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Technical Note

Thermal ramp experiments measure protein conformational stability and propensity to aggregate

Introduction

The native states of proteins are stabilized by formation of many weak non-covalent interactions. Hydrophobic side chains of amino acids tend to pack together, and hydrogen bond donors and acceptors (particularly in the peptide backbone) pair with each other. The denatured protein also forms many interactions (albeit with solvent water molecules) such that the folded state is only marginally stable due to a small difference between the large free energy values exhibited by the native and unfolded states.

Proteins can therefore be denatured by changing their physical or chemical environment - here the application of a thermal stress will be discussed. A widely used measure of the thermal structural stability of a protein is the temperature at which it unfolds from the native (folded) state to a denatured (unfolded) state. For many proteins this unfolding process occurs over a narrow temperature range and the mid-point of the transition is often termed the 'melting temperature' or 'T_m'. This value reflects the thermal structural stability of the protein and is related to the Gibbs free energy of the protein (the energy difference between the native and denatured states).

Formally, T_m can be defined as the temperature at which there is an equal population of folded and unfolded proteins in solution. In order for an experiment to accurately determine an unambiguous T_m a number of criteria must be fulfilled: the protein should have a single co-operative unfolding transition, the transition should be from fully folded to fully unfolded populations, the transition should be reversible and the unfolding and folding transitions should take place in a time period much shorter than the rate at which the temperature is increased during the experiment.

For the large multi-domain proteins that are typically of interest as biopharmaceuticals, these conditions are not often met. As a result, the T_m values measured by various analytical techniques are often 'apparent' or 'observed' transition temperatures rather than melting temperatures according to the formal two-state definition. For practical purposes, the apparent T_m of a given protein in a given solvent environment is a valuable and widely used measure of the thermal stability of a protein, which is useful for instance when looking at the effects of formulation on thermal stability. Here, we therefore define T_m as the midpoint of an observed temperature-induced unfolding transition.

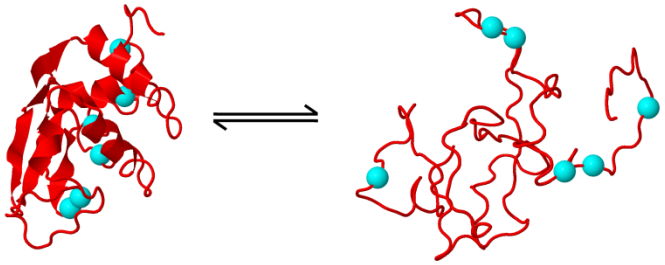

Figure 1: Representation of the equilibrium between the native state (left) and the unfolded state (right) of hen egg white lysozyme (HEWL). The Trp residues (cyan spheres) are buried in the hydrophobic core of the native state but exposed to the polar aqueous solvent in the highly dynamic unfolded state. Figure prepared using coordinates in PDB accession file 3J6K.

Measurement of T_m using the UNit

The UNit has multiple modes that allow the acquisition of T_m data. Typically a thermal ramp experiment involving simultaneous heating of a number of samples over a defined temperature range at a defined rate is used. The change in tertiary conformation of a protein with temperature can be monitored using intrinsic fluorescence. This uses the emission spectra of naturally occurring aromatic amino acids (such as tryptophan (Trp) and tyrosine (Tyr)) as probes of tertiary structure and can be excited using the integral 266 nm laser in the UNit. The change in environment of Trp residues is represented in Figure 1 for an unfolding event of the small globular protein hen egg white lysozyme (HEWL).

It is also possible to obtain similar information within the UNit instruments by addition of extrinsic dyes (such as 8-Anilino-1-naphthalenesulfonic acid – 1,8-ANS – or SYPRO orange) that report on unfolding by changing their fluorescence properties upon binding to exposed hydrophobic regions. This note will focus on the label-

free approach of intrinsic fluorescence which can be used in the UNit with low sample volumes (9 µL).

Using the spectroscopic techniques available in the UNit it is possible to obtain information on the fraction of unfolded protein as the temperature increases (making the same assumptions described earlier) and therefore to obtain further thermodynamic parameters using van't Hoff analysis as described in the Technical Note entitled 'Thermodynamic stability of proteins – temperature-induced unfolding'.

Example thermal ramp data

The UNit is capable of measuring 48 samples at once, so different conditions of the same protein can be studied simultaneously. Example data were acquired for HEWL (1 mg mL^{-1}) formulated with phosphate or citrate-phosphate buffer at a range of pH values. The temperature was increased from 15 - 95 °C at a constant linear rate of 0.3 °C min^{-1}.

Example raw intrinsic fluorescence emission spectra obtained during the experiment are displayed in Figure 2. It is apparent that as the temperature increases the spectra intensity decreases and also the maximum emission wavelength (λ_{max}) moves to longer wavelengths. This shift is caused by the fact that the fluorescence emission of Trp is sensitive to the polarity of its surroundings. Hence, as these residues experience a change in environment, from the buried hydrophobic core of a native state to the exposed, polar state in the unfolded polypeptide chain the λ_{max} moves.

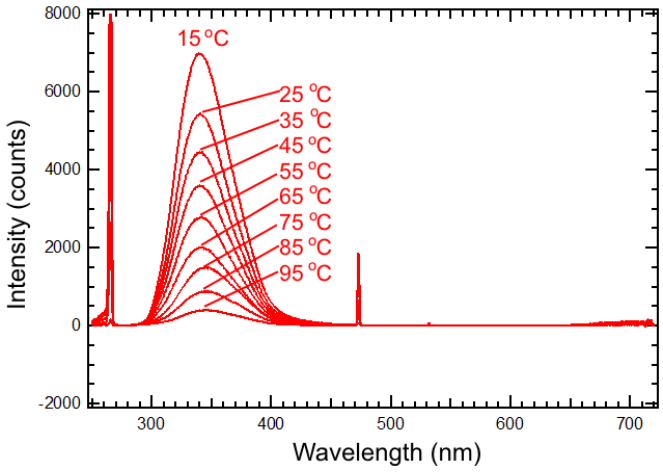

Figure 2: Intrinsic fluorescence emission spectra for HEWL in pH 6 buffer between 15 and 95 °C.

The UNit software automatically follows the changes in spectral properties with temperature and provides parameters that describe this. Definitions of these parameters are found in the Technical Note named 'Intrinsic fluorescence monitors conformational changes in proteins'. In Figure 3 the barycentric mean (BCM), which describes the shift in center of gravity of the intrinsic fluorescence spectra, and hence the conformational change of the protein is plotted against temperature for each of the HEWL samples tested. Each trace has a similar shape and displays a single transition over a narrow temperature range indicative of a co-operative unfolding event for this single-domain globular protein. The temperatures at which these transitions occur differ between the formulations, indicating the protein has different thermal stabilities under these various conditions. By default the UNit Analysis software uses the maximum value of the first derivative of the trace to define T_m, although other analyses, such as curve fits can be applied to obtain these values.

The data for HEWL indicate that the samples with the highest T_m are those with a pH of 4 or 5, suggesting the protein has the highest thermal conformational stability under these conditions.

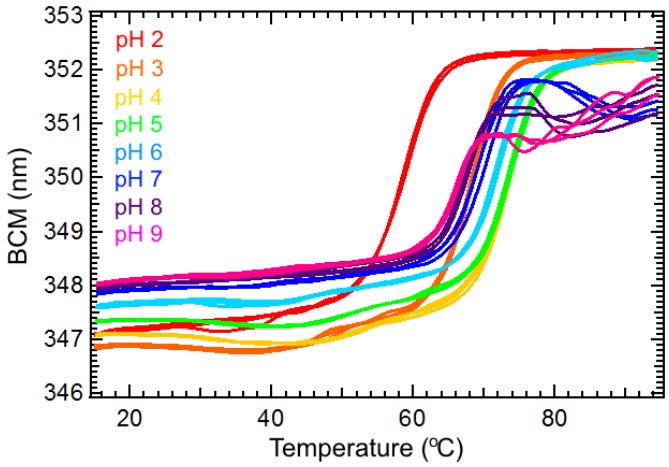

Figure 3: BCM *versus* temperature plots for HEWL in pH 2 - 9 buffers.

Aggregation onset measurements

Protein degradation can occur *via* numerous mechanisms and frequently results in formation of aggregates. Typically, unfolding of proteins results in exposure of hydrophobic residues buried in the native

core. These moieties prefer to pack with each other, and therefore intermolecular interactions are promoted which drive aggregation. Formation of these degenerative intermolecular interactions can be mitigated in some cases by appropriate formulation, for instance by adjusting pH, ionic strength or addition of additives such as detergents, which may limit aggregation even for an unfolded protein ensemble. In certain systems aggregation can occur prior to unfolding as aggregation-prone regions may be displayed on the surface of the native protein.

During a thermal ramp aggregation will occur after unfolding in the first case described above as exposure of aggregation-prone hydrophobic residues is dependent upon conformational stability, whereas aggregation may precede unfolding during a thermal ramp in the second case, where native states aggregate. Therefore, it is important to measure both conformational stability and robustness to aggregation to obtain a comprehensive picture of protein stability. It has been demonstrated that use of independent orthogonal stability screening assays is crucial for the correct assessment of therapeutic protein candidates. [1]

In the UNit, aggregation behavior can be measured in the same experiment (and for the same samples) that provides tertiary conformational information. The intensity of static light scattering (SLS) from the lasers used to illuminate the sample is proportional to the average molecular weight of species in solution. Thus, it is sensitive to aggregation events during a thermal ramp. The UNit uses this data to assign an aggregation onset temperature (T_{agg}) for each sample.

Hence, measurement of both T_m and T_{agg} values for the same samples adds an extra dimension to stability assays that aids formulation optimization.

The data obtained for the example HEWL samples is displayed in **Figure 4**. For samples incubated below pH 6 there is no clear aggregation event, even at high temperatures. The intrinsic fluorescence data (**Figure 3**) indicate that these exact samples unfold during the thermal ramp, suggesting that charge-charge repulsion between denatured monomers under these low pH conditions inhibits the inter-molecular interactions that promote aggregation. In the samples incubated at pH 6-9 there is an increase in SLS intensity observed, indicating that aggregation has taken place. Interestingly, after a sharp increase the intensity drops again during further heating (for instance at ~ 70 °C for the pH 9 sample). This is due to the fact that very large protein aggregates precipitate and drop out of solution. SLS is only sensitive to species in solution, therefore upon precipitation the measured intensity drops. The UNit Analysis software automatically obtains T_{agg} values for data regardless of whether there is a clear post-transition baseline or not.

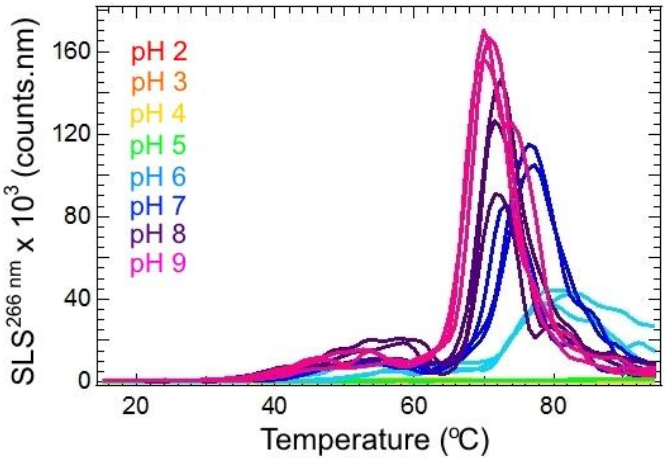

Figure 4: SLS (266 nm) *versus* temperature plots for HEWL in pH 2 - 9 buffers.

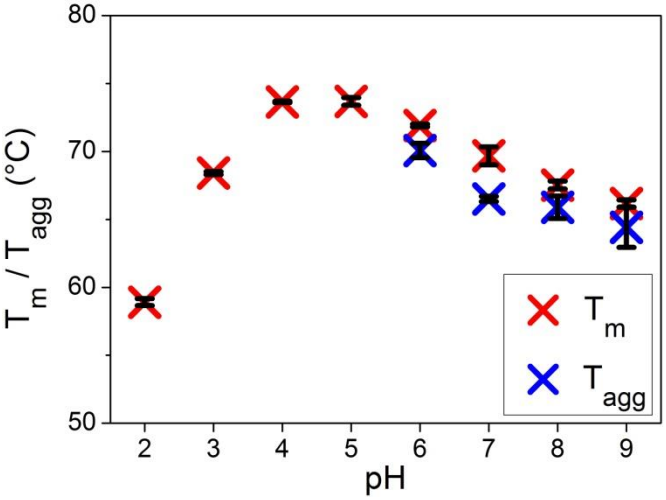

Figure 5: T_m and T_{agg} *versus* pH plot for HEWL samples.

Mechanisms of protein degradation

The ability to obtain simultaneous information on both conformational stability and propensity to aggregate

provides a detailed description of the degradation mechanism of a protein sample. Comparison of the T_m and T_{agg} values for HEWL are shown in Figure 5 and Table 1. It is clear for the samples that show aggregation that this occurs at a similar temperature to unfolding (remember that here we use the midpoint of the unfolding transition, but the onset of aggregation). This indicates that this protein undergoes conformationally-driven aggregation, where hydrophobic residues hidden in the core of the native protein have to be exposed before aggregation occurs.

pH	T_m (°C)	T_{agg} (°C)
2	58.9 ± 0.3	
3	68.4 ± 0.1	
4	73.7 ± 0.1	
5	73.7 ± 0.3	
6	71.9 ± 0.1	70.1 ± 0.5
7	69.7 ± 0.7	66.5 ± 0.2
8	67.5 ± 0.3	65.9 ± 0.8
9	66.2 ± 0.3	64.4 ± 1.5

Table 1: T_m and T_{agg} values for HEWL in pH 2 - 9 buffers measured using the UNit.

Mechanisms of MAb degradation

The UNit approach of simultaneously obtaining both intrinsic fluorescence and SLS data to assess stability is generally applicable to the vast majority of proteins as described in the Technical Note 'Versatility of the multi-parameter UNit approach in characterizing a range of protein formulations'. The example above describes data for a relatively simple, single-domain globular protein, however, most therapeutic proteins are more complex than this. Currently, the most common class of biologics are monoclonal antibodies (mAbs), which are large multi-domain proteins with a characteristic structure.

A thermal ramp was applied to 1 mg mL^{-1} of a monoclonal antibody (MAb1) stored in phosphate-citrate buffer at pH 4.6. The temperature was increased from 15 - 95 °C at a constant linear rate of 0.3 °C min^{-1}. The data obtained for both intrinsic fluorescence (BCM) and SLS are overlaid in Figure 7. It is apparent that the intrinsic fluorescence plot is more complex than that observed for HEWL and contains multiple transitions, indicative of the presence of at least two domains with different thermal stabilities. Indeed, it is known that the thermal stability of the C_H2 domain is more sensitive to low pH conditions than that of the rest of the protein, such as the F_{ab} domain.[2] Interestingly, aggregation is only observed upon unfolding of the higher stability domains of the protein, which presumably contain the most aggregation-prone sequences. It also demonstrates that the least conformationally stable regions of a multi-domain protein are not necessarily the most aggregation-prone and highlights the importance of obtaining multiple stability-defining parameters.

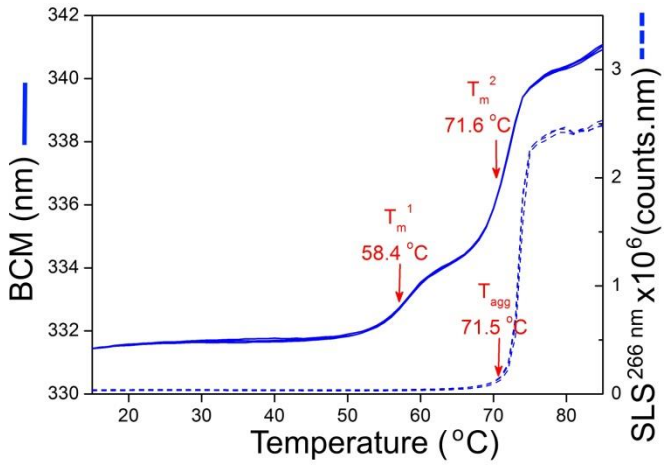

Figure 7: Overlay of intrinsic fluorescence and SLS data obtained during a thermal ramp of MAb1 at pH 4.6. The T_m and T_{agg} values extracted from this data are displayed in red.

Summary and Conclusions

Thermal ramp experiments on up to 48 low-volume (9 µL) protein samples using the UNit, provide multiple stability-defining parameters including T_m and T_{agg}. The ability to obtain simultaneous information on both conformational stability and propensity to aggregate provides far greater detail on the degradation mechanism of a protein sample. High-throughput measurements can be used for a range of applications where protein stability is vital, such as in forecasting the long-term stability of protein formulations or in screening certain protein-ligand interactions.

References

[1] Garidel *et al., Spectroscop. Eur.* (2014) **26**, 9-13.

[2] Vermeer & Norde, *Biophys. J.* (2000) **78**, 394-404

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