Quantify protein stability with ΔG

Introduction

Biologics stability determinations often rely on forced degradation methods to rank constructs or optimize formulations. The most well-established methods rely on temperature stress to disrupt the three-dimensional protein structure. While these methods are good surrogates for room-temperature stability assessments, they do not always distinguish conditions that are of similar stability.

Chemical denaturation methods help address the gap between thermal degradation and longer term stability experiments. Chemical denaturation is an equilibrium experimental method in which increasing concentrations of a denaturant, such as urea or guanidine hydrochloride, are introduced to the protein solution. Chemical denaturation experiments are performed at ambient temperatures. Spectroscopic methods, such as intrinsic fluorescence, are used to determine the Gibbs free energy (ΔG) required to unfold the protein.

Chemical denaturation is not widely used, in part because of the tedious process for manual set-up, and the data analysis challenges for more complex unfolding pathways. This unfortunately means the largest benefit of the method is not used: namely, ΔG measurements are a direct calculation of the amount of unfolded protein present in a sample. In contrast to other stability determinations, ΔG measurements are quantitative.

Unchained Labs addresses the challenges of ΔG determinations with two platforms. UNcle (Figure 1) is an all-in-one stability platform that enables multiple applications with one instrument. Fluorescence, static light scattering (SLS) and dynamic light scattering (DLS) detection methods are used to characterize protein stability. Chemical denaturation is one of the many protein characterization applications that can be performed on UNcle. Preparation of samples is done by the user or an automated liquid handler, incubated offline at ambient temperatures, and then read on UNcle.

Figure 1: UNcle is an all-in-one protein stability measurement platform. Among the built-in application capabilities is the ability to measure ΔG from denaturation curves that have been created offline.

Figure 2: The HUNK fully automates ΔG measurements. The HUNK formulates denaturation curves from protein and formulation stocks, incubates the samples, reads the samples, and analyzes the data.

The data is analyzed automatically upon completing an experiment.

The HUNK (Figure 2) fully automates the chemical denaturation experiment. The operator adds stock protein, formulation, and denaturant, and programs the experiment they want to run. The HUNK formulates the chemical denaturation curve and measures intrinsic fluorescence. Up to 96 differ-

ent ΔG measurements can be made unattended. The software automates the curve fitting required to generate a ΔG value. Experiment set-ups are flexible and can be used to calculate separate ΔGs for two-state and three-state proteins.

In this technical note we show how chemical denaturation experiments can be performed utilizing UNcle or the HUNK and highlight the benefits of each, depending on your workflow.

Methods

Manual formulation

Bovine IgG (Sigma, #I5506) was prepared at 12.5 mg/mL in 17 mM phosphate buffer, pH 8. A 24-point chemical denaturation curve was made by adding and mixing 10 µL protein to varying ratios of phosphate buffer, and the same buffer containing 6M guanidine hydrochloride (GdnHCl). The final volume of each well was 125 µL. The final concentration of Bovine IgG was 1 mg/mL in each well. Samples were covered and incubated overnight at room temperature to ensure equilibrium was reached before detection.

Fluorescence reader detection

Samples were prepared as above, placed in a 96-well black microtiter plate (Greiner, #655209), and read on a SpectraMax i3 plate reader (Molecular Devices) with excitation at 280 nm. Data was exported to Excel and analyzed with HUNK data analysis software, using the ratio of fluorescence at 350:330 nm.

UNcle detection

Samples were prepared as described in the manual formulation method. Nine µL of each sample was pipetted in duplicate into UNis. Samples were read using the UNcle ΔG application with excitation at 266 nm. UNcle Analysis Software fit the data and calculated the ΔG using the ratio of fluorescence at 350:330 nm.

HUNK formulation and detection

Bovine IgG was prepared at 12.5 mg/mL in 17 mM phosphate buffer, pH 8, and loaded into the instrument. Stock solutions of the formulation buffer and 6M GdnHCl were prepared and loaded into the instrument. HUNK automatically formulated the 24-point curve in duplicate by dispensing and mixing the appropriate amounts of formulation buffer, denaturant, and protein, with a constant final volume of 125 µL/well. Curves were incubated overnight to be consistent with the manual experiment. The HUNK read the plate with a 280 nm excitation and recorded emission between 300–500 nm. The data was fit using HUNK analysis software and ΔG values were determined. To compare data with the other detection methods, ΔG was calculated using the ratio of fluorescence at 350:330 nm.

Results

Manual formulation

Bovine IgG was chosen as a model system for this experiment. It is a polyclonal antibody that unfolds rapidly in the presence of a denaturant. When exposed to GdnHCl, it undergoes one distinct transition. In contrast, an engineered monoclonal antibody of higher purity will undergo multiple transitions, often with higher ΔG values.

Chemical denaturation methods have traditionally been performed manually. For comparison purposes, we formulated the 24-point denaturation curve manually with a gradient of GdnHCl concentrations between 0–5.52 M. Samples were equil-

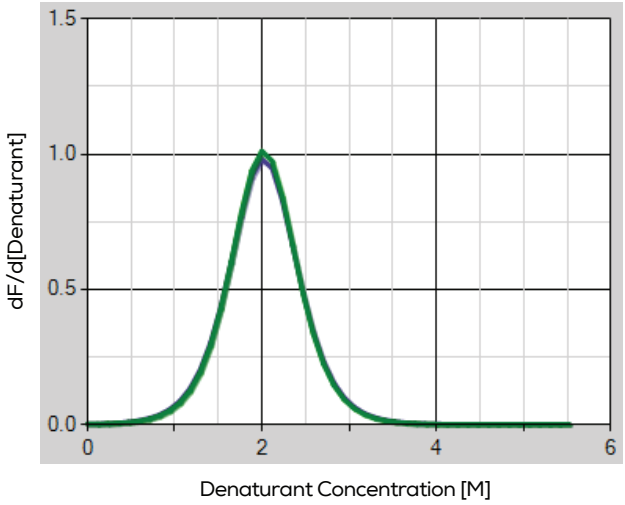

Figure 3: The first derivative of the fluorescence curves, as calculated by HUNK software. Two replicate curves are overlaid, in green and blue. The maximum height at 2 M corresponds to the $C_{1/2}$ value for this curve.

ibrated overnight at room temperature, and then read on a fluorescence plate reader. To calculate the parameters of interest from the plate reader data, a nonlinear least squares regression analysis was performed to generate the denaturation curve. The first derivative yields a peak maximum which corresponds to $C_{1/2}$, the concentration of denaturant at which half of the protein is unfolded. For bovine IgG, there is a single peak at 2 M denaturant (Figure 3).

The equilibrium constant, K, describes the ratio of the concentration of unfolded (denatured) protein to folded (native) protein in a sample, and is related to ΔG:

$$\Delta G = -RT \ln K$$

The m value, which corresponds to the slope, and describes the dependence of free energy on denaturant concentration, is related to the other parameters:

$$\Delta G = m \times C_{1/2}$$

The fraction of denatured protein in a given sample is related to ΔG:

$$F_d = \frac{K}{1+K}$$

There are literature references that can guide a user towards various graphing and calculation methods. For the sake of consistency and simplicity in fitting the manual experimental data, we used HUNK software, which has built-in fitting algorithms. The output of the software can be seen in Figure 4, which shows high reproducibility of the replicate data, even with manual pipetting. These curves also show the transformation of the physically observable values (the ratio of fluorescence intensity at two wavelengths) to the fraction of protein denatured. For bovine IgG, the calculated ΔG was 4.77 kcal/mol, and the sample in the vial contains about 320 ppm of denatured protein.

UNcle detection

The manually-prepared samples were loaded into UNis and read by UNcle. Only 9 µL of sample is required for each point on the curve, so if protein availability is a concern, this method

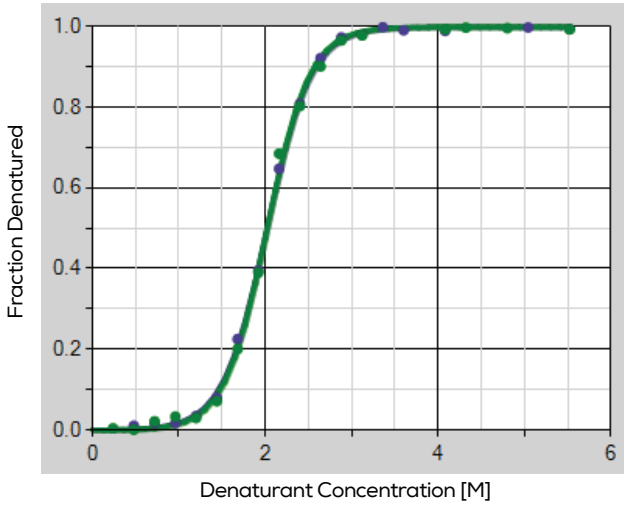

Figure 4: The fraction of protein denatured, as a function of denaturant concentration, as calculated by HUNK software. Two replicate curves are overlaid, in green and blue. The average values and standard deviations for the two replicates are shown in the table.

	Average
ΔG (kcal/mol)	4.77 ± 0.1
m (kcal/mol*M)	2.37 ± 0.05
$C_{1/2}$ (M)	2.02
Amount denatured (ppm)	320 ± 53.7

can provide a significant advantage over other detection methods. UNcle software also automatically analyzes the data immediately upon completion of the experiment. If automated liquid handling capabilities are paired with UNcle, the hands-on laboratory time required can decrease significantly. The data analyzed by UNcle for two replicates is shown in Figure 5, where again the data is highly reproducible, and the ΔG is calculated to be 4.17 kcal/mol. The slight difference in ΔG values between the data collected by the plate-reader versus UNcle is most likely due to the difference in excitation wavelengths between the two systems, from 266 nm to 280 nm. Both are valid wavelengths for exciting fluorescent amino acids, as long as direct comparisons made between measured values and rankings of proteins are confined to a single platform.

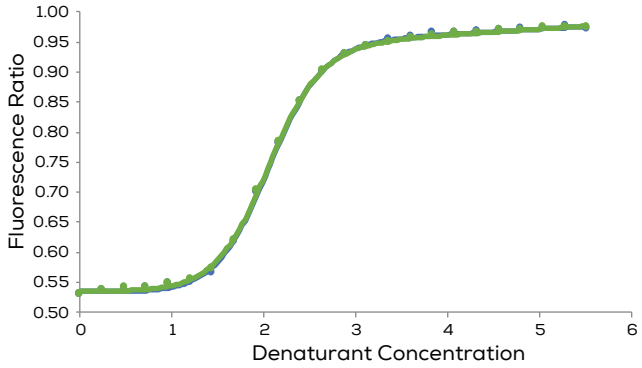

Figure 5: The ratio of fluorescence at 350:330 nm, as a function of denaturant concentration, as calculated by UNcle Analysis software. Two replicate curves are overlaid, in green and blue. The average values and standard deviations for the two replicates are shown in the table.

	Average
ΔG (kcal/mol)	4.17 ± 0.05
m (kcal/mol*M)	2.04 ± 0.03
$C_{1/2}$ (M)	2.04
Amount denatured (ppm)	885 ± 72

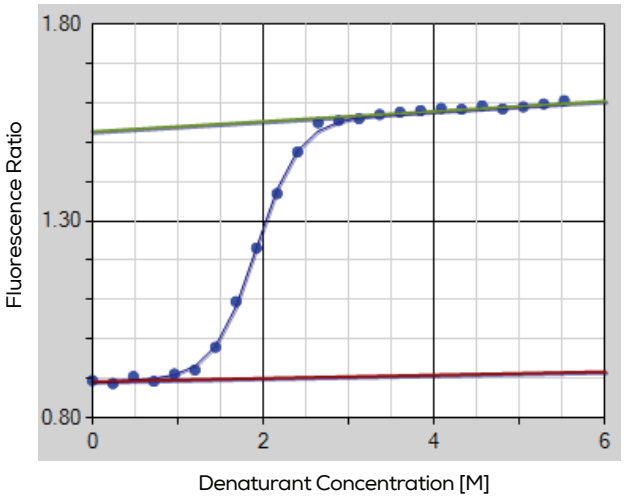

Figure 6: The ratio of fluorescence at 350:330 nm, as a function of denaturant concentration, as calculated by HUNK software. The native and denatured state baselines are shown in red and green, respectively. Average values and standard deviations for two replicates are shown in the table.

	Average
ΔG (kcal/mol)	4.76 ± 0.25
m (kcal/mol*M)	2.45 ± 0.07
$C_{1/2}$ (M)	1.94 ± 0.04
Amount denatured (ppm)	340 ± 136

HUNK formulation and detection

The final method described here for performing chemical denaturation experiments used the HUNK platform. The stock bovine IgG, stock denaturant (6M), and stock formulation were placed in the instrument, and the 24-pt curve was directly generated by the HUNK platform.

After formulating each point on the curve and incubating the samples, the HUNK read the samples and fit the data. As with UNcle, various options are available for analyzing the fluorescence data, but for consistency with the other methods, the 350:330 nm ratio was selected. Values obtained were very similar to the manual experiment read in the plate reader, with a ΔG value of 4.76 kcal/mol (Figure 6).

Comparison between methods

Chemical denaturation and ΔG determinations have not traditionally been broadly used for biologic stability assessments. This is primarily due to the sample volume requirements and the extensive amount of hands on time required to perform the technique. UNcle and HUNK take two different approaches to addressing these issues.

UNcle enables ΔG experiments by providing the ability to quickly arrive at a ΔG with low sample volumes. UNcle minimizes the amount of protein required, as each sample well is only 9 µL. Since protein will typically be diluted 1:10 or more in denaturant and formulation buffer, a 24-pt curve run in duplicate will require a minimum of 50–100 µL of protein. In practice, this volume will increase significantly if automated liquid handling is utilized, as most liquid handlers will have a volume of solution that cannot be accessed (a 'dead volume') and may have to dispense at higher volumes per well to maintain accuracy and precision. UNcle has a capacity of 48 wells at a time, and running multiple curves will necessitate manually swapping UNis in and out of the instrument. UNcle has numerous other protein

Task	Manual	UNcle	HUNK
Hands-on formulation time	1.5 hours	1.5 hours	5 minutes
Reading 2 curves	2 minutes*	2 minutes	1 hour**
Data analysis	2 hours	<1 minute	5 minutes

Table 1: Estimated hands-on time required for the three described methods, for formulating a 24-point curve in duplicate, reading the fluorescence intensity of each sample, and calculating ΔG values.

*The time for reading 48 wells in a plate reader can vary based on the data quality required.
** HUNK times are unattended use. Formulation and detection are performed concurrently for different samples. On average, a ΔG value is generated every 60 minutes.

characterization applications available within the platform, and can easily be utilized for occasional ΔG determinations.

HUNK is designed as a complete solution for ΔG measurements. Liquid handling capabilities are incorporated within the instrument. The operator only needs to add stock solutions and empty plates. HUNK scheduling software tracks when each well is mixed—so rather than requiring an overnight incubation, shorter incubation times can be used where each well is read after the exact same incubation time. As a result, a ΔG result can be generated once an hour on average when running more than one curve. More complicated methods, such as auto-formulation experiments, can also be carried out in HUNK by further exploiting its liquid handling capabilities. The HUNK can be fully loaded and run unattended for up to 96 hours, generating up to 96 ΔG determinations without manual intervention.

Summary

This comparison of three different ways of performing chemical denaturation experiments allows users to compare the hands-on time required for each (Table 1), in order to determine which one best suits their needs at a given time. As project needs can change quickly, it can be advantageous to have a versatile platform like UNcle that enables many different applications for characterizing proteins. However, if chemical denaturation experiments and ΔG determinations are a consistent part of biologic or formulation stability assessments, or the proteins of interest involve multiple domains with more than one transition, the HUNK can streamline this technique and the subsequent data analysis. Making chemical denaturation experiments more accessible, and ΔG measurements more routine can provide a quantitative stability assessment method that may support or further refine thermal screening results. UNcle and HUNK each allow researchers to explore how formulation and constructs changes can provide quantifiable information on changes to biologic stability.

References

1. Freire E, Schön A, Hutchins BM, Brown RK. 2013. Chemical denaturation as a tool in the formulation optimization of biologics. *Drug Discov Today* 18(19-20): 1007–1013.

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