Stability and structural reversibility of proteins exposed to high temperatures

Thermal stability of proteins

During various industrial processes it is necessary for proteins to be temporarily placed under elevated temperatures. It is crucial to understand whether the protein of importance is stable at increased temperatures and whether the effects of increasing the temperature are reversible. The resistance of a protein to denaturation and its ability to refold are also modulated by the chemical conditions under which this process occurs. Therefore, it is advantageous for the design of industrially useful heat-resistant proteins to have a multi-sample platform on which protein thermal denaturation and refolding can be assessed.

The UNit includes a programmable thermal plate that controls the temperature in UNi containing small volumes (< 9 µl) of sample. Due to the low volumes used the temperature changes in the cuvettes are highly responsive allowing a range of software-managed heating and cooling profiles to be executed. 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). Here we describe the benefits of a flexible temperature profile allied to highly responsive real-time analytical measurements for understanding protein stability.

Characterizing protein thermal stability with the UNit

Native protein states are cooperatively stabilized by a large number of weak non-covalent interactions that can be broken by heating. Upon denaturation the interactions (including 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 aberrant intra- and inter-molecular interactions.[1] 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.

To understand the effects that heating has upon protein stability, a model protein, hen egg white lysozyme (HEWL), was studied using the UNit. HEWL is a small protein (14.3 kDa) that contains four intramolecular disulphide bonds (Figure 1)[2] and has a reported pI of 11.3.[3] The thermal stability of the protein under different buffer conditions (50 mM Na citrate, 150 mM NaCl, 100 mM GdnHCl pH 6.4 or pH 3) was assessed initially by performing a linear temperature ramp and monitoring the resulting fluorescence spectra.

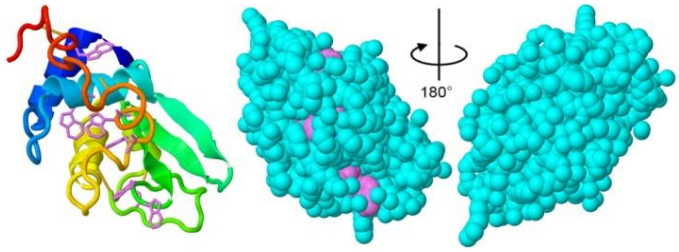

Figure 1. Structure of HEWL (taken from PDB:2LYZ).[2] Ribbon diagram on the left shows disulphide bonds in gold and Trp sidechains in violet. Space-filling models on the right indicate the burial of Trp residues (violet) in the native state.

HEWL contains six tryptophans (Trp), as well as three tyrosines (Tyr), all of which are fully or partially buried in the native state. The aromatic sidechains of these residues are excited in the UNit with the integrated 266 nm laser. The resultant emission spectrum is dependent upon their local environment and hence reflect the tertiary structure of the protein molecule (see the 'Fluorescence data analysis methods' UNit Technical Note).

As the protein is heated during the temperature ramp it unfolds and these buried aromatics become more exposed to the polar solvent surrounding them resulting in a concomitant wavelength shift in the maximum peak intensity.[4] The intrinsic fluorescence data collected are analyzed using the barycentric mean

(BCM), as described in the 'Fluorescence data analysis methods' UNit Technical Note. The BCM value generally increases as a protein unfolds and the Trp and Tyr residues present become more exposed to solvent. The data (Figure 2) show the temperature-dependent transition of the protein from the folded state to the unfolded state and allows automatic calculation of the midpoint of this melting transition (Tm) by the UNit software; the values obtained are similar to those reported elsewhere.[5] It is clear that HEWL is less thermally stable at pH 3.0 than at pH 6.4, likely due to unfavorable charge-charge interactions under these conditions.

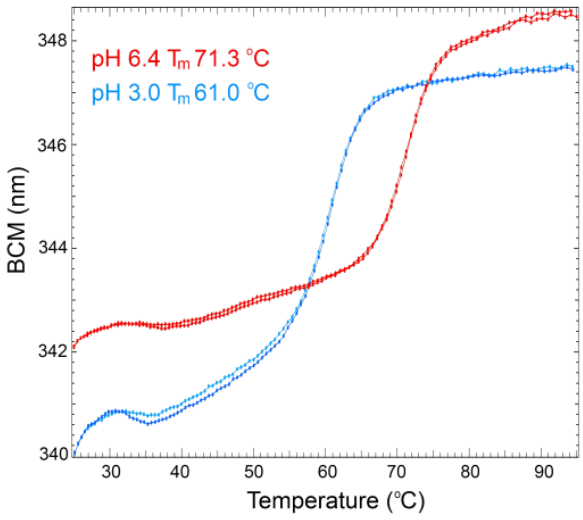

Figure 2. Melting temperature analysis of HEWL at pH 6.4 and pH 3.0 as measured by the UNit.

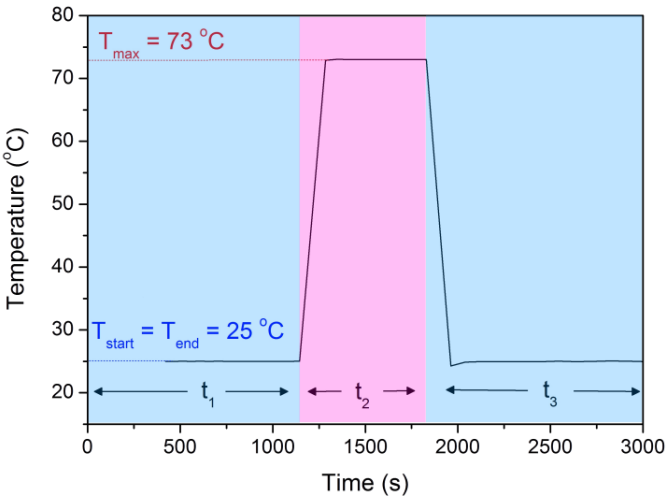

Figure 3. 'Top Hat' temperature profile.

Reversibility of protein unfolding

To assess the effect of high temperatures on the unfolded states of proteins, and to determine whether such temperature-induced changes are reversible, a series of experiments using HEWL were carried out. These studies exploited the programmable thermal plate present in the UNit that allows rapid heating and cooling of the UNis containing multiple samples. The flexibility of the thermal controls allows a number of temperature profiles to be performed. We acquired intrinsic fluorescence and SLS data whilst running a 'Top Hat' temperature profile (Figure 3).

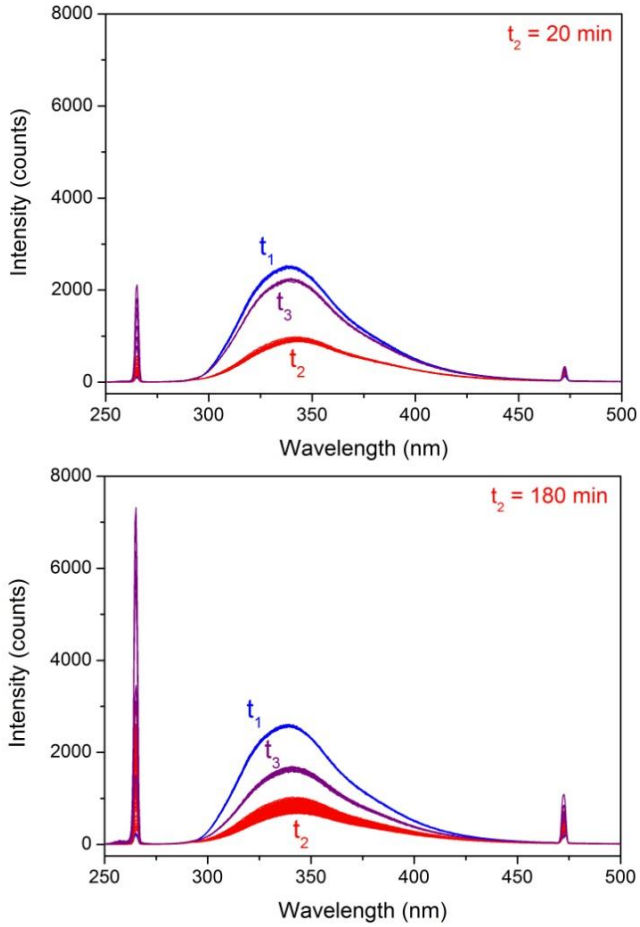

Figure 4. The UNit spectra (showing 266 nm and 473 nm scattered light signals and the protein fluorescence centered around 340 nm) obtained during the three time periods of the 'Top Hat' profile for samples incubated at 73 °C for 20 min (top) and 3 h (bottom).

This temperature cycle allows three different phases of control, where the temperatures and incubation times of each phase can be independently set. Here, we initially incubated the protein samples at close to room temperature (25 °C) for t_1 = 20 min; then the temperature was raised to be above the T_m for HEWL (73 °C) for a range of time periods (t_2 = 10 – 240 min); followed by an incubation period at 25 °C for t_3 = 20 min. Comparison of the data acquired under identical conditions before and after a high temperature incubation provides information on the effect of elevated temperatures on the tertiary structure and aggregation state of the protein

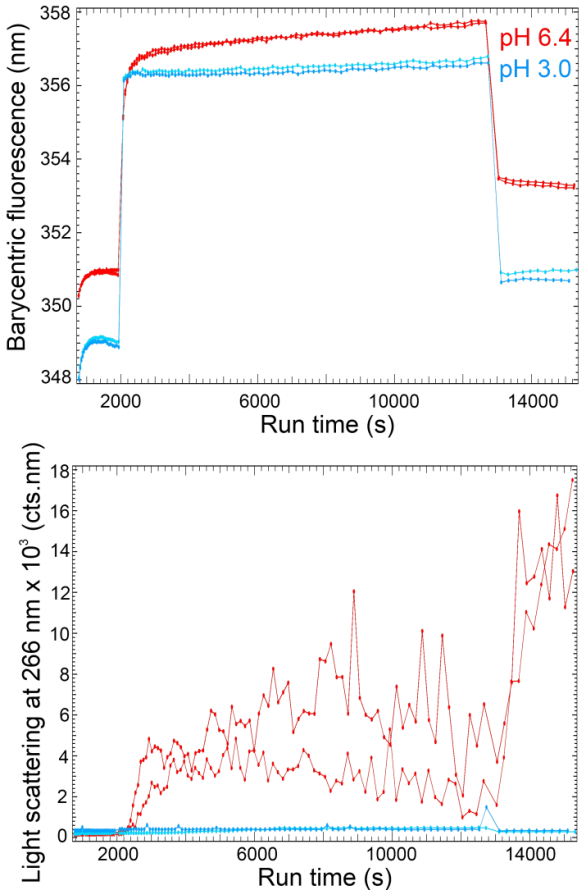

Figure 5. Processed fluorescence (top) and SLS data (bottom) for samples subjected to a 'Top Hat' profile including incubation at 73 °C for 3 h.

The raw spectra acquired during the three time periods (Figure 4) show that during the high temperature phase (t_2) the fluorescence intensity is reduced and the wavelength of the maximum peak intensity (λ_{max}) is shifted to a longer wavelength than is observed at 25 °C (t_1 and t_3). Interestingly, the fluorescence spectra acquired at 25 °C after the high temperature period (t_3) show a reduced intensity to those acquired before the high temperature phase (t_1). The recovery of the protein spectra is further reduced as the t_2 incubation period is increased. This can be observed by comparing the spectra for samples heated at 73 °C for 20 min and 180 min (Figure 4). The larger SLS peaks at 266 nm and 473 nm present in the spectra acquired during t_3 (compared to those present during t_1) indicate that irreversible aggregation has occurred during the high temperature phase.

To allow the process to be followed in real time, the UNit software calculates a value that reflects the tertiary structure of the protein sample from the fluorescence data called the Barycentric Fluorescence, which generally shows an increase as the protein unfolds. The example HEWL data in Figure 5 show that, during heating, the Barycentric Fluorescence value increases as the protein unfolds, but is then reduced during the cooling period. Interestingly the value does not return fully to that recorded before the high temperature step. This indicates that some of the protein present in the sample is irreversibly unfolded or misfolded as evidenced by changes in the environment of the Trp residues present.

The rate of unfolding for HEWL can also be seen to depend upon the buffer conditions in which it is placed. At pH 3.0 there is an almost instant unfolding of the protein, whereas at pH 6.4 a slower phase of unfolding is observed (Figure 5). This is likely due to the greater stability of the protein under these conditions (see Figure 2) which means that the rate of unfolding is slower. The differences in the absolute Barycentric Fluorescence values for HEWL at pH 6.4 and pH 3.0 simply reflect the slightly different spectral properties observed for the Trp residues under these different buffer conditions. The fact that the protein does not fully refold after high temperature treatment is probably due to the build-up of destabilizing chemical modifications in the protein chain whilst it is unfolded.[6,7,8] Furthermore, exposure of hydrophobic residues that are normally buried in the protein interior can lead to aggregation and misfolding which will affect the fluorescence spectra.

The UNit software also allows data to be obtained for SLS at 266 and 473 nm for the samples over time (see Figure 4). The extracted SLS data in Figure 5 shows that there is a very large difference between the samples studied at pH 6.4 and pH 3.0. Whilst there is no change in the scattering observed for the sample incubated at low pH, there is a large change for the protein placed at pH 6.4. This indicates that only the sample buffered at pH 6.4 forms aggregates when incubated above the T_m, even though the protein unfolds under both set of conditions. That the formation of irreversible aggregates is pH-dependent can potentially be explained by intermolecular disulphide bond formation between HEWL molecules.[8] The initial breakage of these bonds in the native state is suppressed at low pH (due to the reduced presence of thiolate ions)[9] and hence during the high temperature stage when the protein unfolds under these conditions, no intermolecular covalent bonds can form. In addition, greater levels of charge-charge repulsion under lower pH conditions further reduce the intermolecular interactions that result from all forms of aggregation.

Time-dependence of chemical modifications

The 'Top Hat' profile was used to acquire multiple data sets where the time period t_2 was altered. This enabled assessment of the effect of the time spent at high temperatures on the reversibility of folding and aggregation of the protein. The overlaid Barycentric Fluorescence data for these profiles (Figure 6) show a general trend where the longer the protein was incubated at high temperatures, the less recoverable the tertiary structure (as measured by the fluorescence spectra). This progression is observed irrespective of the pH conditions studied.

As the fluorescence spectra of proteins can be affected by the bleaching caused by UV lasers[10,11] a control experiment was performed where the temperature was maintained throughout at 25 °C. This enabled an accurate change in Barycentric Fluorescence due to partial unfolding/misfolding/aggregation alone (and not bleaching) to be calculated. The values obtained (Figure 7) show that the change in fluorescence increases with prolonged t_2 values.

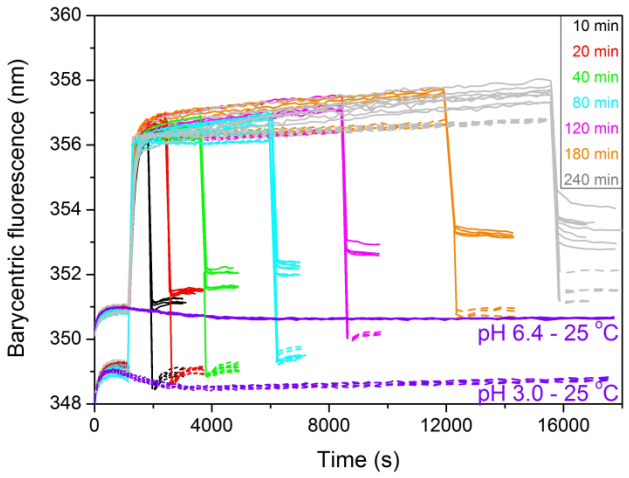

Figure 6. An overlay of all of the processed fluorescence data. Unbroken lines correspond to data obtained at pH 6.4 and broken lines to those data acquired at pH 3.0. The purple traces show data obtained when incubated at only 25 °C throughout the experiment.

The change follows an approximate first-order process in agreement with the literature which indicates that the destabilizing chemical modifications (deamidation, aspartate isomerization, peptide bond hydrolysis) should accrue in this fashion.[6] This is also in agreement with the refolding data presented in the 'Measurement of protein refolding using the UNit Technical Note, which shows that refolding of HEWL at pH 6.4 is dependent on the time the protein spends above its T_m.

The change in SLS at 266 nm (Figure 7) also indicates that there is a first-order time dependence for the formation of irreversible HEWL aggregates. This further suggests that this aggregation is driven by a chemical mechanism such as inter-molecular disulphide bond formation. It is also clear that even when incubated for four hours at a temperature far above its T_m at pH 3.0, HEWL does not aggregate.

Conclusions and summary

Due to the precise temperature control and small sample volumes used in the UNit, programmable heating and cooling profile data for multiple samples under different conditions could be acquired simultaneously. The intrinsic protein fluorescence, allied with the static light scattering measurements at two wavelengths provided information on the ability of a

model protein (hen egg white lysozyme) to withstand high temperature conditions for different time periods. Analysis of the label-free data provided by the UNit gave information on the conformation of the protein at all time points throughout the experiment as well its aggregation state. This allowed for straightforward interpretation of the processes in a complex system. It was seen that due to mechanisms including gradual buildup of chemical modifications, HEWL is increasingly destabilized as it is exposed to high temperatures for longer time periods. Furthermore, at pH 6.4 some of these covalent modifications may result in disulphide scrambling and contribute to the formation of irreversible aggregates.[8]

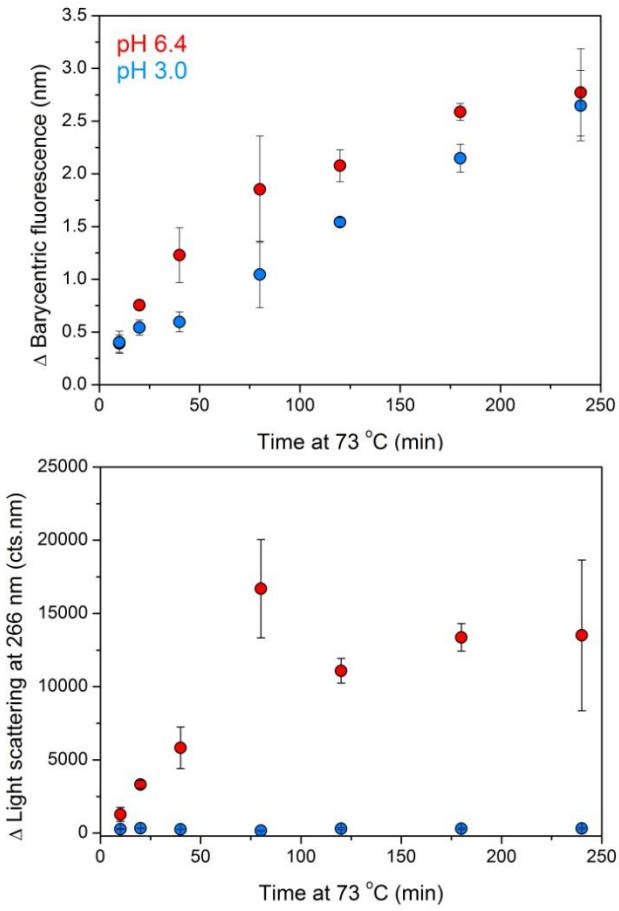

Figure 7. Change in fluorescence (top) and SLS (bottom) properties of HEWL samples after treatment at 73 °C for various time periods.

Further UNit literature related to heating and cooling cycles include: 'Measurement of protein refolding using the UNit' and 'Reversibility of monoclonal antibody unfolding using freeform profiles'.

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