Determine aggregation propensity with ΔG_{trend} and the HUNK

Introduction

The stability of biologics relies heavily on the selection of the optimal construct and optimal formulation. The optimal construct and formulation are those that have long-term stability and a low propensity to aggregate, especially those that aggregate from the denatured state. Automated chemical denaturation can rapidly assess the stability and propensity for aggregation of any construct in any formulation. Importantly, this assessment can be made on day one – that is, as soon as the protein is expressed and formulated – when aggregation levels would be expected to be extremely small. Chemical denaturation is the only way to determine conformational stability (ΔG, the Gibbs free energy of protein unfolding) for monoclonal antibodies and other biologics.

As described in this application note, ΔG measurements are also thermodynamically linked to aggregation. Recent published work (Schön *et. al.*, Analytical Biochem, 488, 45, 2015) demonstrates that changes in ΔG with changing protein concentration, referred to here as ΔG_{trend}, can determine the fraction of total protein that is aggregated and the fraction of the denatured protein that is aggregated. The HUNK, a fully automated platform for chemical denaturation, provides a rapid and early predictive assessment of protein stability. The HUNK platform enables researchers to learn, on day one, which protein construct in which formulation has the lowest propensity to aggregate. This in turn enables researchers to determine the most stable formulations and constructs prior to initiating long-term stability measurements.

Figure 1: The HUNK makes ΔG determinations an easy, walk-away process.

About the HUNK

The HUNK (Figure 1) makes ΔG determinations an easy, walk-away process. The instrument is fully automated and the operator selects methods to perform protein construct screening, formulation screening and optimization, excipient screening, aggregation determinations or ligand binding determinations. Complete stability and aggregation data analysis is performed by the HUNK software. Data may also be exported as desired.

ΔG_{trend}: determine aggregation state changes

All biologics in solution at any temperature exist in an equilibrium between native and denatured states. This relationship is defined by the equilibrium constant (Figure 2), which in turn is dictated by the conformational stability (ΔG). Biologics put into formulation screening and development will

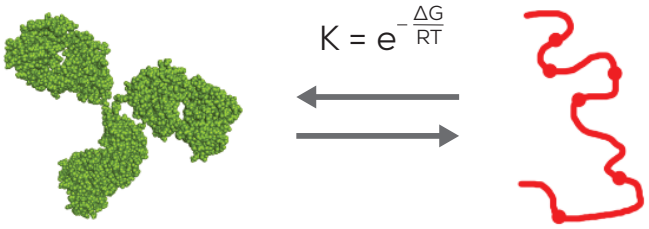


Figure 2: Proteins exist in equilibrium between native and denatured states. The relationship between native and denatured protein is determined by the Gibbs free energy equation $K = e^{-\Delta G/RT}$, where K is the equilibrium constant between the states.

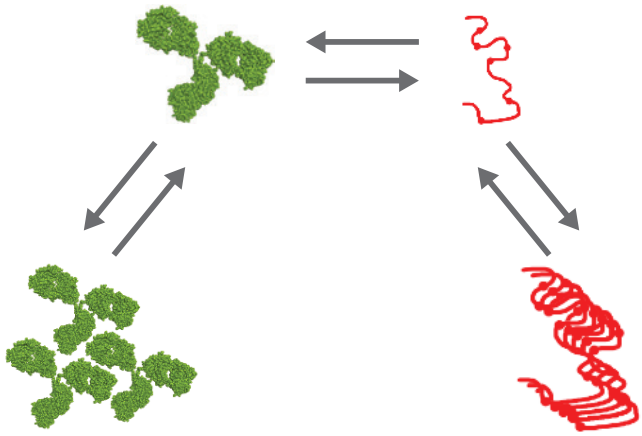


Figure 3: Biologic stability may be limited by either native protein aggregation or denatured protein aggregation. The HUNK can detect and quantitate both.

exist primarily in the native conformation (ΔG > 0). The amount of protein that exists in the denatured form is usually extremely small. Chemical denaturation is an effective way to determine ΔG and in turn, quantify the amount of native and denatured protein. The higher the ΔG, the less protein that exists in the denatured conformation. For example, a biologic with a ΔG of 8 kcal/mol has 1 ppm of denatured protein. Detecting this amount of denatured protein is essentially impossible for instrumentation other than the HUNK, which can quantitate the levels of denatured protein down to the parts per billion level. For biologics, the selected construct and formulation would ideally be optimized to a higher ΔG – i.e., maximize the native protein and minimize the amount of denatured protein.

Denatured states are generally highly prone to aggregation, and ultimately particle formation and precipitation. This aggregation and precipitation is a risk for long-term stability, because they act as a thermodynamic sink: they pull protein in equilibrium towards aggregation, and further decrease the amount of native protein in solution (Figure 3, right). Identifying the level of denatured protein is critically important to understand stability, but provides only a partial picture of aggregation risk because not all denatured protein will aggregate.

Predictive techniques such as chemical or thermal denaturation are critical components in the biological formulation development process to assess stability. Both accelerate the unfolding process, but chemical denaturation is the only approach capable of quantifying thermodynamic stability. The relationship between denaturant concentration and ΔG is linear, chemical denaturation is reversible and chemical denaturation can be performed at more biologically-meaningful temperatures.

In the absence of aggregation, the ΔG of protein unfolding is a unimolecular process independent of protein concentration. If a change in ΔG is observed as a function of protein concentration, then aggregation is present. That is, aggregation is a concentration-dependent phenomenon that can be detected and quantified by chemical denaturation. If native state aggregation occurs, the equilibrium shifts, decreasing denatured protein (Figure 3, left). If denatured state aggregation occurs, equilibrium shifts to the right (increasing denatured protein). Either event will be observable by measuring ΔG_{trend}.

ΔG_{trend} refers to the changes in ΔG as a function of protein concentration. The HUNK can determine ΔG_{trend} for different protein constructs, different formulations of the same construct or any combination of constructs and formulations. Importantly, it can distinguish these events on day one, when other techniques cannot address denatured protein levels or aggregation propensity.

There are three possible relationships between ΔG and protein concentration (Figure 4).

- **ΔG_{trend} is unchanged**: no aggregation is present
- **ΔG_{trend} increases**: protein undergoes aggregation from the native state
- **ΔG_{trend} decreases**: protein undergoes aggregation from the denatured state

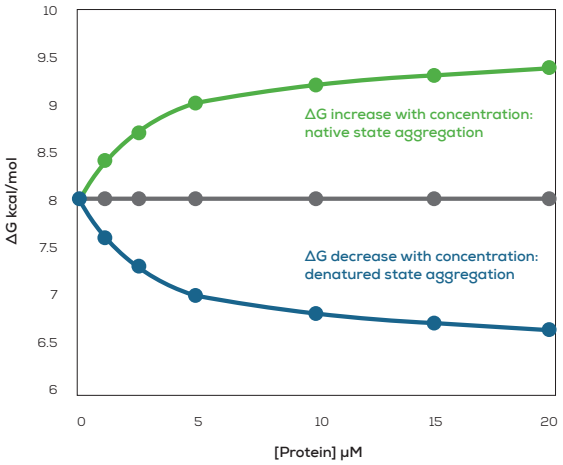


Figure 4: Monitoring the change in measured ΔG with increasing protein concentrations (ΔG_{trend}) enables a quantitative assessment of aggregation, and distinguishes aggregation pathways.

The example below demonstrates how the HUNK can be used for assessing aggregation propensity. The HUNK will use ΔG measurements to report ΔG_{trend}, the fraction of denatured protein, the fraction of total aggregated protein and the fraction of denatured protein that is aggregated.

Prior to determining ΔG_{trend}

Determine the optimal chemical denaturation experimental parameters:

- Most appropriate chemical denaturant (usually urea or guanidine hydrochloride)
- Number of data points in the denaturation curves
- Appropriate protein concentrations
- Required incubation time to reach equilibrium
- Optimal denaturation observable (e.g. fluorescence wavelength, ratio or difference)

Determining ΔG_{trend}

Protein concentrations to test

Test protein concentrations over at least two concentration orders of magnitude. Include a minimum of five different protein concentrations.

Number of data points

If denaturation is two-state (single transition), run a 24-point denaturation curve. If denaturation is three-state (two transitions), run a 32-point denaturation curve. The denaturant concentration range can be adjusted based on the specific protein denaturation profile. Create duplicate or triplicate denaturation curves at each protein concentration.

Protein volume required

The amount of protein required at each concentration will be dependent on the number of points in the denaturation curve, the number of formulations to be tested and the number of curve replicates. For example, a 24-point curve in duplicate with one formulation will require ~700 µL of protein at each concentration to be tested. The HUNK will calculate and display the volume to be transferred per protein concentration; add 100 µL to the displayed volume to account for variations in the protein vial depth.

Experimental detail

The example below is the same biologic in three different formulation buffers, all at pH 5.5. Ten protein stock concentrations were made at a 2-fold serial dilution from 19.2 mg/mL – 37.5 µg/mL. The HUNK protocol performed a 12.5-fold dilution of the protein into formulation plus denaturant, so tested concentrations were 1.54 mg/mL – 3 µg/mL. Higher protein concentrations can be tested, although it was not necessary to understand the ΔG_{trend} and aggregation propensity. The HUNK automatically diluted the protein into a mixture of the matching formulation buffer with increasing concentrations of urea. Each protein concentration was tested in triplicate against a 24-point urea denaturation curve of 1.9–8.28 M urea.

Experimental analysis

ΔG_{trend} values were determined by measuring fluorescence change at a 352 nm/338 nm ratio. The HUNK ΔG_{trend} analysis of concentration normalized data was set with a common baseline and slope to allow trend determinations.

Results

Initial ΔG assessments and ΔG_{trend}

Figure 5 shows the ΔG_{trend} for the biologic in three formulation buffers. Three things that are important to note:

- All three formulations exhibit good stability based on ΔG measurements.
- All formulations have extremely small but quantifiable amounts of denatured protein. The lowest recorded ΔG is 10.8 kcal/mol. This corresponds to about 10 parts per billion (ppb) denatured protein.
- The shifts in ΔG_{trend} signify aggregation. Formulations 1 and 2 exhibit a strong ΔG_{trend}, whereas Formulation 3 does not.

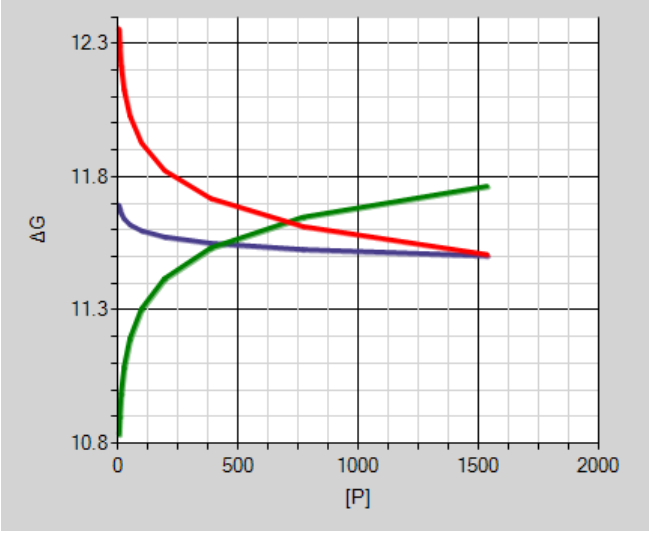


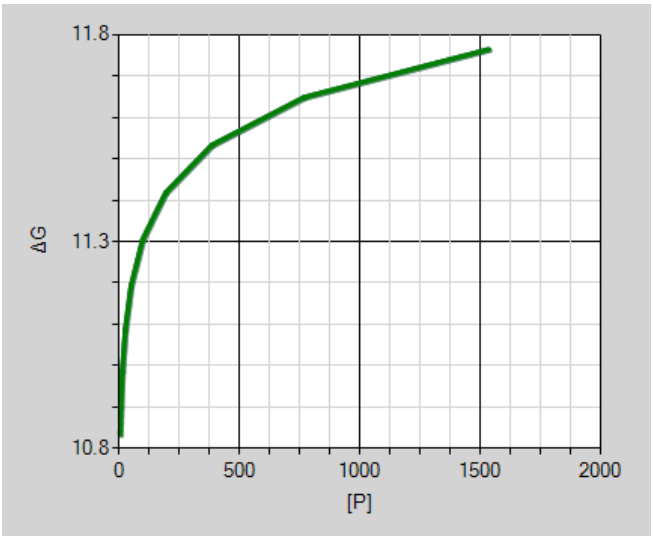
Figure 5: Comparison of ΔG_{trends} for the same biologic in Formulation 1 (green line), Formulation 2 (red line), and Formulation 3 (blue line). Formulation 3 exhibits the smallest change in ΔG (ΔG_{trend} ~0) suggesting that the biologic has the lowest aggregation propensity in this formulation.

Based on the changes in ΔG_{trend}, the protein construct:

- Undergoes native state aggregation in Formulation 1 (increasing ΔG_{trend})
- Undergoes denatured state aggregation in Formulation 2 (decreasing ΔG_{trend})
- Undergoes minimal aggregation in Formulation 3 (minimal change in ΔG_{trend})

Formulation 3 appears to be the optimal one as it is highly stable (high ΔG, with less than 4 ppb of denatured protein) and undergoes minimal aggregation. The specifics of the changes in ΔG are described in more detail below.

A



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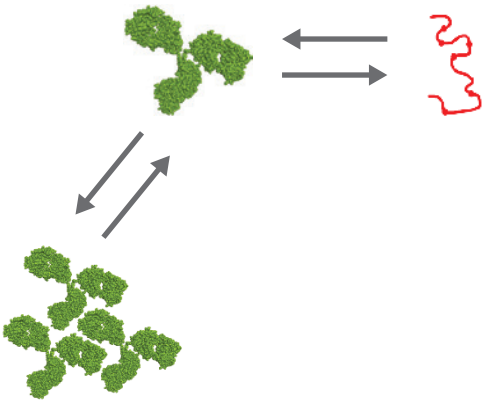


Figure 6: **A**: ΔG_{trend} for Formulation 1, which undergoes native state aggregation (increase in ΔG with increasing protein concentration). **B**: Native state aggregation shifts equilibrium to the left, resulting in an increase in ΔG.

Native state aggregation

Native state aggregation is observed in Formulation 1. This is indicated by an increase in ΔG with increasing protein concentration. Native state aggregation shifts equilibrium to the left (more folded protein) resulting in an increase in ΔG (Figure 6)

Based on the relationship between ΔG and the equilibrium constant, a higher ΔG means there is less denatured protein present as illustrated in Figure 7.

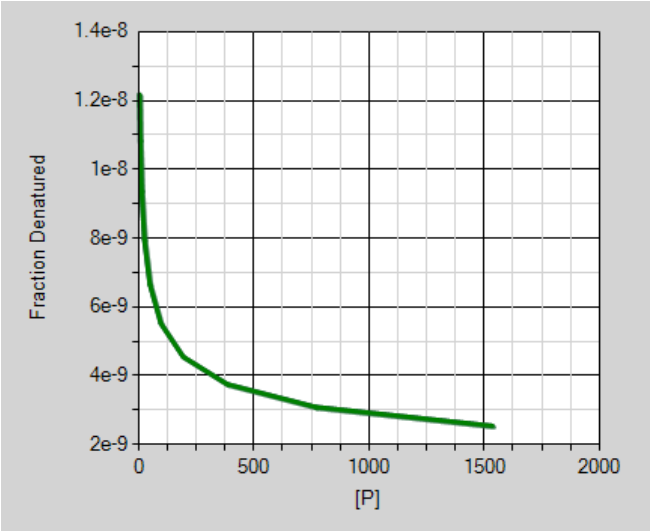


Figure 7: An increase in ΔG_{trend} (native state aggregation) indicates a decrease in denatured protein.

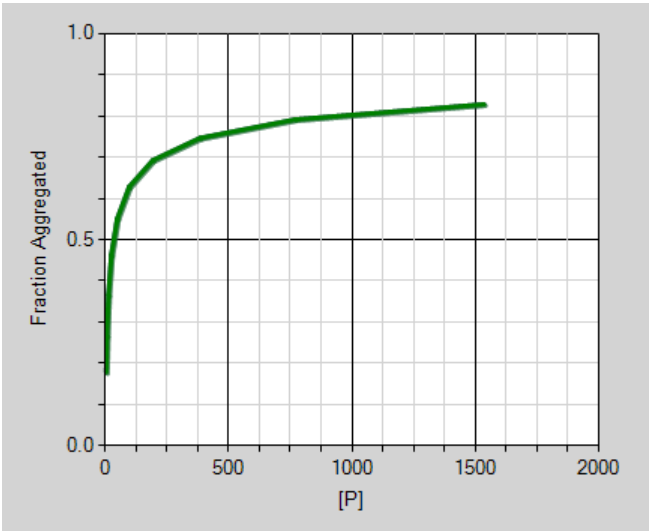
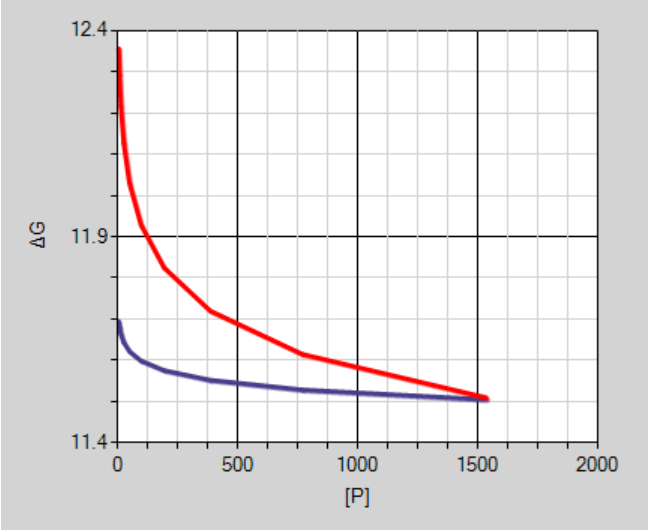


Figure 8: The fraction of the native protein that is aggregated can be determined based on the changes in ΔG_{trend}.

Because ΔG has been measured at multiple concentrations, the fraction of the native protein that is aggregated can be determined (Figure 8).

In situations where all constructs or formulations undergo native state aggregation, the severity of increasing aggregation may be used in conjunction with overall stability to assess the best formulation condition for optimization. Native state aggregation may be addressable by salt or other additives to minimize the ΔG_{trend} and to reduce the rate of aggregation.

A



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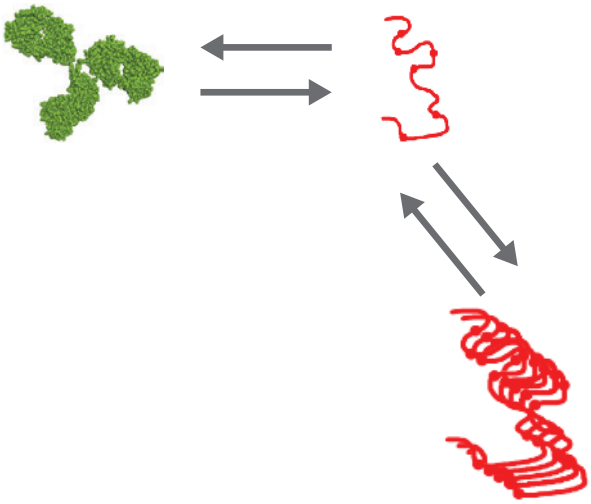

Figure 9: **A**: ΔG_{trend} for Formulations 2 (red line) and 3 (blue line) , which undergo denatured state aggregation (decrease in ΔG with increasing protein concentration). **B**: Denatured state aggregation shifts equilibrium to the right (more denatured protein) resulting in a decrease in ΔG.

Denatured state aggregation

Denatured state aggregation is observed in Formulations 2 and 3. This is indicated by a decrease in ΔG with increasing protein concentration (Figure 9A). Formulation 3 exhibits a smaller decrease in ΔG and therefore indicates less denatured state aggregation.

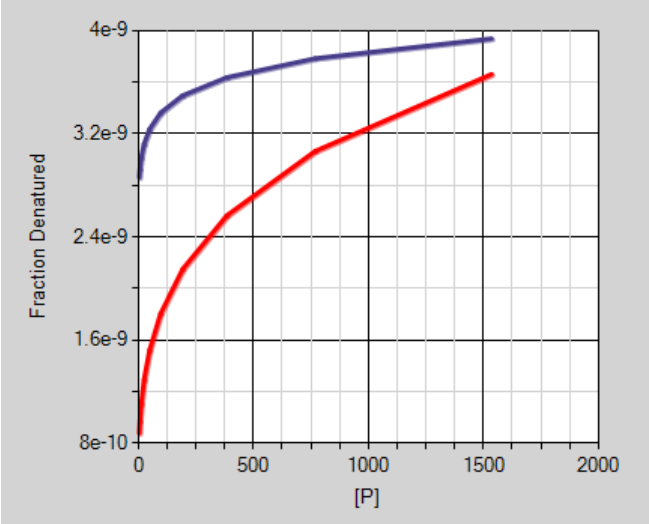

Figure 10: A decreasing ΔG_{trend} indicates an increase in denatured protein (Formulation 2, red line). Formulation 3 (blue line) did not have a significant ΔG_{trend}, and has a more consistent fraction of denatured protein.

Based on the relationship between ΔG and the equilibrium constant, a lower ΔG means there is more denatured protein present, as illustrated in Figure 10. For Formulation 2, the equilibrium has shifted to more denatured protein. Hence we see an increase in the fraction of denatured protein. For Formulation 3, there is very little ΔG_{trend}. With little or no change in ΔG, there is little or no change in the fraction of denatured protein. Hence, Formulation 3 shows very little change in the fraction of denatured protein.

Because ΔG has been measured at multiple concentrations, the fraction of aggregated protein is known (Figure 11). Formulation 2 exhibits an increase in aggregated protein by an order of magnitude. Formulation 3 also exhibits an increase in aggregation, but significantly less than Formulation 2.

Figure 12 shows the fraction of denatured protein that is aggregated in Formulations 2 and 3. Both formulations have increasing amounts of denatured protein that is aggregated, however Formulation 2 has significantly more denatured protein that is aggregated than Formulation 3.

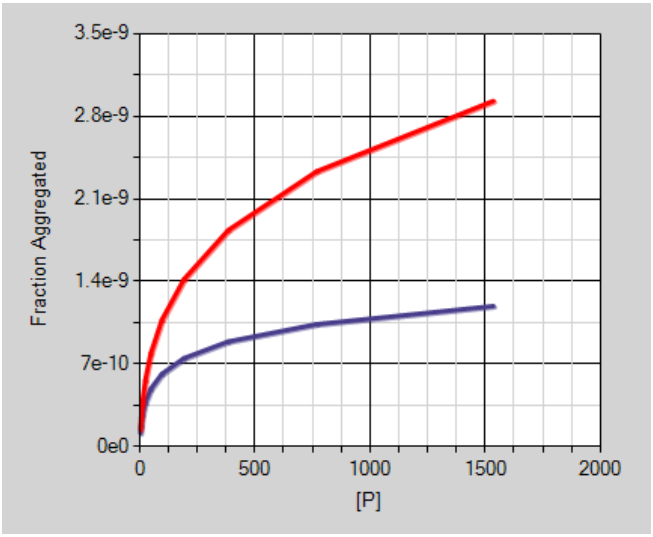

Figure 11: Changes in the fraction of aggregated protein for a biologic as protein concentration increases. Formulations 2 and 3 (red and blue line, respectively) have an increase in aggregated protein, with Formulation 3 aggregating to a lower degree and at slower rate.

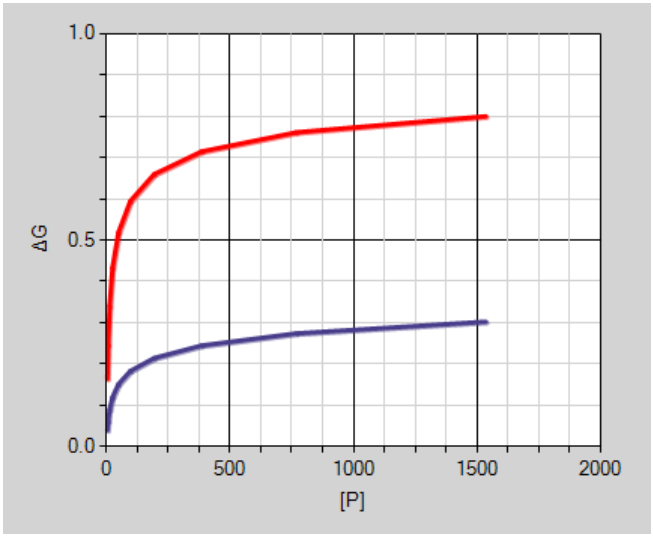

Figure 12: Fraction of the denatured protein that is aggregated for a biologic in Formulations 2 (red line) and 3 (blue line). The amount of denatured protein that is aggregated is higher in Formulation 2 than Formulation 3.

Summary: incorporating ΔG_{trend} into formulation screening

It is significant to note that all three formulations are highly stable in the experiment. By measuring ΔG_{trend}, the HUNK follows changes in ΔG with increasing protein concentration, and key pieces of information are learned:

- Conformational stability of the protein (ΔG)
- Amount of denatured protein present at day one
- Determination of the aggregation pathway (native or denatured)
- Aggregation propensity at day one

By following the change in ΔG, more information on the construct and formulation are obtained early in the screening process. This enables more optimization early: for example, the addition of excipients to stabilize the protein and prevent aggregation.

No other technique enables the measurements of denatured or aggregated protein at day one at room temperature. Chemical denaturation, coupled with the sensitivity of the HUNK, improves an understanding of aggregation propensity earlier in the formulation development and testing process, and helps ensure that only the best biologic and formulation candidates are put forward to long-term SEC stability measurements.

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