ± 10 kcal/mol) can be determined with sufficient accuracy since they are largely independent of an extrapolated ΔC_p value.

For situations like those in **Figure 1**, a precise thermodynamic description of the transition can be obtained by combining the DSC data with ICD data. Chemical denaturation experiments for gp120 were performed at 25 °C using urea as denaturant. **Figure 2** shows triplicate measurements of the chemical

denaturation of gp120 at 25°C. The denaturation of gp120 has a midpoint at 2.7 M urea and is characterized by ΔG° and m values of 7.3 kcal/mol and 2.7 kcal/(mol $\times$ M) respectively. The m -value is equal to the rate of decrease of ΔG with increasing denaturant concentration ($\Delta G = \Delta G^\circ - m[\text{denaturant}]$). In addition, the magnitude of m has been shown to be correlated with the change in solvent accessible surface area during denaturation and therefore also with ΔC_p (13).

Chemical denaturation measurements provide directly the value for ΔG° at 25 °C. Using the standard equation for ΔG° , this value can be combined with the values for $\Delta H_{T_m}^\circ$ and T_m obtained by DSC and derive the following equation for ΔC_p :

$$\Delta C_p = \frac{\Delta G_{298.15}^\circ + \Delta H_{T_m}^\circ \times \left(\frac{298.15}{T_m} - 1 \right)}{298.15 - T_m - 298.15 \times \ln \left(\frac{298.15}{T_m} \right)} \quad (1)$$

According to the above equation, the ΔC_p value of gp120 equals 3.4 kcal/(K $\times$ mol). This ΔC_p value can be used to develop a full thermodynamic description of the transition. **Figure 3** shows the resulting plot of ΔG° , ΔH° and $-T\Delta S^\circ$ as a function of temperature calculated by combining DSC and ICD data. This method can be applied to any unfolding transition provided that the thermal denaturation is reversible and that the unfolding process is the same over the whole temperature interval.

3.2 Case 2: The Structural Stability of Proteins that Exhibit Irreversible Thermal Denaturation. The thermal denaturation of HEW lysozyme is reversible at low pH values but irreversible at neutral pH as shown in Figure 4a, where the irreversibility is reflected by the lack of any transition in the repeated scan of the same sample. In addition, the exothermic drop in the heat capacity at the end of the scan corresponds to precipitation of the aggregated protein. At pH 7.5 HEW lysozyme denatures irreversibly with a midpoint temperature of 72.4 °C and a ΔH° of 112 kcal/mol. Thermal unfolding of HEW lysozyme is reversible at pH values lower than 5.0 where thermodynamic data has been previously obtained by DSC (2, 16-19).

In contrast to thermal denaturation, the chemical denaturation of HEW lysozyme at pH 7.5 is reversible as demonstrated by the overlapping denaturation and renaturation curves in Figure 4b. ICD experiments at 25 °C yield a ΔG° of 10.7 kcal/mol. A combination of DSC and ICD allows definition of the entire pH dependence of HEW lysozyme stability. **Figure 5** shows ΔG° at 25 °C as a function of pH. The data at pH 2 - 4 were obtained by DSC (16, 19). The data at pH 2 ($\Delta G^\circ = 5.0$ kcal/mol) , 2.5 and 4.0 were obtained by digitizing and analyzing figure 2 in ref (16). The ΔG° value of 7.1 kcal/mol at pH 2.0 was calculated from the enthalpy and ΔC_p determined by Pfeil and Privalov (19). The data from pH 4 – 7.5 were obtained by ICD. The experiments demonstrate that for the pH region in which data can be obtained by both DSC and ICD, the ΔG° values coincide. ICD allows continuation of the curve to those regions in which temperature

denaturation becomes irreversible. The combination of DSC and ICD allows determination of the complete pH dependence of ΔG° at 25°C or other low temperatures.

3.3 Case 3: DSC and ICD on Extremely Stable Proteins. Some proteins, especially those from hyper-thermophilic organisms undergo thermal denaturation at temperatures above the operating range of DSC instruments. Also, for some highly stable proteins complete denaturation cannot be achieved by urea or GdnHCl alone. In those situations a plausible alternative is to perform experiments with one denaturant in the presence of various constant amounts of a second denaturant. An example is GdnHCl denaturation in the presence of different concentrations of urea. In this case, ΔG is described by the following equation:

$$\Delta G = (\Delta G^\circ - m_{\text{urea}}[\text{Urea}]) - m_{\text{GdnHCl}}[\text{GdnHCl}] \quad (2)$$

We tested the validity of the approach with HEW lysozyme, which at pH 7.5 is in the determination limit of GdnHCl when used alone. GdnHCl experiments were performed in the presence of 1, 2, and 3 M urea concentrations. The experiments were performed in triplicate and are shown in **Figure 6**. The three sets of experiments were analyzed globally in order to solve Eq. 2 for ΔG° , m_{urea} and m_{GdnHCl} . **Figure 7** shows the results from the global fit. The resulting values

for ΔG° and m_{GdnHCl} for lysozyme in the absence of any urea are 10.7 ± 0.4 kcal/mol and 3.0 ± 0.1 kcal/(mol $\times$ M), respectively. The value for m_{urea} is 1.6 ± 0.1 kcal/(mol $\times$ M). The values are in agreement with the ones obtained with GdnHCl alone and with previously reported values (20, 21).

A thermostable protein that experiences irreversible denaturation is thermolysin which undergoes thermal denaturation at around 90 °C at pH 7.5 (22). In this case, either urea or GdnHCl by themselves are not able to achieve complete denaturation. As in the case of HEW lysozyme, GdnHCl in the presence of different constant concentrations of urea was used to determine ΔG° , m_{urea} and m_{GdnHCl} . **Figure 8a** shows the GdnHCl denaturation of thermolysin in the presence of 3 M urea together with renaturation scans recorded 11, 24, and 48 hours after dilution into wells containing different concentrations of denaturant (see section 2.3 for details of the method). **Figure 8b** shows the C1/2 values obtained from the renaturation curves as a function of time. The C1/2 value for the renaturation of thermolysin requires almost 48 hours after dilution of the denatured protein before the C1/2 value for the renaturation scan coincides with that obtained in the denaturation experiment.

Complete datasets for the structural stability of thermolysin were obtained from GdnHCl denaturation experiments performed in the presence of 2.5, 3, and 3.5 M urea concentrations. The denaturation curves are shown in **Figure 9**. **Figure 10** shows a global fit of the fraction of denatured thermolysin as a function of the

concentrations of GdnHCl and urea. The value for ΔG° obtained from global non-linear least squares analysis are 17.4 ± 0.8 kcal/mol and the values for m_{GdnHCl} and m_{urea} are 3.4 ± 0.2 and 2.6 ± 0.3 kcal/(mol $\times$ M), respectively. As expected, the stability of thermolysin at room temperature is extremely high, around 80,000-fold higher than HEW lysozyme at neutral pH ($K^{\text{thermolysin}}/K^{\text{lysozyme}} = e^{(-\Delta\Delta G^\circ/RT)}$).

4. CONCLUSIONS

There are situations, like the three considered in this paper, when stability data obtained by DSC can benefit from data obtained by ICD. The integration of two orthogonal techniques capable of measuring ΔG° provides a more robust and complete thermodynamic characterization of protein stability, especially in those situations in which a single technique can only provide incomplete data.

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FIGURE LEGENDS

Figure 1. The temperature dependence of the heat capacity function of gp120. The dashed lines are linear extrapolations of the baselines that correspond to the native and denatured states of gp120. This behavior is often seen in DSC of proteins and precludes accurate determination of ΔC_p .

Figure 2. The fraction of denatured gp120 obtained as a function of urea concentration for three different protein samples in PBS, pH 7.5, at 25 °C. The protein concentration was ~ 30 $\mu\text{g/mL}$. Analysis of the data yields $\Delta G = 7.30 \pm 0.08$ kcal/mol, $m = 2.70 \pm 0.05$ kcal/(mol $\times$ M), and $C1/2 = 2.71 \pm 0.03$ M.

Figure 3. The temperature dependence of the Gibbs energy of stability for gp120 and its enthalpic and entropic components. The values were calculated by combining data from DSC and ICD.

Figure 4. (a) The temperature dependence of the heat capacity function of HEW lysozyme at pH 7.5. The transition is irreversible as reflected by the lack of any transition in the repeated scan of the same sample. (b) Chemical denaturation and renaturation scans of lysozyme at pH 7.5, using GdnHCl as denaturant. The overlapping curves demonstrate the reversibility of the chemical denaturation of lysozyme. Both ICD experiments in the figure were performed in the presence of a constant concentration of 2 M urea (see method section 2.3 for details).

Figure 5. Gibbs energy of stability for lysozyme as a function of pH. The values in the region pH 4 – 7.5 indicated by the filled circles were determined by ICD. The values indicated by the filled squares below pH 4.0, where thermal denaturation of lysozyme is reversible, were calculated from values for ΔH° and ΔC_p determined by DSC in refs (16, 19) (see text for details).

Figure 6. GdnHCl denaturation of lysozyme at pH 7.5 in the presence of 1 (■), 2 (▲), and 3 M (●) urea. The protein concentration was ~ 30 µg/mL.

Figure 7. Global fit of the fraction denatured lysozyme as a function of the concentration of GdnHCl and urea in phosphate buffer, pH 7.5. The global fit is used to determine simultaneously ΔG° , m_{GdnHCl} and m_{urea} by non-linear least squares.

Figure 8. Chemical denaturation and renaturation scans of thermolysin using GdnHCl as denaturant at pH 7.5, (10 mM hepes, 100 mM NaCl, 10 mM CaCl₂) in the presence of a constant concentration of 3 M urea. The renaturation scans were recorded 11, 16, 24, and 48 hours times after the protein in high denaturant was diluted into wells containing buffer with different concentrations of denaturant. (For ease of viewing, the curve obtained after 16 hours has not been included in the figure.) b) C1/2 obtained from the renaturation scans recorded 11, 16, 24, and 48 hours after dilution of the denatured protein. The C1/2 value obtained from the denaturation scan has been added for comparison (48 h).

Figure 9. The fraction denatured thermolysin as a function of the concentration of GdnHCl in the presence of different urea concentrations: 2.5 (■), 3 (▲), and 3.5 M (●). The conditions are the same as described in the legend to **Figure 8**.

Figure 10. Global fit of the fraction of denatured thermolysin as a function of the concentration of GdnHCl and urea. As in Figure 7, the global fit allowed simultaneous determination of ΔG° , m_{GdnHCl} and m_{urea} .

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