Meet Hunky: Stability in hi-def

Introduction

Hunky is the only automated platform for quantifying biologic stability and aggregation pathways with ΔG. By automating the previously labor-intensive and tedious steps of sample preparation, incubation, fluorescence data acquisition, and data analysis, Hunky makes chemical denaturation experiments simple and hands-off (Figure 1). ΔG is a quantitative stability measurement that describes the ratio of folded to unfolded protein present in a given sample at biologically relevant temperatures (Ref. 1). Additionally, aggregation propensity and aggregation pathway information can be determined by analyzing ΔG as a function of protein concentration (Ref. 2). Automation and low sample requirements make ΔG measurements for stability and aggregation a good fit at several stages throughout the biologic development process.

Hunky provides 4 applications to assess protein stability and aggregation. The $C_{1/2}$ application provides a quick structural stability screen by determining the concentration of denaturant it takes to unfold half of the protein ($C_{1/2}$). The ΔG application takes the measurement a step further by quantifying stability and determining the amount of denatured protein present in a protein sample at equilibrium at ambient temperatures. By providing stability data orthogonal to traditional thermal ramp stability measurements, the $C_{1/2}$ and ΔG applications aid in ranking and fine-tuning the stability of biologic candidates.

By measuring ΔG at multiple concentrations aggregation propensity and aggregation pathways can be determined. The Agg Path application is a quick measure of aggregation that provides immediate insight into the aggregation risk for protein samples. The ΔG_{trend} application captures a detailed picture of aggregation propensity and the aggregation pathway providing the data needed to move forward with confidence that aggregation is not a risk.

Figure 1: Hunky: Fully automated isothermal chemical denaturation for stability and aggregation assessment.

This technical note highlights how Hunky can be used in your lab to quantify molecule stability, aggregation propensity and aggregation pathway by automating isothermal chemical denaturation with fast and rigorous experimental applications.

Stability and aggregation with chemical denaturation

Biologics exist in an equilibrium between the native and denatured states in solution. This equilibrium is governed by the equilibrium constant K, where $K = e^{-\Delta G/RT}$ (Figure 2). Conformational stability is dictated by the Gibbs free energy, ΔG. A higher ΔG value describes an equilibrium that favors the native state, and therefore less denatured protein is present in the sample. Since ΔG is a quantitative measurement the fraction of denatured protein (F_d) can be calculated by $F_d = K/(1+K)$. For example a protein with ΔG = 8.2 kcal/mol would have 1 ppm of denatured protein,

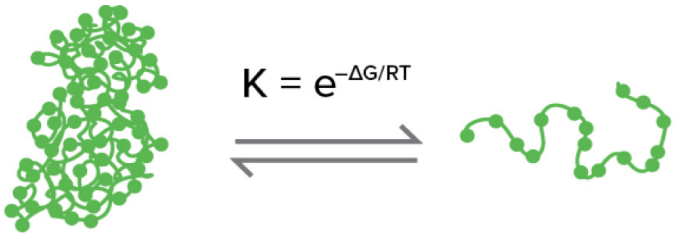

Figure 2: All biologics, even the best formulations, exist in equilibrium between the native and denatured state. Chemical denaturation makes it possible to measure ΔG for the protein in your sample vial at biologically relevant temperatures.

while a protein with ΔG = 4.1 kcal/mol has 1,000 ppm of denatured protein present (Table 1). The ΔG for proteins in the native state at equilibrium and room temperature can be measured by performing isothermal chemical denaturation experiments.

ΔG kcal/mol	Stability	Fraction denatured
9.6	Small amount of denatured protein	1/10,000,000
8.2		1/1,000,000
6.8		1/100,000
5.5	Moderate amount of denatured protein	1/10,000
4.1		1/1,000
2.7	Large amount of denatured protein	1/100
1.3		1/10
0		1/2

Table 1: ΔG quantifies stability. Since $K = e^{-\Delta G/RT}$ and $F_d = K/(1+K)$ the fraction of denatured (F_d) can be calculated making it clear which molecules and sample conditions are stable.

Chemical denaturation experiments measure the change in intensity and peak position of the intrinsic protein fluorescence as the concentration of a denaturant (most commonly urea or guanidine hydrochloride) is increased. The raw fluorescence spectra are then analyzed by the barycentric mean (BCM), wavelength ratio, single wavelength, or wavelength difference analysis methods to create the denaturation curve (Figure 3A). The analysis of a denaturation curve produces the two parameters, $C_{1/2}$ and m, needed to calculate the native state ΔG by $\Delta G = C_{1/2}{*}m$ (Ref. 1). $C_{1/2}$ is the concentration of denaturant at the midpoint of the unfolding transition, which represents the state in which half of the protein population is unfolded. ΔG and $C_{1/2}$ report on the structural stability of proteins, where higher ΔG (or $C_{1/2}$) values indicate a more stable protein. For proteins that undergo multiple unfolding events the ΔG for each transition is calculated (Figure 3B). The nonlinear least squares regression analysis of chemical denaturation curves is done automatically in Hunky Analysis software, making it easy to extract ΔG and $C_{1/2}$.

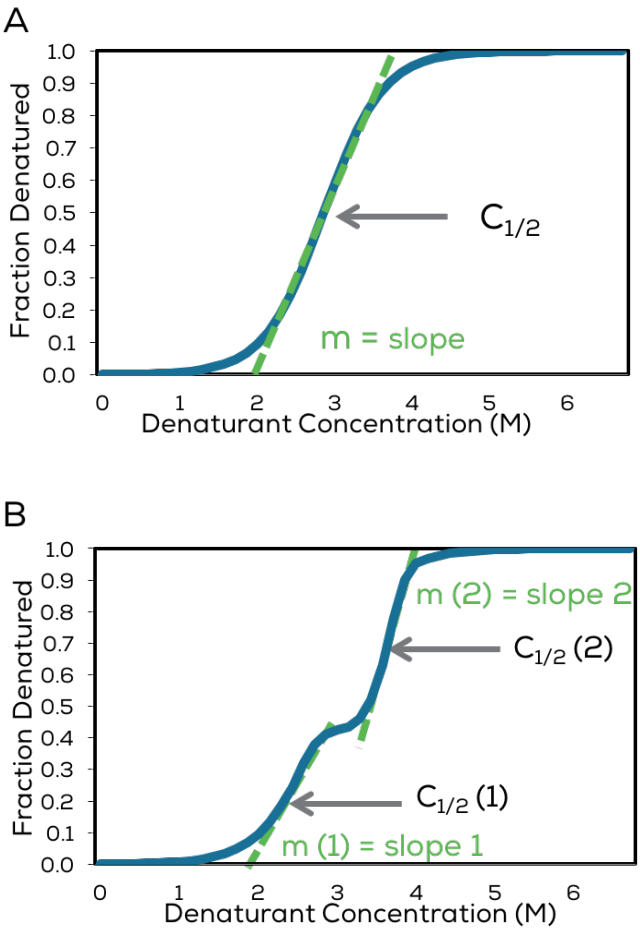

Figure 3: Biologics can undergo single (A) or multiple unfolding transitions (B). Hunky Analysis software automatically calculates the parameters for each transition, for up to 4 states.

Ideally stability is optimized by maximizing ΔG of a given sample to lower the amount of denatured protein in the tube, since the denatured state is often highly prone to aggregation. Aggregates can serve as a thermodynamic sink, shifting the equilibrium away from the native state, creating a risk for long-term stability. Reducing the amount of denatured protein in the sample tube by maximizing ΔG can reduce aggregation risks, but this provides only a partial look at aggregation. To determine a sample's aggregation propensity and aggregation pathway, ΔG must be measured at low and high protein concentrations.

Protein unfolding is a unimolecular, concentration-independent process in the absence of aggregation. In this case ΔG will not change as protein concentration increases. If aggregation occurs, however, the measured ΔG values show a dependence on protein concentration as equilibrium is shifted away from the desired native state (Ref 2). The direction of change, either positive or negative, is indicative of the aggregation pathway (Figure 4). Native state aggregation shifts the equilibrium towards the native state, increasing ΔG at higher protein concentrations (Figure 5). Aggregation from the denatured state has the opposite effect, shifting equilibrium away from the native state and lowering ΔG at higher protein concentrations. Aggregation propensity and pathway are measured either with two points with Agg Path, or over a multi-point concentration series with ΔG_{trend}. Both methods provide unique aggregation information not provided by other techniques that makes it possible to address aggregation on day one.

Hunky Applications

$C_{1/2}$

Structural stability can be quickly assessed by measuring $C_{1/2}$. The $C_{1/2}$ application collects denaturation curves with a minimal number of points, increasing throughput and lowering the volume of protein required. The 8-point denaturation curves generate robust and accurate $C_{1/2}$ values for qualitative stability analysis. $C_{1/2}$ can be paired with thermal stability measurements (T_m or T_{onset}). Adding an orthogonal stability measurement collected at biologically relevant temperatures allows for more informed decisions on which candidates to move forward.

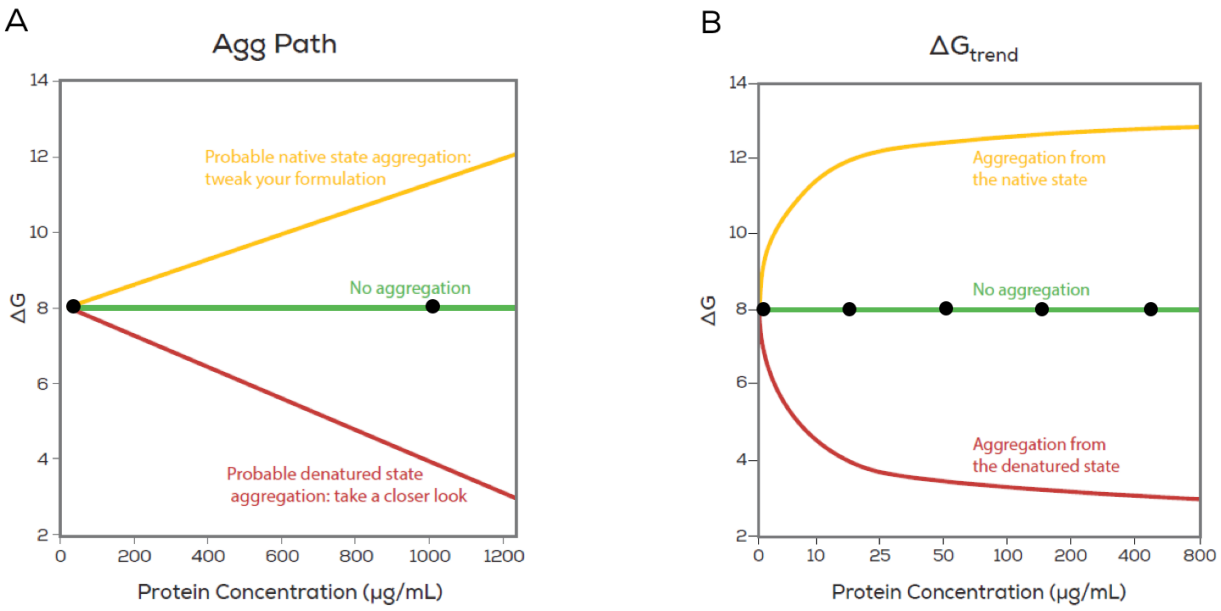

Figure 4: If aggregation is present, ΔG changes as a function of protein concentration. The aggregation pathway is determined by an increase or decrease in ΔG at higher concentrations. Knowing the aggregation pathway makes it easier to address aggregation risks. Hunky measures aggregation pathway and propensity with the 2-point Agg Path experiment (A) or the detailed ΔG_{trend} experiment (B).

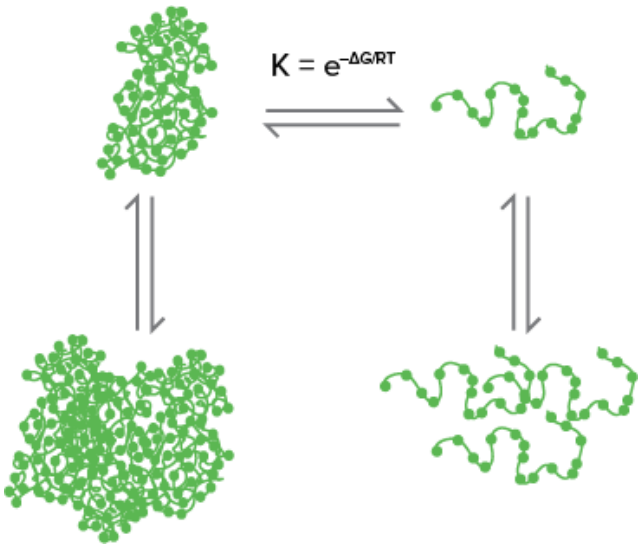

Figure 5: Aggregation can act as a thermodynamic sink, shifting the equilibrium away from the desired native state.

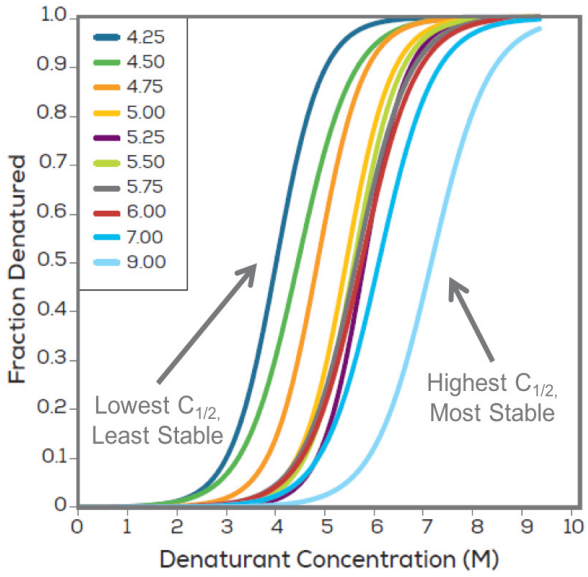

Figure 6: Measuring $C_{1/2}$ provides a quick structural stability screen to rank or bin samples and requires less protein sample than a ΔG measurement.

Method: The $C_{1/2}$ for 10 cyctochrome c samples formulated in buffers of varying pH was determined by collecting 8-point denaturation curves on Hunky with the $C_{1/2}$ application. Cytochrome c (Sigma-Aldrich #C7752) was prepared at an initial concentration of 912.5 µg/mL in a Universal buffer comprised of 10 mM histidine-HCl, 10 mM sodium phosphate dibasic and 10 mM sodium succinate dibasic at pH 5.0. Additional stocks of the Universal buffer were prepared with 10 mM histidine-HCl, 10 mM sodium phosphate dibasic and 10 mM sodium succinate dibasic buffers and titrated to pH 4.25, 4.50, 4.75, 5.00, 5.25, 5.50, 5.75, 6.00, 7.00 and 9.00 to create the 10 buffers for the $C_{1/2}$ screen. Hunky prepared each denaturation point by mixing protein, formulation buffer and formulation buffer with denaturant for a final protein concentration of 73 µg/mL. Cytochrome c was denatured with 10 M Urea. Denaturation curves were analyzed by BCM with a 2-state fitting model in the Hunky Analysis software.

Results: Cytochrome c undergoes a single unfolding transition when denatured with Urea (Figure 6). Generally, as the formulation pH increased, the $C_{1/2}$ increased resulting in the observed shifting of the denaturation curves to higher denaturant concentrations. Cytochrome c stability was ranked in tiers with cutoffs at $C_{1/2}$ < 5 M for low stability, 5 M ≥ $C_{1/2}$ < 6 M for moderate stability and $C_{1/2}$ ≥ 6 M. Cytochrome c was found to be most stable ($C_{1/2}$ ≥ 6 M) at pH 7.00 and 9.00, moderately stable from pH 5.00 to 6.00, and least stable below pH 5.00.

ΔG

Protein stability can be quantified with ΔG. The native state ΔG is determined by analyzing chemical denaturation curves with 24 or more points. The automated analysis provides $C_{1/2}$, m-value, ΔG and the fraction of denatured protein. A higher ΔG indicates a more stable molecule with less denatured protein present. ΔG can provide higher resolution between similar molecules or sample conditions that are often indistinguishable by T_m or T_{onset} improving stability ranking and refinement.

Method: Adalimumab was prepared in 10 mM sodium acetate pH 4.0, 10 mM sodium acetate pH 4.3 and 10 mM sodium acetate pH 4.6 at 625 µg/mL and 1 mg/mL. The thermal stability (T_m) of Adalimumab at 1 mg/mL was determined on Uncle (Unchained Labs) by measuring the change in intrinsic fluorescence over a thermal ramp of 20 – 95 °C with a 0.25 °C/min ramp rate. The resulting fluorescence emission spectra were analyzed with Uncle Analysis software to calculate T_m. The ΔG of 50 µg/mL Adalimumab was measured for each formulation by collecting and analyzing 32-point

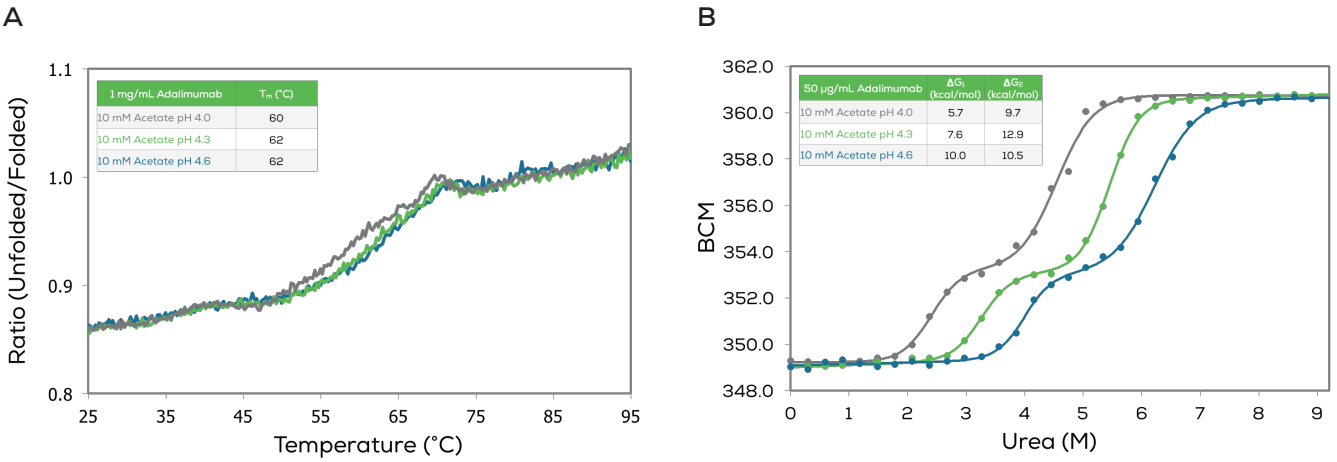

Figure 7: The melting temperature (T_m) of Adalimumab in 10 mM acetate over a small pH range spanning 0.6 pH units does not provide enough resolution to create a stability ranking (A). ΔG measurements of the same formulations made it possible to differentiate between Adalimumab formulations with small pH differences, identifying 10 mM acetate pH 4.6 as the most stable formulation (B).

denaturation curves on Hunky. Each Adalimumab formulation was denatured with a linear gradient of Urea from 0 M to 9.2 M at 25 °C in triplicate and incubated for 3 hours with synchronization before fluorescence data acquisition. Denaturation curves were analyzed with Hunky Analysis software, using the BCM fluorescence analysis method and a 3-state fitting model.

Results: Adalimumab underwent a single unfolding transition in all 3 formulations over a 20 – 95 °C temperature ramp. Melting temperatures were similar for the three formulations, ranging between 60 – 62 °C, making it difficult to identify the most stable formulation based solely on T_m (Figure 7A). To clarify the stability ranking, ΔG was measured on Hunky. All Adalimumab formulations underwent two distinct unfolding transitions when denatured with 10 M Urea (Figure 7B). The ΔG of the first transition, ΔG(1), increased with pH from ΔG(1) = 5.7 ± 0.2 kcal/mol at pH 4.0 to ΔG(1) = 10.0 ± 0.2 kcal/mol at pH 4.6. The difference in ΔG(1) corresponds to the contribution of 72 ppm or 0.05 ppm of denatured protein to the vial of protein at equilibrium at room temperature for the pH 4.0 and pH 4.6 formulations, respectively. A second unfolding event occurs at higher concentrations of denaturant, which again shows Adalimumab at pH 4.0 to be the least stable with ΔG(2) = 9.7 ± 0.2 kcal/mol. Stability analysis with ΔG identified 10 mM acetate pH 4.6 as the most stable formulation studied and fills in the blanks left by T_m.

Agg Path

Aggregation propensity and aggregation pathway can be determined by Agg Path. Agg Path requires ΔG to be measured at two concentrations, at least 40-fold different from each other, for quick aggregation assessment. ΔG is collected as described previously. The Hunky Analysis software automatically performs a global fit of the denaturation curves and reports the ΔG at each concentration, and the magnitude of change in ΔG. If no change is reported there is no aggregation risk. A positive or negative change indicates probable aggregation from the native state or denatured state, respectively. Measuring Agg Path provides early insight into aggregation so pitfalls can be avoided prior to initiating long-term studies.

Method: Stock solutions of Adalimumab were prepared in 10 mM acetate pH 4.0, 10 mM acetate pH 5.0 and 10 mM citrate, 100 mM phosphate pH 5.2 at 162.5 µg/mL and 10.8 mg/mL. Hunky was used to prepare denaturation curves at final concentrations of 13 µg/mL and 864 µg/mL Adalimumab, and to determine aggregation pathways with the Agg Path application. The 3 Adalimumab formulations, at low and high concentrations, were denatured with a 32-point linear gradient of Urea from 0 M to 9.2 M at 25 °C in triplicate and incubated for 3 hours with synchronization before fluorescence data acquisition. Denaturation curves were fit with Hunky Analysis software using the BCM

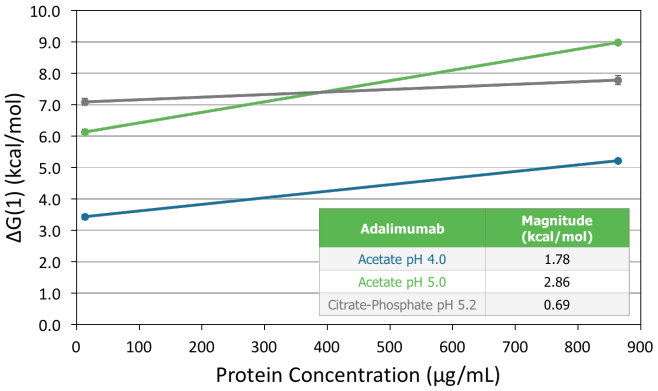

Figure 8: The magnitude of change between low and high concentration ΔG(1) measurements identified 10 mM citrate, 100 mM phosphate pH 5.2 as the most stable and least aggregation prone Adalimumab formulation studied.

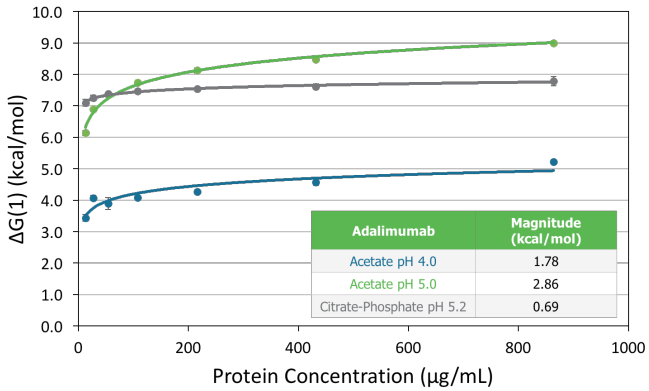

Figure 9: The Agg Path results for Adalimumab were confirmed by ΔG_{trend}.

fluorescence analysis method and a 3-state fitting model.

Results: The change in ΔG(1), the first unfolding transition, between 13 µg/mL and 860 µg/mL Adalimumab was analyzed for each formulation (Figure 8). ΔG(1) for 13 µg/mL Adalimumab ranks 10 mM acetate pH 4.0, 10 mM acetate 5.0 and 10 mM citrate, 100 mM phosphate pH 5.2 as the least to most stable. The low ΔG(1) for 10 mM acetate pH 4.0 indicates the need for stability optimization or elimination from further consideration. Additionally, Agg Path shows that Adalimumab is prone to aggregate from the native state with ΔG(1) increasing by 1.78 kcal/mol at 864 µg/mL for this formulation. Agg Path helps to differentiate between 10 mM acetate 5.0 and 10 mM citrate, 100 mM phosphate pH 5.2, which both have high ΔG(1) at low protein concentration. A drastic increase of 2.86 kcal/mol in ΔG(1) is observed for 10 mM acetate pH 5.0, while a small increase of 0.69 kcal/mol occurs in 10 mM citrate, 100 mM phosphate pH 5.2. Adding Agg Path to the analysis of these formulations allowed for the quick identification of 10 mM citrate, 100 mM phosphate pH 5.2 as the formulation on the right track with the highest stability and lowest aggregation risk.

ΔG_{trend}

Similar to Agg Path, ΔG_{trend} determines aggregation propensity and aggregation pathway. ΔG_{trend} requires ΔG to be measured at a minimum of 5 concentrations covering at least a 40-fold concentration difference. By collecting these additional points, a more robust aggregation picture is formed. In addition to the magnitude of change, ΔG_{trend} provides the rate of change in ΔG as a function of concentration and ensures that a wide enough concentration window has been collected. The aggregation pathway information provided by ΔG_{trend} lets researchers know if their sample is prone to native state aggregation, which may be addressed by formulation tweaks, or if it is prone to denatured state aggregation and deserves closer examination.

Method: Stock solutions of Adalimumab were prepared in 10 mM acetate pH 4.0, 10 mM acetate pH 5.0 and 10 mM citrate, 100 mM phosphate pH 5.2 at 162.5 µg/mL, 337.5 µg/mL, 675 µg/mL, 1.35 mg/mL, 2.7 mg/mL, 5.4 mg/mL and 10.75 mg/mL. Hunky was used to prepare denaturation curves at final concentrations of 13, 27, 54, 108, 216, 432 and 864 µg/mL Adalimumab and determine aggregation propensity with the ΔG_{trend} application. The 3 Adalimumab formulations, at all 5 concentrations were denatured with a 32-point linear gradient of Urea from 0 M to 9.2 M at 25 °C in triplicate and incubated for 3 hours with synchronization before fluorescence data acquisition. Denaturation curves were globally fit automatically with Hunky Analysis software using the BCM fluorescence analysis method and a 3-state fitting model.

Results: The Adalimumab formulations studied by Agg Path were also analyzed by ΔG_{trend}. The

additional data gives shape to ΔG_{trend} curves, providing the rate of change in ΔG with concentration as well as ensuring that a sufficient concentration range has been covered to reach a plateau in ΔG (Figure 9). The same experimental conclusions hold true in the analysis of ΔG_{trend}. Adalimumab is the least stable in 10 mM acetate pH 4.0 and carries a risk of aggregating from the native state. 10 mM acetate pH 5.0 and 10 mM citrate, 100 mM phosphate pH 5.2 are more stable formulations with ΔG(1) > 6 kcal/mol, but the citrate-phosphate formulation shows a smaller magnitude of change in ΔG(1) making it a better candidate for further optimization. ΔG_{trend} confirms the conclusions drawn from Agg Path analysis.

Summary

Hunky fully automates chemical denaturation experiments from start to finish, making it easy to access the unique stability and aggregation information ΔG provides. The 4 experimental applications span from low protein sample volume, quick screening experiments with $C_{1/2}$ and Agg Path to robust stability quantification and aggregation measurements with ΔG and ΔG_{trend}. Hunky allows researchers to quantitate stability and avoid potential aggregation issues earlier than ever before.

References

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2 Schön A, Clarkson BR, Siles R, Ross P, Brown RK, Freire E. 2015. Denatured state aggregation parameters derived from concentration dependence of protein stability. Analytical Biochemistry 488: 45-50.

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