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Isothermal chemical denaturation of large proteins: Path-dependence and irreversibility

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ABSTRACT

State functions (e.g., ΔG) are path independent and quantitatively describe the equilibrium states of a thermodynamic system. Isothermal chemical denaturation (ICD) is often used to extrapolate state function parameters for protein unfolding in native buffer conditions. The approach is prudent when the unfolding/refolding processes are path independent and reversible, but may lead to erroneous results if the processes are not reversible. The reversibility was demonstrated in several early studies for smaller proteins, but was assumed in some reports for large proteins with complex structures. In this work, the unfolding/refolding of several proteins were systematically studied using an automated ICD instrument. It is shown that: (i) the apparent unfolding mechanism and conformational stability of large proteins can be denaturant-dependent, (ii) equilibration times for large proteins are non-trivial and may introduce significant error into calculations of ΔG, (iii) fluorescence emission spectroscopy may not correspond to other methods, such as circular dichroism, when used to measure protein unfolding, and (iv) irreversible unfolding and hysteresis can occur in the absence of aggregation. These results suggest that thorough confirmation of the state functions by, for example, performing refolding experiments or using additional denaturants, is needed when quantitatively studying the thermodynamics of protein unfolding using ICD.

Introduction

The ability to assess and modulate a protein's stability is a driving force behind many aspects of biotherapeutic development, from lead candidate selection to formulation development to downstream processing. Historically, the most common and accessible method to measure protein thermal stability has been differential scanning calorimetry (DSC). This technique consists of thermally perturbing a solution over a wide range of temperature to obtain accurate measures of T_m and ΔCp, which may be used to determine the relevant state functions (e.g., ΔH) for protein unfolding under appropriate conditions. The major drawback of this technique is the tendency for thermal denaturation to occur in an irreversible manner, especially through protein aggregation. In general, this invalidates thermodynamic models that require reversibility and prevents the user from determining the relevant state functions. Isothermal chemical denaturation (ICD) is an alternative technique to obtain thermodynamic parameters that tends not to have this same drawback.

ICD involves systematically perturbing the structure of proteins in solution by the step-wise addition of chaotropes, such as urea. The ability of such chemical agents to denature proteins was established over a century ago [1,2]. However, it took until 1964 for Tanford to demonstrate that the backbone and side chains of polypeptides had a lower free energy in the presence of urea or guanidine solutions than they did in water, favoring protein unfolding [3]. Shortly thereafter, Greene and Pace reported that the standard free energy of denaturation, ΔG^0, varied linearly with the concentration of denaturant [4]. Thus, they proposed the linear extrapolation model (LEM):

$$\Delta G = \Delta G^0 - m[D] \quad (1)$$

where ΔG^0 is the change in free energy upon denaturation in the absence of denaturant and m is the dependence of ΔG on the denaturant (e.g., urea), [D] is the denaturant concentration. ΔG^0 can be calculated as:

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$$\Delta G^0 = m^* c_{1/2} \quad (2)$$

where $c_{1/2}$ represent the concentration of denaturant at which 50% of the protein molecules are folded and 50% of the protein molecules are unfolded (i.e., $\Delta G = 0$). Subsequent studies have applied this model to the unfolding of dozens of proteins and have reported the calculated ΔG^0 values [5–10].

It is important to note that the accuracy of ΔG^0 and the validity of the results is dependent on the reversibility of the denaturation process, which was demonstrated in early reports using small, globular proteins [11–15]. More recent literature has applied the LEM to larger proteins, such monoclonal antibodies (mAbs), or protein subunits, without explicitly demonstrating reversibility and/or comparing the impact of critical experimental parameters [16–18]. In other cases, irreversibility of protein denaturation was reported, but was solely attributed to aggregation of the unfolded state. Here, we utilize an automated, high-throughput ICD instrument, which is capable of precisely controlling sample conditions (e.g., incubation time).

We present several examples to demonstrate important caveats associated with the accurate determination of chemical stability for large, therapeutically-relevant proteins. These include: (i) confirming that the protein is fully unfolded (e.g., comparing multiple denaturants), (ii) confirming that unfolding is under equilibrium conditions and is not path-dependent (e.g., comparing multiple incubation times), (iii) ensuring that the probe used is appropriately monitoring the underlying physical phenomenon, (iv) ensuring that the unfolding is reversible. The significance of these four criteria demonstrated in this work draw attention to the experimental conditions used for the quantitative reporting of ΔG^0 values.

Materials & methods

Proteins and reagents

mAb-PTI and mAb-PFA are both CHO-expressed monoclonal IgG1κ antibodies. mPt-PFR is a recombinant protein. All three proteins were produced and/or purified by Pfizer Inc. (New York, NY, USA). Bovine serum albumin (BSA) was purchased as a lyophilized powder from Sigma (St. Louis, MO, USA). Ultrapure urea and succinic acid were obtained from Fisher Scientific (Waltham, MA, USA); molecular-grade guanidinium HCl (GdnHCl) and l-histidine were obtained from Sigma (St. Louis, MO, USA).

Buffer preparation

The formulation buffers consisted of: 20 mM succinate, pH = 6.0; 20 mM histidine, pH = 6.0; 20 mM histidine, pH = 6.4 for mAb-PTI, mAb-PFA, and mPt-PFR respectively. For each protein, two denaturant buffers were prepared that were identical in composition and pH to the formulation buffer, but also contained either ~7 M GdnHCl or ~9 M urea. These buffers were prepared fresh throughout the study to prevent degradation of the denaturants in solution. All buffers were filter sterilized with a 0.2 μm Nalgene filter (Thermo Scientific, Waltham, MA, USA). The denaturant concentration was verified by measuring the refractive index of all solutions using a Leica Auto Abbe Refractometer (Model 10500B) equilibrated at 25 °C using a Neslab RTE-111 circulating water bath temperature controller and inserting the values into the online calculator: http://sosnick.uchicago.edu/gdmcl.html.

Circular dichroism

Near-UV Circular Dichroism (CD) spectroscopy measurements were performed on a Jasco J-815 spectrometer. All measurements were carried out using a 10 mm rectangular quartz cell and the temperature was maintained at 20 °C using an automated water bath. Near-UV CD spectra were measured from 250 to 360 nm. Molar circular dichroism was calculated as:

$$\Delta\varepsilon = (\Theta^* MW)/(32980^* C^* l) \quad (3)$$

Where $\Delta\varepsilon$ is molar circular dichroism in $M^{-1}cm^{-1}$, θ is the ellipticity (raw CD output) in mdeg, MW is the molecular weight in g/mol, C is the protein concentration in g/L, and l is the path length of the cuvette in cm. Protein concentrations were ~0.5 mg/mL.

Isothermal chemical denaturation

Automated ICD studies were performed using GdnHCl and/or urea as the denaturant(s), using an Unchained Labs HUNK system (Pleasanton, CA, USA), which measures fluorescence emission. The excitation wavelength was set to 290 nm to monitor tryptophan fluorescence and the emission intensities were recorded between 310 and 500 nm. In some cases, an excitation wavelength of 280 nm was used to monitor intrinsic protein fluorescence and the emission intensities were recorded between 300 and 500 nm. The gain setting was set to 100 and the excitation and emission bandwidths were both set to 10 nm. This instrument automatically prepares and measures all samples starting from stock buffer, denaturant, and protein solutions. In general, 24-point linear gradients were automatically generated from the formulation buffers and denaturant buffers. Unless otherwise specified, protein stock solutions were prepared at 1 mg/mL to account for the 12.5-fold dilution that occurs upon mixing with varied amounts of buffer and denaturant. Protein concentrations were constant for all samples in a given experiment. The incubation time between mixing with denaturant/buffer and the measurement was user-specified and automatically controlled to be identical for all samples (data points) in a given experiment. Refolding experiments were performed by first incubating the protein for at least 24 h in a high (i.e., denaturing) concentration of either urea or guanidine, and then mixing with a denaturant-free buffer in the same manner as preparing samples for denaturation (i.e., varying the denaturant concentration while maintaining a constant protein concentration).

Data analysis was performed with the Unchained Labs software, which was provided with the instrument. For each protein and buffer combination, the λ_{max} for the fluorescence signal of the native state (e.g., 350 nm) and the unfolded state (e.g., 362 nm) was manually determined. The ratio of the fluorescence intensity (e.g., 362 nm/350 nm) can be plotted against denaturant concentration as the raw data, or used to calculate the fraction of the proteins that are unfolded (Fraction Denatured) using Eq (4).

$$FD = (S_{obs} - S_N)/(S_D - S_N) \quad (4)$$

where F_D is the fraction of protein that is denatured (Fraction Denatured), S_{obs} is the observed signal (e.g., peak intensity ratio); S_N is the signal corresponding to the native state, explicitly defined as:

$$S_N = m_F[D] + b_N \quad (5)$$

where m_F is the dependence of the native state signal on the denaturant concentration, and b_N is the intercept of the native state signal; S_D is the signal corresponding to the denatured state, explicitly defined as: