UNCHAINED LABS

Technical Note

Measurements of protein refolding using the UNit

The importance of measuring protein refolding

Protein thermal stability is vital for commercial interests as many processes, such as those in the food industry, involve use of enzymes at high temperatures and require knowledge of how proteins behave when heated.[1] Furthermore, analysis of refolding is industrially important because a common bioprocessing step during biopharmaceutical production involves protein denaturation followed by refolding.[2,3,4] The robustness of a protein to denaturation and its ability to refold are also modulated by the conditions under which this process occurs. Therefore, it is important for the design of industrially useful heat-resistant proteins to have a multi-sample platform on which protein denaturation and refolding can be assessed.

Native protein states are cooperatively stabilized by a large number of weak non-covalent interactions that can be perturbed by methods such as heating, addition of chemical denaturants or mechanical stress, all of which lead to unfolding. Upon denaturation the interactions (such as hydrophobic and electrostatic forces) between amino acids within the primary sequence that drive protein folding and stabilize the protein in the native state, can also lead to aggregation as the residues involved become free to form inter-molecular interactions.[5] Heat-induced denaturation of proteins is further complicated by the fact that it can lead to chemical and covalent changes to the protein chain in the unfolded state. Therefore, understanding the refolding process is a complex task.

Characterizing protein refolding with the UNit

Thermal denaturation and refolding of a protein was studied using the UNit instrument. The UNit includes a heating/cooling plate that controls the temperature in a micro cuvette array (UNi) containing small volumes (< 9 µl) of sample. This enables a range of software-managed heating and cooling profiles to be performed, whilst the effects on the sample can be determined by simultaneously assessing protein structure *via* intrinsic fluorescence measurement and aggregation state using static light scattering (SLS) at two wavelengths (266 and 473 nm).

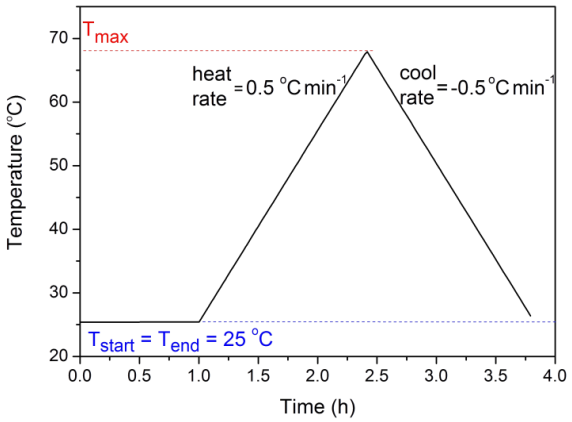

Figure 1. Sawtooth temperature cycle profile.

For this study, a 'sawtooth' profile was used (Figure 1) that included a pre-measurement incubation at 25 °C followed by a linear temperature ramp (at 0.5 °C min^{-1}) up to a defined maximum temperature and then a linear decrease back to 25 °C at the same rate. The maximum temperature (T_{max}) was varied in a range of experiments so that the effect of this value on thermal denaturation could be assessed. Hen egg white lysozyme (HEWL), a small protein (14.3 kDa) that contains four intramolecular disulphide bonds (Figure 2)[6] and has a reported pI of 11.35 was studied.[7] HEWL contains six tryptophans (Trp), as well as three tyrosines (Tyr), all of which are fully or partially buried in the native state.

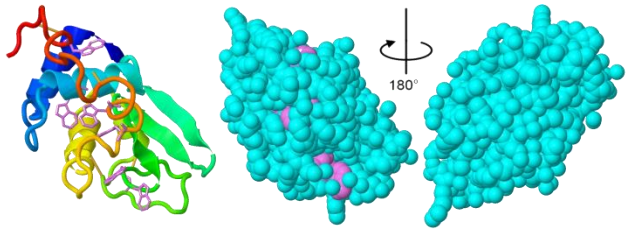

Figure 2. Structure of HEWL (taken from PDB:2LYZ).[6] Ribbon diagram on the left shows disulphide bonds in gold and Trp sidechains in violet. Space-filling models on the right indicate the burial of Trp residues (violet) in the native state.

The aromatic sidechains of these residues are excited at 266 nm and, with their resultant emission spectrum being dependent upon their local environment, reflect the tertiary structure of the protein molecule (see the 'Fluorescence data analysis methods' the UNit Technical Note). As the protein is heated and unfolds these buried aromatics become more exposed to the polar solvent surrounding them resulting in a concomitant wavelength shift in the maximum peak intensity.

Using HEWL and the 'sawtooth' thermal profile it should be possible to answer a number of questions about thermal refolding: How reversible is thermal unfolding? How badly can the protein be treated and still refold? Are the melting temperatures, and hence the unfolding and refolding mechanisms, the same in both the heating and cooling branches of the profile?

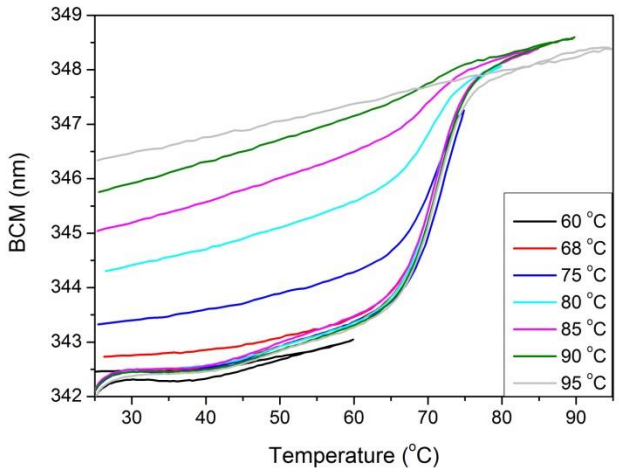

Figure 3. Averaged BCM data obtained for HEWL samples using sawtooth temperature profiles with the T_{max} value for each experiment indicated.

Thermal refolding of lysozyme

Initially, HEWL was screened for thermal reversibility under a range of conditions (pH 4.6/5.5/6.4/7.2/8.0/8.4 in 0/50/150 mM NaCl with addition of GdnHCl/Arg/DTT/EDTA) using multiple small volume samples in the UNit. Then, a number of thermal 'sawtooth' cycles using $T_{start} = T_{end} = 25$ °C and a range of T_{max} values (60, 68, 75, 80, 85, 90, 95 °C) were performed using eight replicates of 0.5 mg ml^{-1} HEWL in 50 mM Na citrate (pH 6.4), 150 mM NaCl, 100 mM GdnHCl, each experiment was performed in duplicate. The intrinsic fluorescence data collected was analyzed using the barycentric mean (BCM), as described in the 'Fluorescence data analysis methods' the UNit Technical Note. This value generally increases as a protein unfolds and the Trp and Tyr residues present become more exposed to the polar solvent and λ_{max} moves to longer wavelengths. The averaged BCM data for each set of conditions is shown (for illustration purposes only) in Figure 3.

It was observed that the heating arm of each profile gave a similar response, independent of T_{max}, whereby at lower temperatures, an initial shallow linear slope (presumably due to temperature-dependent changes in the intrinsic fluorescence of the folded protein) is succeeded by a steeper gradient as the transition to an unfolded state occurs. The point at which this rate of change is greatest is taken as the melting temperature (T_m) as described in the 'Comparison of the UNit with standard methods to determine T_m' Technical Note, which under these conditions is measured as 71.9 ± 0.3 °C, similar to those recorded elsewhere.[8] For those datasets that continue heating beyond this point, the post-transition slopes are all very similar. The cooling arm of the experiment is dependent on the T_{max}, as incubation at higher temperature results in reduced recovery of folded protein at the end of the experiment.

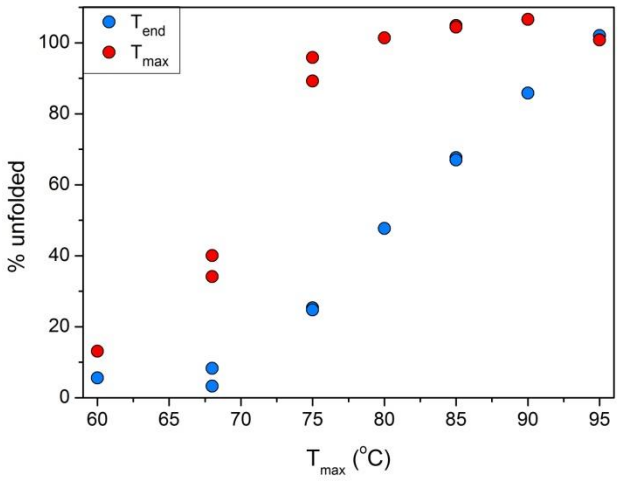

Figure 4. Estimated amount (from BCM data) of unfolded HEWL present at the T_{max} (red) and T_{end} (blue) of each experiment.

Reversibility of protein unfolding

To analyses the reversibility of the process, data obtained from the sawtooth heating profiles at the start of the experiment were compared to those of the same temperature at the end of the experiment. This provides a method to assess the effect of heating the protein and gives an endpoint analysis of the reversibility of heat-induced unfolding of HEWL. To provide context and values for 100 % unfolded samples, BCM was measured at a range of temperatures for a sample of HEWL that was irreversibly denatured. Furthermore, a native sample was exposed to an equivalent number of laser shots so that photobleaching could be accounted for. Thus, maximum ΔBCM value ($BCM_{Tstart} - BCM_{Tend}$) could be estimated using the values from the fully denatured and fully native datasets. This enabled the evaluation of the percentage of unfolded protein at both the T_{max} achieved in each experiment and also at the conclusion of the temperature cycle (Figure 4).

The data indicate that as the T_{max} rises above the T_m (71.9 °C) almost all of the proteins show disruption of tertiary structure. The amount of protein unfolded after cooling back to 25 °C shows that recovery is higher in samples heated to lower T_{max} values, for instance, although virtually all of the tertiary structure of HEWL is disrupted upon heating to 75 °C, only 25.0 % remains disrupted at the end of the experiment, after cooling.

It is likely that the decrease in reversibility as a function of increased exposure of the protein to temperatures above the T_m value is due to covalent chemical modifications that occur as the protein is denatured under these conditions. At the pH studied it is known that the intramolecular disulphide bonds present in HEWL can be broken and, upon cooling, can form scrambled covalent interactions that lead to misfolded monomer and formation of intermolecular contacts, resulting in aggregation.[9] This is confirmed by the UNit, which simultaneously measures SLS at two wavelengths (266 nm and 473 nm) and can be used to assess the levels of aggregation present in the sample, as described in the 'Characterization of protein aggregation using the UNit' Technical Note. The change in SLS from T_{start} to T_{end} for each experiment is shown in Figure 5. It is clear that the samples heated to temperatures above the T_m of HEWL contain aggregates even after returning to the starting temperature.

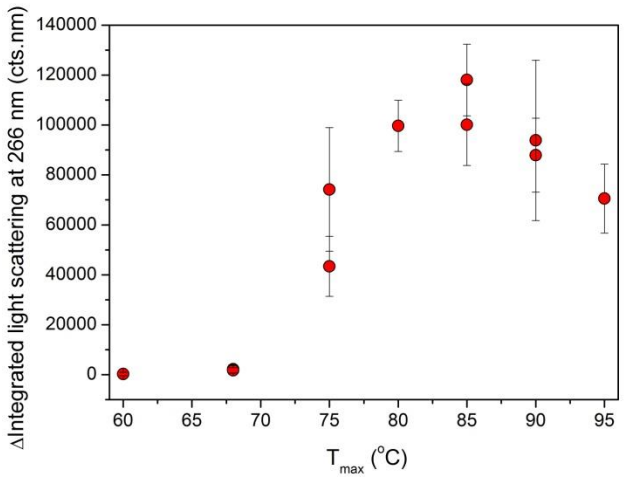

Figure 5. The change in SLS at 266 nm from the start of the experiment to the endpoint.

In addition to aggregation induced by heat denaturation, it is possible that the accumulation of other chemical modifications (such as deamidation, aspartate isomerization and peptide bond hydrolysis) will eventually result in a native state that is so destabilized that an unfolded or misfolded state will be more thermodynamically stable even at low temperatures.[9,10,11]

Measurement of protein stability

The T_m values upon cooling can be calculated and compared with the corresponding data obtained upon heating (Figure 6). Under the conditions studied, the T_m calculated during heating is consistent across all experiments (71.9 ± 0.3 °C), however, the value of the T_m measured during cooling is lower for each experiment, giving an overall reduced average of 69.5 ± 0.6 °C. This is likely due to chemical modifications to the protein that are promoted (alongside unfolding) at high temperatures, meaning that the transition upon cooling is between modified unfolded and folded states of different stabilities to those found during the heating transition. [9,11]

A number of heat-induced chemical changes (such as deamidation) destabilize the native state, but do not irreversibly denature the protein, and hence result in a reduced T_m value. It can be observed that the cooling T_m values for the fraction of proteins that refold decrease

as their exposure to high temperatures increases. This implies that as the incubation time and temperature are raised there is an accumulation of such chemical changes that decrease protein stability.[9,10,11]

The reversibility of the process can also be assessed by the relative heterogeneity of unfolding and folding melting temperatures as judged by the gradients of the transition slopes (Figure 7). These data indicate that when T_{max} is below T_m (and hence less than half of the protein population is unfolded as judged by intrinsic Trp fluorescence) the values for unfolding and folding are approximately equal. This suggests that under these conditions the process of HEWL unfolding is mechanistically reversible, presumably because fewer chemical modifications have accrued as the protein spends less time unfolded at the highest temperatures.

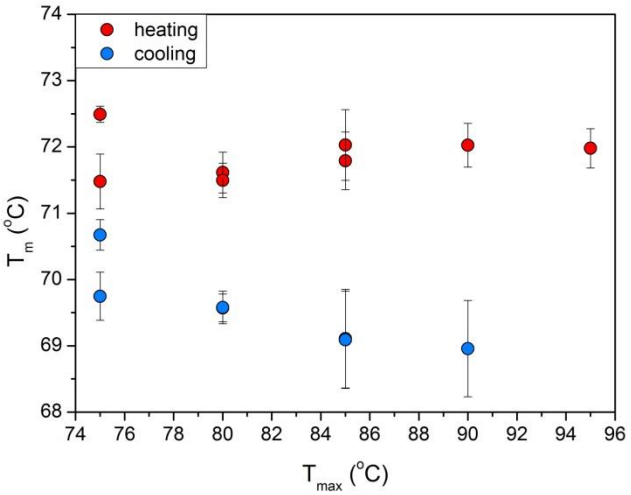

Figure 6. Measured T_m values for heating (red) and cooling (blue) branches of sawtooth profile.

However, as the T_{max} increases there is a noticeable difference in the unfolding and refolding transition gradients. The refolding occurs over a broader temperature range as the T_{max} increases, up to 95 °C where no refolding is measureable. This change in folding transition could be because under the higher temperature conditions there is a greater range of chemically-altered species, many with reduced native stabilities, resulting in the observation of a broader slope.[9,11]

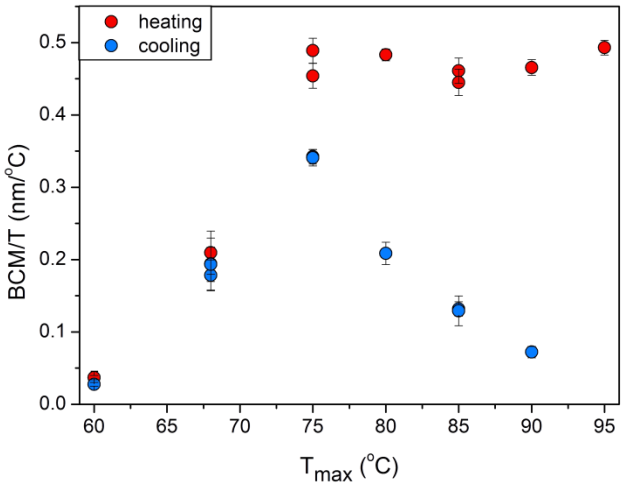

Figure 7. Slopes of melting transition for both heating (unfolding, red) and cooling (refolding, blue) branches of sawtooth experiments calculated from BCM data.

Conclusions and summary

A sawtooth heating profile on the UNit has enabled the examination of the reversibility of HEWL thermal refolding. The low volumes used allow the determination of sample response to temperature change with high time resolution. Indeed, the combination of intrinsic fluorescence and SLS measurements has provided valuable information on the complex processes undergone by the protein. The UNit instrument enabled the use of small sample volumes and acquisition of multiple datasets in a highly practical and time-efficient manner. The technique also provided an economical method for screening a range of conditions during one experiment.

Under the conditions studied, heating of HEWL results in conformational unfolding and generation of both chemical modifications (in the shape of formation of non-native and intermolecular disulphide bonds) that result in irreversible denaturation as well as chemical modifications that reduce the stability of the native state, in a manner that is proportional to the exposure of the protein to extreme temperatures. The biophysical data acquired using the UNit agree well with previous studies that indicate a loss of enzymatic activity of HEWL upon heating due to chemical degradation.[9,10,11]

References

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