Application Note
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AN-850-0144

Structural Characterization and Comparison of Temperature Stress on a Protein Library Across pH and Concentration using Microfluidic Modulation Spectroscopy

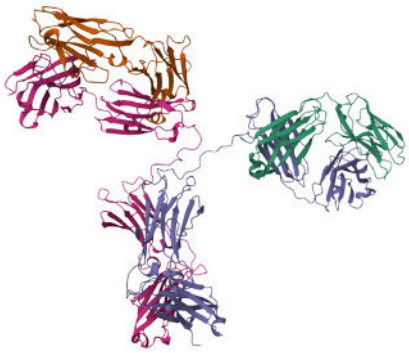
- ○ Biosimilars
- ○ mAbs
- ○ ADCs
- ○ AAVs
- ○ Ligand Binding
- ✓ Protein/Peptide Analysis
- ○ VLPs
- ○ Nucleic Acid
- ○ Fusion Proteins
- ○ Enzyme Analysis

- ✓ Aggregation
- ⬢ Quantitiation
- ✓ Structure
- ✓ Stability
- ✓ Similarity

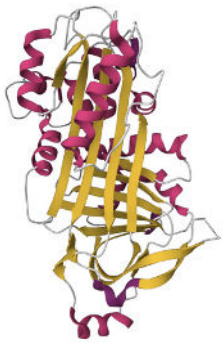
Abstract

Thermal stress is the most common stress condition utilized for gauging protein stability. However, techniques to measure the effects of thermal stress such as UV melts, circular dichroism (CD) and differential scanning calorimetry (DSC) typical only provide an overall melt temperature, T_m, and thermodynamic parameters, with very low granularity on structural changes associated with the melt. Microfluidic Modulation Spectroscopy (MMS) is an automated mid-IR technique capable of measuring protein secondary structure at very high resolution across a broad range of concentrations and buffer conditions. MMS can be used to measure and monitor the structural changes that are occurring, leading to protein unfolding, and then thermally induced aggregation. In this study, we started by measuring thermal unfolding for lysozyme across a range of pH values and found the T_m to be pH dependent. We next melted an IgG across a range of concentrations and found the T_m to be modestly concentration independent. Finally, we used MMS to determine the structural differences of the two T_m values determined for ovalbumin.

Lysozyme
(PDB ID: 1DPX)



IgG
(PDB ID: 1IGT)



Ovalbumin
(PDB ID: 1OVA)

Introduction

The melting temperature (T_m) of biologic drugs is critical to characterization as it sheds light on functionality, manufacturability, and formulation stability in particular. Furthermore, T_m is a versatile parameter that can report on the stabilizing effects of small molecule drug candidates on target proteins or RNA. T_m refers to the temperature at which the molecule unfolds and is specifically the temperature at which half the molecules are unfolded and half are folded. Therefore, the higher the T_m, the more stable the macromolecule. Historically, instruments that measure T_m provide just the T_m with no other information or context on the structure.[1] However, here we present a way to determine T_m using MMS, giving full secondary structure information at each temperature along the ramp so we can observe which structural components are changing, and use 2D heat maps to see the effects of different drugs or formations on the stabilities of different parts of the biologic.

MMS interrogates the amide I band of the IR spectrum to sensitively probe protein structure while modulating against the reference buffer for accurate, real-time background subtraction in aqueous-based samples.

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Introduction, continued

As it is so sensitive, this technique is particularly useful for quality control and is compatible with many different formulation buffers. The MMS system, Aurora Tx, was used for this study and is equipped with a high-power Quantum Cascade Laser that is significantly more intense than traditional FTIR light sources. The combination of more light and modulating background subtraction makes MMS about 30 times more sensitive than FTIR and 5 times more sensitive than CD to small changes in structure.[2] Given that MMS is a flow system, our unique temperature control of the cell means that samples are only exposed to the increased temperature right before sample measurement. This gives a T_m that is relative to the real-time temperature increase.

Figure 1. Aurora Tx used for structure and T_m characterization.

Methods

Lysozyme sample preparation:
Hen Egg White Lysozyme (HEWL) from Sigma (#L6876) was prepared at 2 mg/mL in 100 mM citrate buffer at pH 3, 100 mM glycine buffer at pH 4, 100 mM acetate buffer at pH 5, and 100 mM phosphate buffer at pH 7.

IgG sample preparation:
Lyophilized IgG from bovine serum (Sigma #15506) was prepared at 0.5, 1, and 2 mg/mL in phosphate buffer saline (PBS) pH 7.4.

Ovalbumin sample preparation:
Ovalbumin was dissolved at 5 mg/mL in PBS buffer and incubated in a water bath at 60, 65, 70, 75, 80°C, and boiling water for 5 mins, cooled to room temperature and run on the Aurora in addition to a control sample that was not exposed to increased temperature.

Thermal Ramping Methods:
All samples were run on an Aurora Tx from 25-95°C at a ramp rate of 1°C/min and a modulation speed of 1 Hz. Samples are maintained in a room temp 96 well plate, while the flow cell is ramped from 25-95°C.

The backing pressure was adjusted to maintain 60 µL/min flow rate, and the instrument collects a full spectrum from 1590-1710 cm^{-1} every 17 seconds, yielding about 250 spectra for the full ramp.

Results and Discussion

Formulation development is the process for determining the optimum buffer and excipients for a biologic drug to be the most stable, soluble, and active. One of the most important features in determining biologic stability is the pH. The pH condition that results in the highest T_m typically corresponds to the condition with the highest thermal stability for the macromolecule. Figure 2A shows the melt curve of lysozyme at pH 3 focusing on just the signal at 1656 cm^{-1}, which corresponds to the amount of alpha-helical structure. The first derivative plot demonstrates how the T_m can be calculated from the curve by showing a peak at the temperature that has the largest slope and, therefore, the fastest rate of alpha-helical unfolding. Figure 2B depicts the melt curves for lysozyme collected at four different pH values. The sample at pH 4 results in the highest T_m, indicating it is the most stable formulation pH, and the pH 3 sample has the lowest T_m, making it the least thermally stable. This result is in agreement with other reports from the literature comparing the stability of HEWL at different pH values.[3]

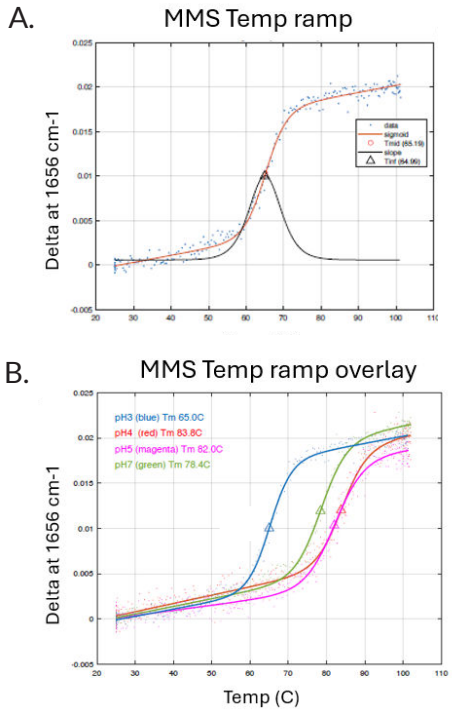

Figure 2 (A). Melt curve for 2 mg/mL lysozyme at pH 3. The first derivative plot indicates the T_m. (B). Overlay of the melt curves for pH 3, 4, 5, and 7.

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Results and Discussion, continued

Unlike traditional techniques used for measuring T_m, MMS provides structural information across the entire melt curve. This is exemplified in Figure 3A for lysozyme (pH 3), where the y-axis is temperature, the x-axis is wavenumber, yellow regions represent loss in structure, and dark blue represents increase in structure. Figure 3B shows the higher order structure (HOS) at 3 temperatures, demonstrating conversion of alpha-helix to turn structure due to the thermal stress.

T_m values measured for the 0.5, 1, and 2 mg/mL IgG samples were all approximately 81°C. Shown in Figure 3C is the 2D plot for 0.5 mg/mL and it is clear that the unfolding of IgG occurs at a much higher temperature compared to lysozyme. The major components that we see are native beta-sheet converting to intermolecular beta-sheet (beta-).

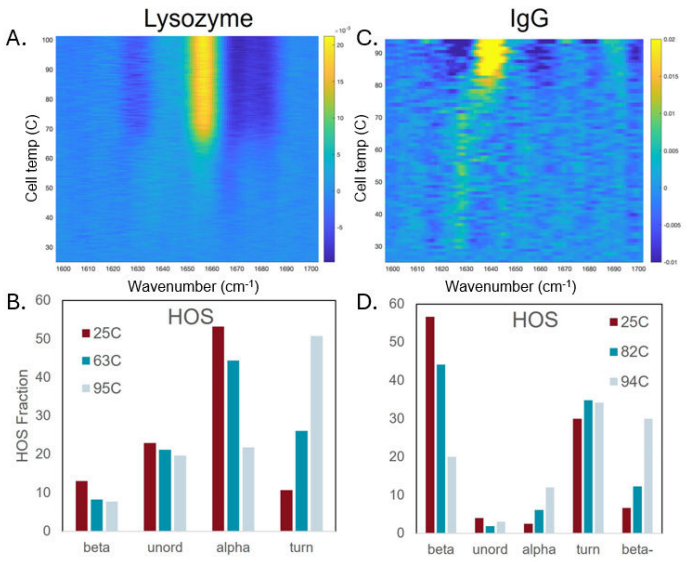

Figure 3 (A). MMS thermal melt plot as an inverted heat map. (B). The higher order structure (HOS) bar graph for lysozyme shows the loss in alpha-helix and increase in turn structure caused by temperature. (C). 2D MMS melt plot for IgG and (D). the structure changes observed for IgG, specifically loss in native beta-sheet and gain in intermolecular beta (beta-).

The next study compares MMS melt data with a more traditional T_m technique, differential scanning fluorimetry (DSF), using Sypro Orange fluorescent dye in the buffer as a probe. Figure 4A shows the first derivative of the DSF data depicting a melt curve of native ovalbumin with two transition temperatures. By pre-stressing the sample with temperature or pressure, the melt curve could appear very different, but yield the same T_m (Figure 4B). We tested ovalbumin in MMS to tease out which structural components were affected by thermal stress.

Figure 4C shows the stability plot and how each secondary structure is affected by 5 mins incubation at each temperature and below in Figure 4D is the MMS thermal ramp corroborating the loss in native beta-sheet structure and gain in intermolecular beta-sheet structure. Both T_m values are visible and now we can see T_m1 corresponds to a loss in alpha-helix and T_m2 corresponds to a loss in native beta-sheet structure and increase in aggregated beta-sheet structure. This result suggests that ovalbumin is likely to aggregate at its T_m2 rather than its T_m1, providing valuable structural information on top of the melting temperatures.

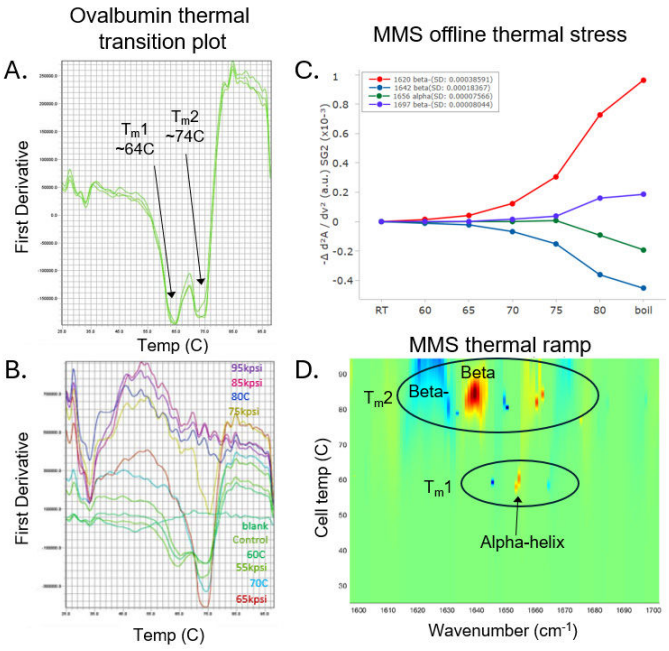

Figure 4 (A). DSF melt curve for ovalbumin using a Thermo 7500 instrument with Sypro Orange dye to visualize protein unfolding. (B). Pre-stressing the ovalbumin for 5 mins at various temps, or 10 mins using Pressure BioScience's HUB 880 Explorer led to different looking curves, but all had the same T_m. (C). MMS data can show which secondary structure is affected by the temp applied. (D). MMS thermal ramp shows both T_m values and loss in structure is shown as yellow/red, gain in structure as blue/dark blue.

Conclusions

In this study, we used MMS for structural characterization and for thermal melting, correlating structure and stability for various proteins with different types of structures. In the first example, we showed that lysozyme at pH 4 is the most thermally stable of the pH values that were tested, and pH 3 is the least stable. Additionally, comparing lysozyme to IgG, lysozyme has a much lower T_m.

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Conclusions, continued

Temperature stress unfolds lysozyme by converting alpha-helical structure to turn structure, whereas the same temperature stress causes IgG native beta-sheet to form intermolecular beta-sheets. In the last example, pre-stressed ovalbumin melt curves using traditional methods result in the same T_m but appear very different, while MMS thermal ramp data can distinguish both T_m values and correlate the type of secondary structures to each T_m. For ovalbumin, the alpha-helix structures melt at the first T_m and the native beta-sheet structures melt at the second T_m and convert to aggregated beta-sheet. Overall, this data demonstrates MMS adds a high level of structural granularity to melt experiments that was not previously accessible to formulation scientists, providing valuable insight into how various formulation components stabilize different structural motifs in biologic drugs.

References

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