Application Note
APR 2025
AN-850-0149

Advancing Protein Stability Analysis: Enhanced Structural Characterization of Thermal Unfolding with Microfluidic Modulation Spectroscopy (MMS)

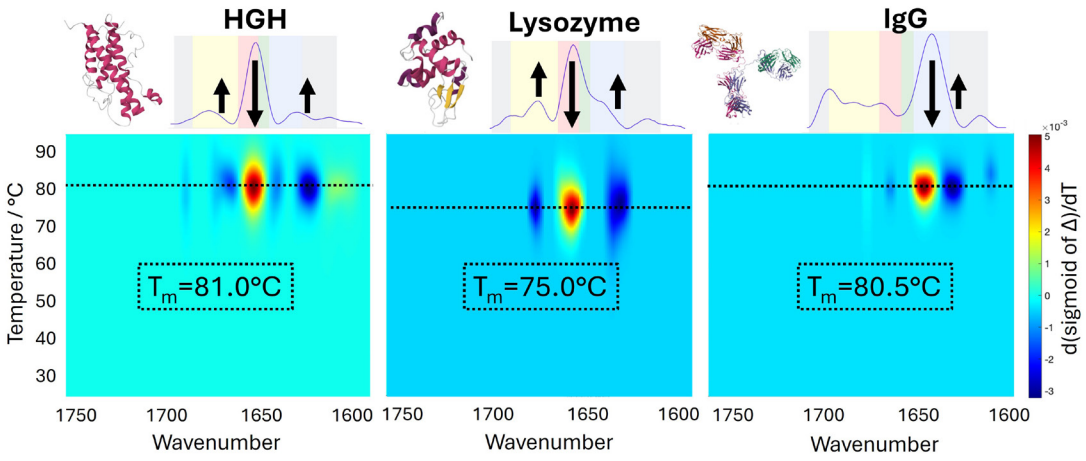
- ○ Biosimilars
- ☑ mAbs
- ○ ADCs
- ○ AAVs
- ○ Ligand Binding
- ☑ Protein/Peptide Analysis
- ○ VLPs
- ○ Nucleic Acid
- ○ Fusion Proteins
- ○ Enzyme Analysis

- ☑ Aggregation
- ⬢ Quantitiation
- ☑ Structure
- ☑ Stability
- ⬢ Similarity

Abstract

Understanding protein thermal stability is essential for the development and formulation of biologics. In this study, we evaluate the structural unfolding of proteins during thermal melting using Microfluidic Modulation Spectroscopy (MMS) with the AuroraTX system and compare the results to the traditional thermal ramp methods Differential Scanning Fluorimetry (DSF) and Differential Scanning Calorimetry (DSC). By monitoring structural transitions in real time, MMS provides detailed insights into secondary structure changes during thermal ramps.

Using Human Growth Hormone (HGH), hen egg white lysozyme (HEWL), and Immunoglobulin G (IgG) as model proteins of varying size and complexity, we demonstrate the capability of MMS to resolve distinct melting behaviors and domain-specific unfolding events. Additionally, we assess the reversibility of thermal unfolding by comparing inline melt curves to spectra of offline thermally stressed samples. Our results highlight the added value of MMS in capturing structural changes during thermal denaturation, offering a complementary perspective to traditional thermal stability assays.



We show the thermal unfolding behavior of differently structured proteins, measured with thermal ramp MMS. The figure presents MMS heat maps of HGH, Lysozyme and IgG from 25 to 95°C between 1760-1588 cm^{-1}. The MMS heat maps show the first derivative of the difference spectra at each temperature compared to the room temperature spectra where the hot spots (red) indicate a spectral (and structural) loss, and the cold spots show a spectral (and structural) gain.

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Introduction

Thermal ramping of proteins is a critical method to assess the stability of a protein's tertiary and secondary structures when subjected to increasing temperatures. As temperature rises, non-covalent interactions stabilizing the protein, such as hydrogen bonds, hydrophobic interactions, and van der Waals forces, are disrupted due to the increased interaction contribution from entropy (ΔS), leading to unfolding or "melting." This process is characterized by a specific melting temperature (T_m), at which half of the protein population is unfolded.

Two commonly used techniques to study protein thermal melting are Differential Scanning Fluorimetry (DSF) and Differential Scanning Calorimetry (DSC). DSF monitors changes in protein fluorescence, often using environment-sensitive dyes that bind exposed hydrophobic regions upon unfolding, to determine T_m DSC, on the other hand, directly measures the heat capacity of the protein solution as a function of temperature, providing detailed thermodynamic parameters such as T_m and enthalpy of unfolding (ΔH).[1,2]

Protein melting information is vital for assessing protein stability during formulation, storage, or therapeutic development, as thermal sensitivity can impact efficacy and shelf-life. An aspect often overlooked is the structural component in the melting behavior of proteins. Since protein structure directly relates to its stability, insights into the structural reorganization of proteins along thermal melting would be vital for drug development.

MMS has been demonstrated as a powerful tool for studying protein structure. It is a mid-IR-focused spectroscopy technique that allows determining structural differences of biomolecules as low as 0.67% by auto-referencing between sample and reference buffer using a microfluidic flow scheme.[3] With the AuroraTX, MMS is now also able to record melting curves of proteins, peptides or oligonucleotides and identify the structural changes along thermal unfolding.

In this work, we will show the performance of the AuroraTX compared with DSF and DSC in terms of protein stability assessment. In addition, we will study the reversibility of the observed structural changes by comparing melt data with offline thermally stressed samples of the same proteins. For both studies, Human Growth Hormone (HGH), hen egg white lysozyme (HEWL), and Immunoglobulin G (IgG) will serve as model proteins covering different structural complexities and stabilities. For instance, lysozyme, a small and highly stable enzyme, typically exhibits a sharp melting transition, while IgG, a large multidomain antibody, shows multiple unfolding transitions corresponding to its individual domains. HGH, a moderately stable protein, unfolds in a single cooperative transition.[4–6]

Methods

✓ **Samples:**

In this work, we used 1 mg/mL and 0.5 mg/mL samples of Human Growth Hormone (HGH), hen egg white lysozyme (HEWL), and Immunoglobulin G (IgG), each dissolved in PBS. For ensuring buffer-matching between sample and reference, each sample was dialyzed in PBS using Float-A-Lyzer cassettes. The samples used for MMS studies were further matched by loading protein sample into a 10 kDa Amicon spin filter with excess dialysis buffer, spinning down, and utilizing the flow through as the reference. For the isothermal MMS spectra, 1 mg/mL of each sample was stressed offline by incubating at the corresponding temperature for 60 minutes and subsequently cooled down to room temperature.

✓ **Measurements:**

As a T_m control, DSF curves were collected of the 1 mg/mL samples with a ramp rate of 1 °C/min. The MMS spectra were recorded with an AuroraTX (RedShiftBio), measuring differential absorbance spectra of each sample against its buffer reference across the Amide I band region (1760-1588cm^{-1}). For the isothermal measurements, triplicate spectra were collected and averaged for each 1 mg/mL sample. For the thermal melt, 0.5 mg/mL of the same samples were measured at a ramp rate of 1 °C/min with recording 4 spectra per 1 °C between 25 and 95° C.

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Methods, continued

✓ Data analysis:

The MMS data were analyzed using the RedShiftBio Analytics software package *delta*.

For the isothermal datasets, the raw differential absorbance was converted to absolute absorbance by normalizing for concentration, macromolecular displacement of buffer, and optical path length. The second derivatives of the absolute absorbance spectra are automatically generated along with the "similarity plot" by inverting and baseline subtracting the second derivative plot. The delta plot reflects the difference spectrum of the corresponding sample compared to the user-defined control, employing the second derivative data.

For the thermal ramp datasets, the second derivative spectra were used to track the difference between the absorbance at each temperature vs the room temperature control. For each wavenumber of the spectra, this results in a melting curve reporting on different structural components, e.g. alpha helical (~1657 cm^{-1}), intramolecular beta sheet (~1641 cm^{-1}) or intermolecular beta sheet (~1627 cm^{-1}). Those wavenumbers that showed a melting transition were fit with a sigmoid of which we took the first derivative to determine the corresponding T_m. The collective results for all wavenumbers were then plotted as a 2D heatmap where the hot and cold spots reflect the structural changes and associated T_m values.

Results

1. Human Growth Hormone (HGH) and Hen Egg White Lysozyme (HEWL)

1.1 Thermal melt: MMS vs DSF

Figure 1 presents a comparative analysis of the thermal unfolding behavior of Human Growth Hormone (HGH) and hen egg white lysozyme (HEWL), assessed using Microfluidic M odulation Spectroscopy (MMS) and Differential Scanning Fluorimetry (DSF). Figure 1A (top panels) shows the MMS results, while Figure 1B (bottom panels) displays the corresponding DSF melt curves. Within each part, the left panels correspond to HGH and the right panels to HEWL.

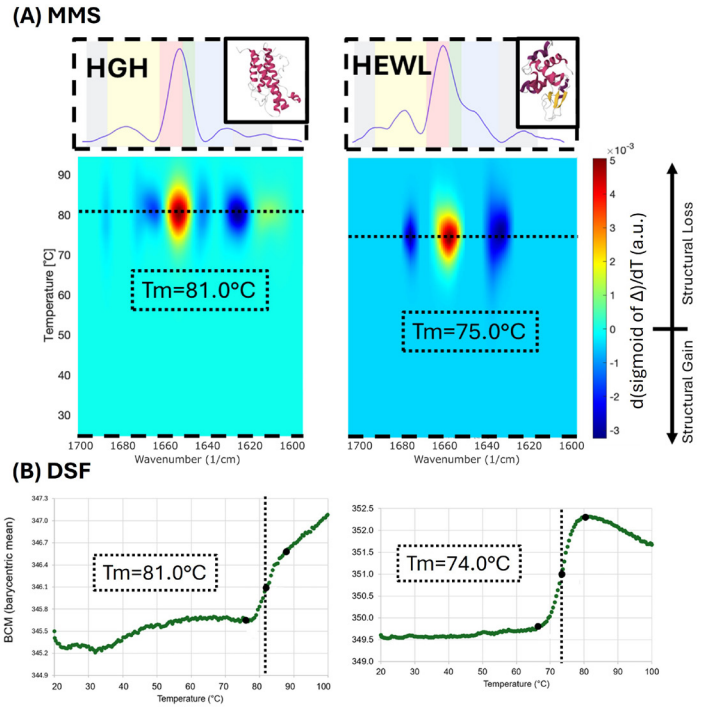

Figure 1: Thermal melt results of HGH (left) and HEWL (right), measured with MMS (panel A) and DSF (panel B). Top of panel A shows the inverted and baseline-corrected second derivative MMS spectra at room temperature. Bottom part of panel A shows the MMS heat maps from 25 to 95°C. The MMS heat maps show the first derivative of the difference spectra at each temperature compared to the room temperature spectra where hot spots (red) indicate a spectral (and structural) loss, and the cold spots show a spectral (and structural) gain. The DSF curves in panel B were recorded from 20 to 100°C. For both methods, the determined melting points T_m are displayed in the corresponding inset.

In the MMS data (Figure 1A), the inverted and baselined second derivative spectra at room temperature reveal the secondary structure composition of the native proteins. Gaussian fitting of these spectra indicates that HGH consists primarily of alpha helical content (61%), along with 17% beta sheet, 14% turn, and 8% unordered structure. HEWL exhibits a more balanced distribution with 46% alpha helix, 23% beta sheet, 25% turn, and 6% unordered structure, reflecting its more structurally diverse fold.

Thermal ramp MMS measurements, shown as 2D heatmaps in the 1720–1588 cm^{-1} spectral window, clearly capture the unfolding transitions. The T_m values, determined from these data, are 81° C for HGH and 75° C for HEWL. These values are in excellent agreement with the DSF results (Figure 1B), which report T_m values of 81 °C for HGH and 74 °C for HEWL, demonstrating close concordance (±1 °C) between structural and fluorescence-based thermal assays.

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Results, continued

Beyond T_m determination, the MMS data reveal detailed spectral signatures of structural changes during unfolding. Both proteins show a dominant hotspot at 1657 cm^{-1}, indicative of alpha helical loss. However, their cold spots, corresponding to spectral increases and structural gains, differ in character and distribution. HGH displays two distinct cold spots in the beta sheet region: at 1645 cm^{-1} (associated with intramolecular beta sheet formation) and 1622 cm^{-1} (intermolecular beta sheet formation). In addition, it shows a broad spot around 1668 cm^{-1} (turn structures). In contrast, HEWL exhibits one broader cold spot in the beta sheet region across 1617–1638 cm^{-1}, reflecting a mixture of intra- and intermolecular beta sheet content, along with a turn-associated cold spot at 1668 cm^{-1}.

These results indicate that while both HGH and HEWL undergo comparable global unfolding transitions marked by alpha helix loss, their structural rearrangements differ in detail. MMS uniquely reveals these mechanistic differences through its ability to resolve specific spectral and structural features during thermal unfolding.

1.2 Reversibility

Figure 2 provides additional insights into the thermal unfolding behavior of HGH and HEWL, extending the data previously shown in Figure 1. Part (I) displays the MMS results, while part (II) presents the corresponding DSF melt curves. As before, the left panels show data for HGH and the right panels for HEWL.

Panel (I.II) of Figure 2 re-iterates the MMS melt maps, which highlight temperature-dependent spectral changes across the Amide I region. Panel (II) shows the corresponding DSF curves, again confirming the melting points observed earlier (T_m(HGH) = 81 °C; T_m(HEWL) = 74–75 °C).

Importantly, panel (I.I) introduces isothermal MMS spectra of offline thermally stressed samples measured at room temperature following incubation at three defined temperature points: T_{ref} (room temperature), T_{onset} (start of transition), T_m (melting point), and T_{end} (end of transition). The resulting delta plots show the difference in spectra between each thermally treated sample and its room temperature reference.

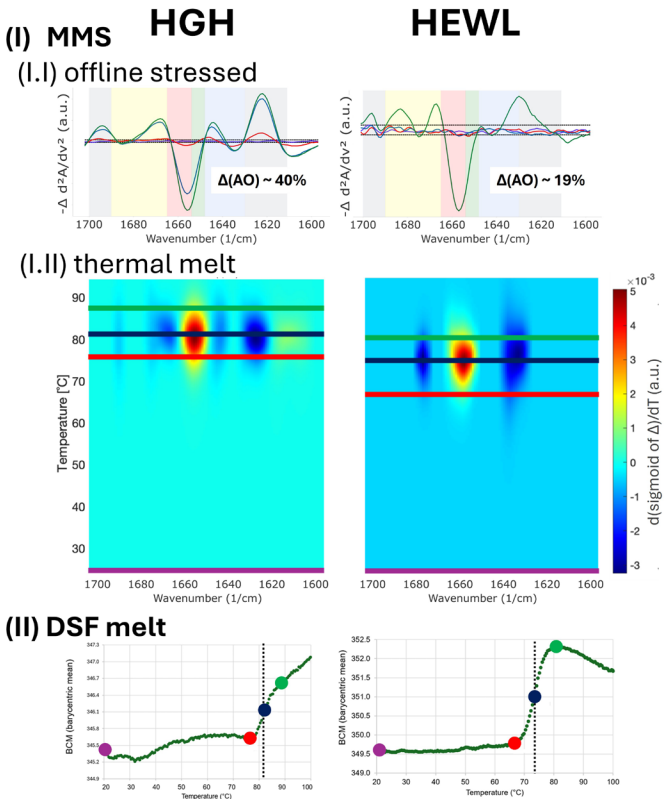

Figure 2: Offline thermally stressed HGH (left) and HEWL (right) measured with MMS (panel I.I), compared with thermal melts of HGH (left) and HEWL (right), studied with MMS (panel I.II) and DSF (panel II). Panel I.II shows the MMS heat maps from 25 to 95°C. The MMS heat maps show the first derivative of the difference spectra at each temperature compared to the room temperature spectra where hot spots (red) indicate a spectral (and structural) loss, and the cold spots show a spectral (and structural) gain. The DSF curves in panel II were recorded from 20 to 100°C with the relevant temperature points marked in purple (room temperature), red (T_{onset}), blue (T_m) and green (T_{end}). The corresponding temperatures are marked with horizontal lines in the MMS heatmap following the same color coding. Panel I.I shows the MMS difference spectra between offline thermally stressed samples and the room temperature control. The color coding of the spectra corresponds to the temperatures marked in the melting curves.

Overall, the spectral changes observed in the offline stressed samples match with the spectral regions where we find hot and cold spots in the MMS heat maps. For HGH, the delta plots demonstrate consecutive and irreversible structural changes beginning already at T_{onset}, which persist after cooling back to room temperature. This irreversibility is reflected in the Similarity Score (ΔAO), the change in spectral area overlap, with HGH showing a ~40% difference between T_{end} and T_{ref}. In contrast, HEWL exhibits significantly smaller changes (~19% ΔAO), with notable differences emerging only between T_m and T_{end}. Below the T_m, the room-temperature spectra of thermally stressed HEWL remain nearly identical to their reference, indicating structural reversibility up to the midpoint of unfolding.

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Results, continued

These findings suggest that although HGH exhibits higher thermal stability based on its T_m, its unfolding is associated with more pronounced and irreversible structural rearrangement. HEWL, while melting at a lower temperature, retains considerable structural reversibility during the early stages of thermal stress. Thus, the offline isothermal MMS data reveal not only the extent but also the reversibility of protein structural changes, providing crucial mechanistic insights into the thermal behavior of proteins.

2. Immunoglobulin G (IgG)

2.1 Thermal melt: MMS vs DSF

Figure 3 presents thermal melt measurements of Immunoglobulin G (IgG) acquired using MMS (top) and DSF (bottom), following the same format as shown previously for HGH and HEWL in Figure 1.

The inverted and baselined second derivative spectrum at room temperature reveals that IgG is predominantly composed of beta-sheet structures, as indicated by a strong absorption centered at 1641 cm^{-1}. Gaussian deconvolution of this spectrum quantifies the secondary structure composition as 63% beta-sheet, 30% turn, 6% unordered, and only 1% alpha-helical content, consistent with the expected architecture of IgG.

DSF analysis (Figure 3, bottom panel) reveals two distinct unfolding transitions, with melting temperatures T_{m1} = 71 °C and T_{m1} = 81 °C. These transitions are in line with the known modular architecture of IgG, where individual domains—such as CH2/CH3 and Fab—exhibit distinct thermal stabilities.

In contrast, MMS analysis, which currently employs a mono-sigmoid fitting approach per wavenumber, identifies a single thermal transition at 80.5 °C. This result closely aligns with the second DSF transition (T_{m2}), suggesting that the primary structural rearrangement detectable by MMS occurs during melting of the Fab domain. The associated hotspot in the MMS melt map is observed at 1641 cm^{-1}, reflecting a loss of intramolecular beta sheet content.

A corresponding cold spot at 1627 cm^{-1} indicates the formation of intermolecular beta sheet structure, often associated with early aggregation phenomena.

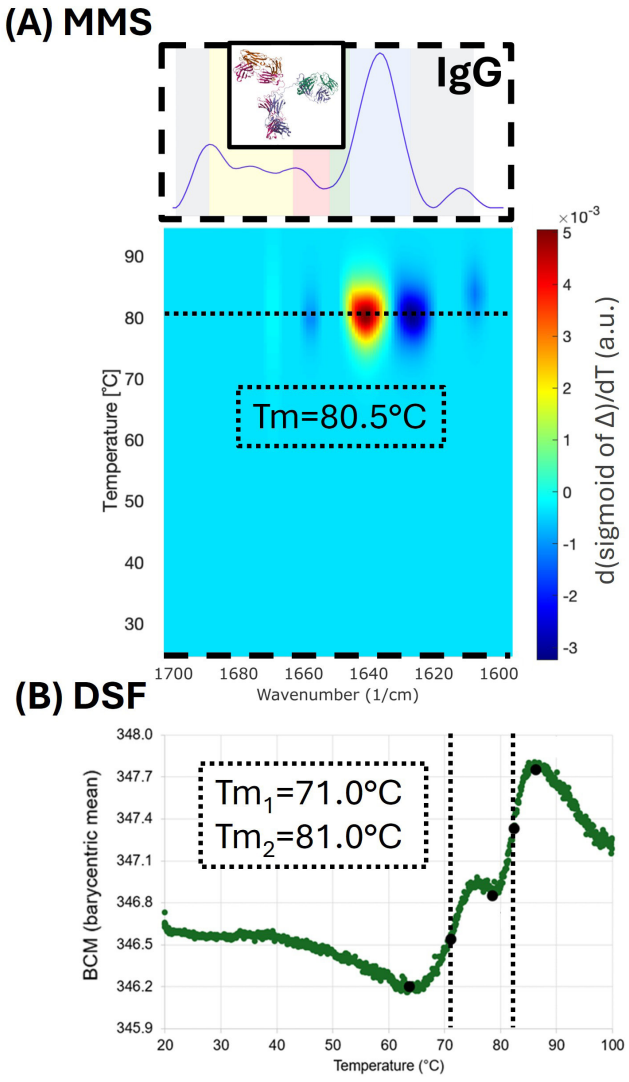

Figure 3: Thermal melt results of IgG measured with MMS (panel A) and DSF (panel B). Top of panel A shows the inverted and baseline-corrected second derivative MMS spectrum at room temperature. Bottom part of panel A shows the MMS heat maps from 25 to 95°C. The MMS heat map shows the difference spectra at each temperature compared to the room temperature spectra where hot spots (red) indicate a spectral (and structural) loss, and the cold spots show a spectral (and structural) gain. The DSF curves in panel B were recorded from 20 to 100°C. For both methods, the determined melting points T_m are displayed in the corresponding inset.

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Results, continued

It is important to note that while mono-sigmoid fits are effective for capturing dominant transitions at specific wavenumbers, they can obscure multiple overlapping transitions that occur within the same spectral region. To address this limitation, a multi-sigmoid fitting strategy has been developed and is applied in section 2.2 to further resolve and interpret the complex, domain-specific unfolding behavior of IgG. This approach allows for a more nuanced analysis of structurally heterogeneous proteins and enhances the utility of MMS in characterizing multistate thermal transitions.

2.2. Multi-sigmoid MMS analysis vs DSC vs DSF

To resolve the multistate thermal transition behavior of Immunoglobulin G (IgG), we applied a multi-sigmoid fitting approach to the MMS melt data, focusing on the most prominent hot spot at 1641 cm^{-1} and cold spot at 1627 cm^{-1}. The results are presented in the top panel of Figure 4.

The middle panel shows a Differential Scanning Calorimetry (DSC) thermogram for IgG, while the bottom panel reproduces the DSF melting curve from Figure 3. DSF reveals two melting transitions at 71 °C (T_{m1}) and 81 °C (T_{m2}), consistent with IgG's known domain-specific unfolding events. When the MMS data are fitted with a multi-sigmoid, these two transitions are also resolved, confirming that MMS can accurately detect complex thermal unfolding behavior.

Importantly, the intensity of the spectral change is not evenly distributed between the two transitions. The change associated with T_{m2} is approximately ten times larger than that at T_{m1}. This explains why the earlier mono-sigmoid fit in Figure 3 predominantly reflected T_{m2}—it represents the major structural reorganization, typically attributed to Fab domain unfolding.

Unlike DSF, MMS provides quantitative insights into the magnitude of structural transitions, making it a more informative method for analyzing protein unfolding. This is further validated by the DSC data, which also reveal the same T_m values, along with a similar relative ratio of transition intensities. The agreement across all three methods confirms that MMS not only matches in terms of transition temperatures but also meets the quantitative precision of DSC.

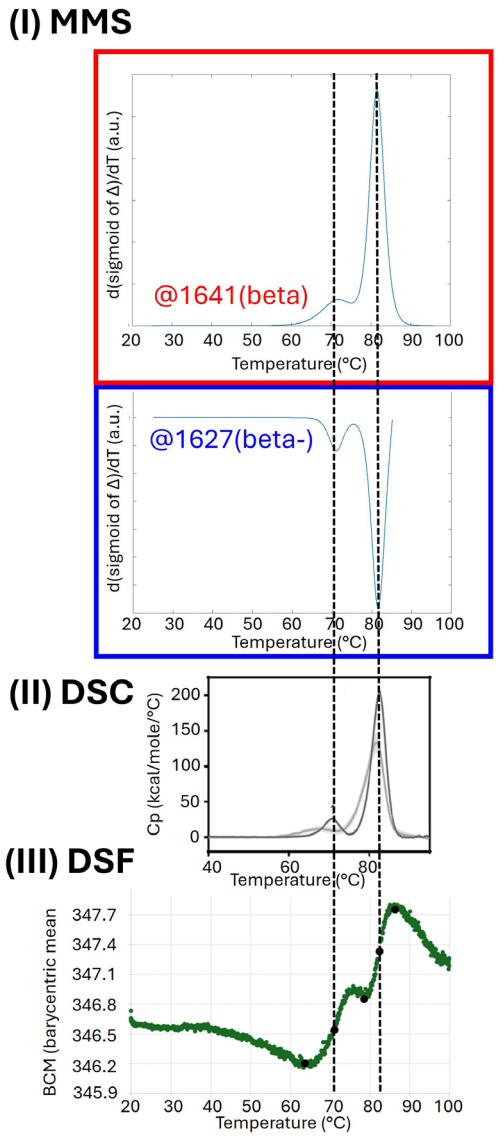

Figure 4: Multi-sigmoid fitting of MMS thermal melts of IgG (panel I), compared with DSC (panel II) and DSF (panel III) data. The MMS and DSF data correspond to the measurements presented in Figure 3. The DSC figure is adapted from [7] showing the same IgG as tested here (darker trace) and an ADC of that IgG (lighter trace). The MMS curves in panel I correspond to vertical slices at 1641 cm^{-1} (beta sheet band) and 1627 cm^{-1} (intermolecular beta sheet) of the heatmap shown in Figure 3, fitted with a multi-sigmoid fit.

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Conclusions

This study demonstrates the powerful capabilities of Microfluidic Modulation Spectroscopy (MMS) using the AuroraTX platform for detailed characterization of protein thermal unfolding. Compared to traditional methods like DSF and DSC, MMS not only accurately determines melting temperatures but also provides unique insights into secondary structure changes throughout thermal transitions. By resolving multistate unfolding events and quantifying the extent of structural rearrangements, MMS proves to be a highly sensitive and quantitative method for protein stability analysis.

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