Structure–energy relations in hen egg white lysozyme observed during refolding from a quenched unfolded state†

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We use infrared spectroscopy to study the evolution of protein folding intermediate structures on arbitrarily slow time scales by rapidly quenching thermally unfolded hen egg white lysozyme in a glassy matrix, followed by reheating of the protein to refold; upon comparison with differential scanning calorimetric experiments, low-temperature structural changes that precede the formation of energetic native contacts are revealed.

The folding of proteins into their functional state is one of nature's most ubiquitous phenomena. Understanding the mechanisms underlying protein folding is important for the rational modification and design of novel proteins, the understanding of human degenerative diseases that are tied to protein misfolding and/or aggregation,[1,2] and the development of improved formulations to prevent protein degradation during storage.

Current optical methods for studying folding mechanisms with sub-μs resolution do not probe folding from a fully denatured state.[3,4] Standard stopped-flow techniques, on the other hand, are able to prompt folding from fully-denatured samples, but have a time resolution of the order of 1 ms.[3,4] Consequently, important events in the refolding of fully denatured states that occur on the μs time scale are not observable, which creates an important gap in fundamental understanding. Recent developments in microfluidics have allowed denaturant dilution in less than 20 μs, offering the prospect of vastly improved time resolution using stopped-flow approaches.[3,4] Here we present an experimental method that is capable of detecting the changes in intermediate structure during the folding process on arbitrarily slow (arrested) time scales, and starting from the fully thermally-denatured state.

To this end, a recently introduced quench-and-refold approach[5] was adapted to spectroscopic scrutiny. The approach consists of the following steps: (1) thermally unfold/reversibly denature the protein, (2) trap the unfolded protein into a glassy matrix by quenching the sample in liquid nitrogen (LN_2), and (3) refold the protein by slowly heating the sample across the glass transition temperature. At this stage, infrared spectroscopy (IR) is used to collect spectra of the protein at various temperatures. Intermediates are observed and captured by taking advantage of the very slow kinetics in the viscous liquid state near the glass temperature (T_g).[6]

Angell and Wang first introduced the quench-and-refold approach and provided proof-of-concept using differential scanning calorimetry (DSC).[5] In conventional DSC studies, a protein is considered fully denatured upon the completion of energy absorption, as indicated by a peak centered at the protein denaturation temperature (T_d). Refolding of a protein is also indicated by the energy released, calculated as the area under the peak.[7,8] If unfolding and refolding energies are the same, the protein is said to have refolded completely. Combining the quench-and-refold approach with DSC, detection of stages during refolding is possible.[5] Here, we show that combining DSC and IR offers new insight into energetic and structural signatures of the refolding process, and their non-trivial relationship to each other.

The model protein must have the ability to refold completely and be a slow folder (> 100 ms), allowing immersion in LN_2 with a 120 K/s quenching rate for small samples[9] to suppress folding during quenching so as to trap the unfolded protein into a glassy state. Electrospray is one possible technique to quench fast-folding proteins.[9] Hen egg white lysozyme was chosen as the model protein, as it is commonly used in experiments and is known to exhibit intermediates.[10,11]

The solvent must not allow ice to form upon cooling or heating, because this would cause abrupt changes in solution composition and sample temperature. The solvent itself should not denature the proteins at room temperature and should support the thermally denatured protein at higher temperatures without permitting aggregation to occur. The solvent's melting and glass transition temperatures (T_m, T_g) are also important in experimental design. The sample solution must be homogenous at the protein unfolding temperature, thus the solvent T_m cannot be too high. On the contrary, T_g should not be too low; our LN_2 cooled cold-stage (FTIR600 stage with a minimum temperature of −196 °C) needs to be able to keep the quenched sample well below (~20 °C) the T_g.

Hen egg white lysozyme dissolved in water alone aggregates upon thermal treatment above its T_d; therefore an excipient is needed. Summers and Flowers II[12] showed that ionic liquids can support reversible protein folding. Angell *et al.* demonstrated the generality of this behavior in protic ionic liquids within an optimal range of proton activity.[13,14] Both groups also demonstrated that ionic liquids can prevent protein aggregation.[5,12] Accordingly, ethylammonium nitrate (EAN)

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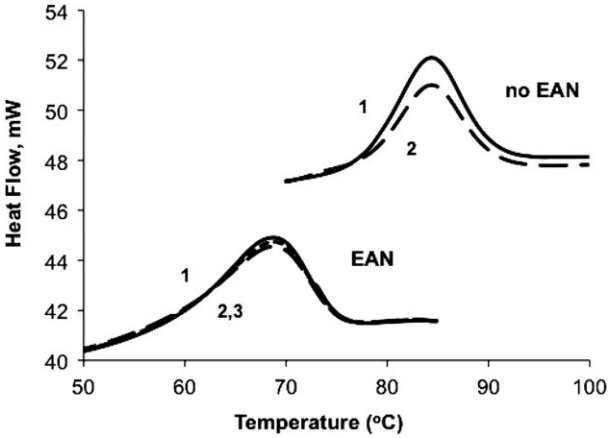

Fig. 1 Successive DSC heating curves of 200 mg/ml lysozyme in 40 wt% sucrose and 60 wt% H_2O (no EAN) and in a solution of 40 wt% EAN, 27 wt% H_2O and 33 wt% sucrose (EAN) showing the reversibility of lysozyme (unfolding–refolding) and prevention of aggregation using EAN in this formulation with sucrose.

is added to the solvent to prevent protein aggregation as the protein is thermally treated. This is demonstrated in Fig. 1.

Sucrose is added to the mixture to prevent ice crystallization and so permit vitrification:[15] DSC heating scans show no evidence of an endothermic peak below 0 °C.†

As will be explained below, DSC and IR measurements reveal an important gap in the temperature at which refolding-related signals are manifested upon reheating. Since the protein concentrations differ by an order of magnitude (200 mg/ml in DSC, 20 mg/ml in IR), it was decided to test whether lower biomolecular concentrations, with concomitant decrease in protein–protein interactions, would shift the DSC onset of refolding towards lower temperatures. Fig. 2 shows DSC heating curves of quenched unfolded and quenched folded protein for three different lysozyme concentrations. The DSC heating curve of the quenched unfolded protein shows an extended enthalpic relaxation with no distinct peak roughly between 0 and 60 °C, providing evidence of non-cooperative folding. Area calculations for the enthalpic

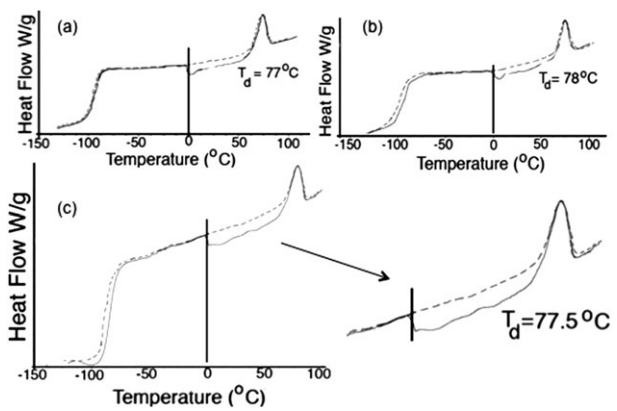

Fig. 2 Comparison of DSC upscans of solutions quenched from 30 °C (with lysozyme folded, dashed line) and quenched from 86 °C (lysozyme unfolded, solid line): (a) 50 mg/ml lysozyme (b) 100 mg/ml lysozyme and (c) 200 mg/ml in a solvent mixture with equal weight each of EAN, H_2O and glucose.

relaxation and the unfolding peak were found to be similar, thus indicating that the quenching of lysozyme using LN_2 is able to successfully trap a fully unfolded protein. Note that calorimetric signatures of refolding of the quenched-unfolded sample all begin at ~0 °C. Thus, changing the protein concentration does not cause a systematic shift in the calorimetric onset of refolding.

Fig. 2 shows no evidence of phase separation in the matrix. However, the matrix solution thermodynamics in the presence of varying amounts of protein—the conditions under which unmixing can occur and its possible role in initiating refolding—merit detailed scrutiny and will be the subject of future investigations.

Fourier transform IR spectroscopy is useful for protein secondary structure detection and quantification. The best-characterized and most-studied region is the amide I′ band[16] (1600–1700 cm^{-1} region) that arises primarily from the peptide carbonyl stretch vibration. Since the carbonyl group forms hydrogen bonds to peptide NH groups in a variety of different secondary structural environments,[17] the amide I′ band involves multiple single peaks having different frequencies, each peak being related to a specific secondary structural element (*e.g.* α-helix, β-sheets). Qualitative monitoring of structural changes can be done by following changes in absorption at a specified frequency. Quantitative analysis involves deconvolution of the broad amide I′ band and curve fitting (*e.g.* with a superposition of Gaussian or Lorentzian peaks). Peak areas and positions are then used to provide a quantitative estimate of relative weights assigned to specific secondary structural elements.[18]

Though the previous DSC-based quench-and-refold studies used H_2O,[5] our IR experiments use D_2O due to H_2O's overlapping absorbance with the amide I′ region,[19] and the fact that this absorbance exceeds that of the sample, making accurate measurements of the signal difficult. A solvent composition of 2 wt% lysozyme, 26 wt% D_2O, 39 wt% EAN and 33 wt% sucrose was used. In this solvent, lysozyme unfolds at ~80 °C and the solvent matrix T_g is around −100 °C (Fig. 2). Although the solvent formulation is not exactly the same in the DSC and IR experiments, previous calorimetric measurements show that T_g varies by at most 10 °C in the presence or absence of EAN.[20] Quantitative analysis of IR spectra at room temperature (25 °C) shows that the native structure of lysozyme in our solvent mixture (42 % α-helix, 19% β-sheet, 26% turn, 13% random)† and in a neutral buffer are very similar (40 % α-helix, 19% β-sheet, 27% turn, 14% random).[21]

Fig. 3 presents IR spectra that support the previously observed[5] ability to trap unfolded protein by quenching using LN_2: note, in particular, that spectra right before quench (90 °C; thermally infolded) and right after quench (−135 °C quench) are very similar. Once the unfolded protein is trapped in a glassy matrix, it is slowly heated. IR scans are collected to observe protein structural changes and intermediates as the protein refolds to the native state. It can be seen that the protein spectra before thermal treatment (25 °C) and after the entire quench-and-refold process (25 °C end) are very similar, providing spectral evidence of the reversibility of lysozyme unfolding and subsequent refolding.

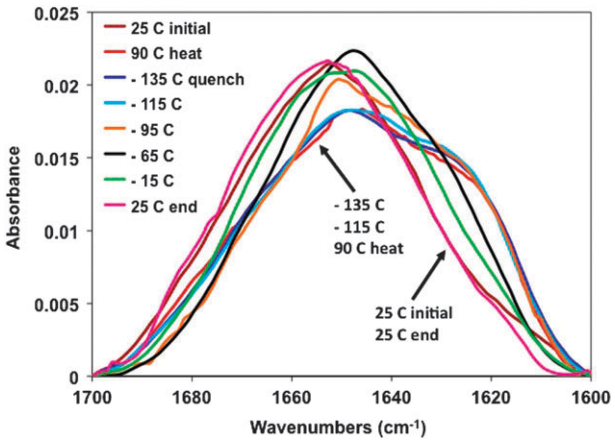

Fig. 3 IR spectra of lysozyme sample heated in the oven to 90 °C for 10 minutes, and then immediately quenched with LN_2. The sample is then heated from −135 °C to 25 °C at 20 °C/min with 3 minute annealing intervals every 5 °C to collect spectra.

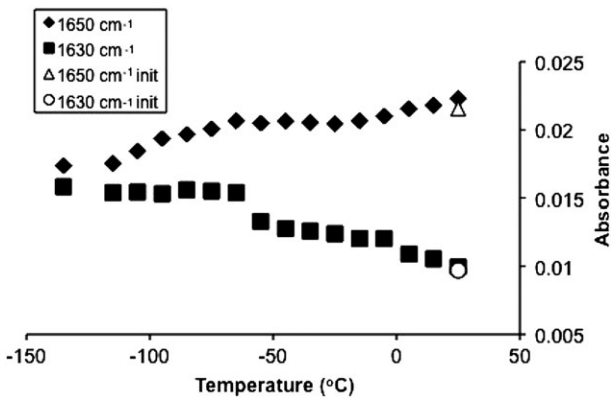

Fig. 4 Absorbance level at 1650 cm^{-1} (α-helix) and 1630 cm^{-1} (β-sheets). Error bars for 4 scans are within the size of each data point.

Fig. 4 plots the temperature dependence of absorbance levels at chosen frequencies. It confirms the existence of multiple conformations in the lysozyme folding process.[22] Absorbance peaks at 1650 cm^{-1} are generally associated with α-helices, while peaks at 1630 cm^{-1} are associated with intramolecular β-sheets.[17] At ∼−100 °C, α-helices start forming. In contrast, the β-sheet content begins to decrease at a higher temperature, ∼−65 °C.

Comparison of DSC and IR data reveals an interesting difference, in that DSC reheating curves do not show any enthalpic relaxation (refolding) until 0 °C (see Fig. 2 and ref. 5), while spectral shifts and qualitative changes are observed at much lower temperatures (see Fig. 4). By contrast, an IR upscan through the unfolding temperature range complements the calorimetric findings, the α-helix signature disappearing over the same temperature range as the heat of unfolding is observed.† The DSC scans of Fig. 2 establish that the difference in refolding behavior is not due to the difference in protein concentration.

Since the integral of the exothermic deviation observed between 0 and 60 °C is approximately equal to the unfolding enthalpy, the energy of the protein just before the calorimetric action starts is indeed that of the unfolded protein. If this is the case, then the quench-and-refold approach supplemented by IR appears to be able to capture the formation of secondary structural elements before energetic native contacts of the hydrophobic core fall into place. Such energy-neutral rearrangements have not been observed previously in IR. Structural changes such as the fast hydrophobic collapse,[3] can occur at very short time scales and in most cases during other experimental technique's "dead time". In our studies, these very short time scales appear to be associated with phenomena occurring at low temperatures. T-jump kinetic studies of hydrophobic collapse reveal a lack of activation energy at such short time scales.[23] The lack of corresponding calorimetric signal suggests that the refolding events seen in the IR at low temperatures are likewise energy-neutral.

In sum, we have developed a sensitive method to track the protein intermediate structures upon folding from the fully unfolded state back to the native state. The main advantage of this technique is the opportunity to observe subtle structural changes as they evolve over a continuum in time, due to the slow kinetics found in the viscous liquid state near T_g. The combined use of IR and DSC reveals the ability of IR to capture early structural changes. Their possible relation to the recently reported fast (∼20 μs) hydrophobic collapse[3] remains to be established.

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