Differential Scanning Calorimetry Study of a mAb that Precipitates upon Thermal Denaturation

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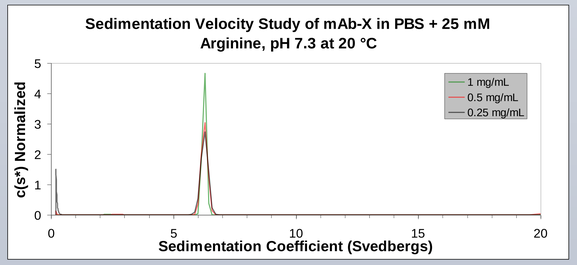
mAb-X precipitates upon thermal unfolding. Can we study its unfolding transition?

When mAb-X is thermally denatured, it precipitates heavily at concentrations around 1 mg/mL. This prevents us from studying the mechanism of thermal denaturation because the aggregation signal one observes in Microcal's VP-DSC generates a strong exothermic spike in the heat capacity profile, obscuring the data.

This signal is not observed in the capillary DSC due to the geometry of its sample cell – therefore, if heavy aggregation occurs upon sample unfolding, it may not be immediately appreciated by the investigator. This is critical knowledge because in general heavy aggregation will decrease a protein's Tm. Furthermore, if a more detailed analysis of the transition is desired, it would have to be modeled explicitly in the data analysis. Therefore, it is desirable to find conditions where the aggregation process is attenuated, allowing us to study the thermal denaturation mechanism separate from the aggregation process. The aggregation signal can be used to screen conditions that attenuate precipitation – this is much harder to accomplish using the capillary DSC.

To assess the aggregation state of mAb-X, we carried out a series of sedimentation velocity experiments, from 0.25 to 1.0 mg/mL protein loading concentration, and analyzed the data using the *c*(*s*) method.



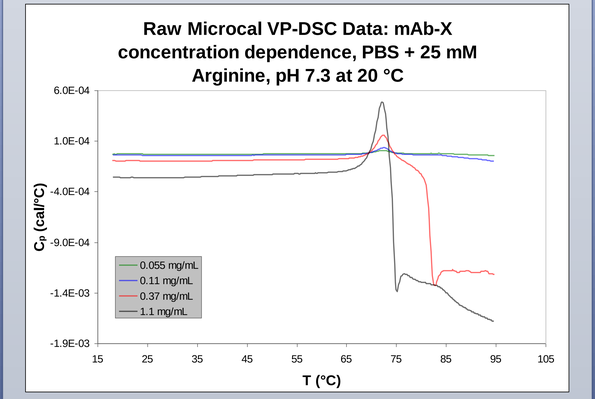
These experiments show that mAb-X exists in solution at 20°C predominantly as a single species with a sedimentation coefficient of 6.3 S, independent of loading concentration. This sedimentation coefficient, along with the fitted diffusion coefficient, show that mAb-X is > 99% monomer. This result holds over a range of pHs and excipient concentrations. These experiments serve as a control that the precipitation observed when mAb-X is thermally denatured arises from the unfolding of the antibody monomer, not a fraction of aggregated protein that was already present in the protein preparation.

Introduction and background on DSC.

Differential Scanning Calorimetry measures the heat capacity, Cp, of a molecule. It is applied in the biotechnology industry to 1) study the thermal stability of a wide array of protein therapeutics and vaccines, and 2) demonstrate comparability of higher order structure (HOS) for material from different processes or manufacturing sites.

Specifically, DSC measures the difference in heat flow between a sample and reference chamber, as both chambers are slowly heated. Both chambers are enclosed by an adiabatic insulator. The sample chamber contains the molecule of interest in an appropriate buffer, while the reference chamber contains the identical buffer. For a folded protein, more heat will flow into the sample chamber to break the non-covalent bonds associated with its tertiary structure than flows into the reference chamber. This is an endothermic process and results in a positive deflection of Cp. If less heat flows into the sample chamber than the reference, this causes a negative deflection in Cp.

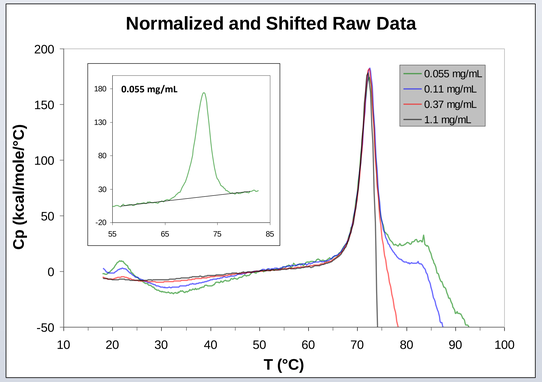
Thermal denaturation of mAb-X monitored by our MicroCal VP-DSC



As mAb-X is unfolded, the Cp rises due to increased heat flow into the sample chamber. However, a sharp drop in Cp is seen as the protein is unfolded. This is referred to as the aggregation signal and indicates the sample is precipitating in the sample cell. However, this signal appears to attenuate as the protein concentration is decreased and the rate of aggregation slows.

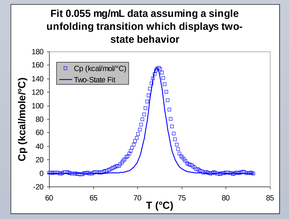
The aggregation artifact is attenuated at decreased mAb-X concentrations.

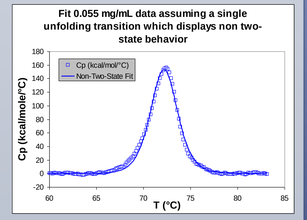
Below we show the raw data after normalization for loading concentration and shifting each profile to a common point at 50 °C. The inset shows a zoomed view of the 0.055 mg/mL data. The black line is a fit of the pre- and post-transition baselines, and demonstrates there is no significant ΔCp between folded and unfolded protein.



It is clear the aggregation artifact is significantly attenuated at the lowest concentration, clearly establishing pre- and post-transition baselines. The excellent sensitivity of the MicroCal VP-DSC allowed us to go low enough in concentration to collect data that are analyzable using rigorous thermodynamic models to study the mechanism of protein unfolding.

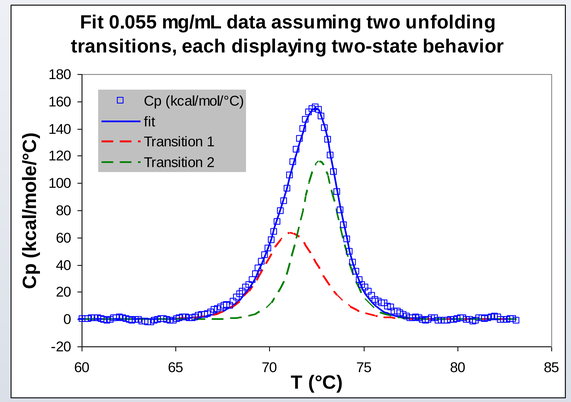
Fitting the 0.055 mg/mL DSC data assuming a single unfolding transition.





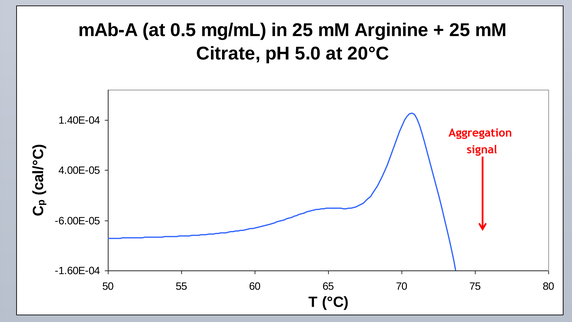
While the data show only a single unfolding transition, it is clear they cannot be described by a single, two state transition. Relaxing the thermodynamic requirement that $\Delta H_{VH} = \Delta H_{Cal}$ significantly improves the fit. *However, this is not a true thermodynamic model*. This fit returns $\Delta H_{VH} / \Delta H_{Cal} = 0.42 \pm 0.01$, i.e. *there must be additional transitions*.

Fitting the 0.055 mg/mL DSC data assuming two unfolding transitions.



Next we fit the data assuming the presence of two transitions. For example, this could represent the unfolding of two independent domains within the protein, or it could represent the presence of a significant concentration of a single unfolding intermediate – *thermodynamic measurements alone cannot distinguish which path is followed, they can only enumerate the number of distinct molecular species present in the system*. This model describes the data very well, and returns well resolved enthalpies and Tms for the two transitions. If this model is correct, we may be able to find a set of conditions that will resolve these two transitions.

pH 5 preferentially weakens one Tm over the other such that both transitions can be observed in the primary data.



In the future we will lower the protein concentration to generate data that can be analyzed to determine if both transitions are adequately described by a two-state model.