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Steady state and time-resolved IR spectroscopy of the native and unfolded states of bovine ubiquitin: protein stability and temperature-jump kinetic measurements of protein folding at low pH

Christopher S. Colley,[a] Ian P. Clark,[b] Samuel R. Griffiths-Jones,[a] Michael W. George*[a] and Mark S. Searle*[a]

[a] *Department of Chemistry, University Park Nottingham, UK NG7 2RD E-mail: mark.searle@nottingham.ac.uk and mike.george@nottingham.ac.uk*

[b] *Central Laser Facility, Rutherford Appleton Laboratory, Chilton, Didcot, Oxfordshire, UK OX11 0QX*

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Infrared spectroscopy has been used to characterise the folded and unfolded states of bovine ubiquitin (a small protein of 76 residues) under acidic conditions (pH ~ 1); fast time-resolved measurements of protein unfolding, initiated by a laser-induced temperature-jump of ~ 8 °C, shows rapid refolding and β-sheet secondary structure formation on a timescale of a few milliseconds.

Our understanding of the mechanism of chemical reactions has benefited greatly from time-resolved kinetic measurements to examine the order of bond breaking events and the relative strengths of covalent interactions.[1] The protein folding reaction, in which a disordered polypeptide chain can assemble into a compact 3D structure, is a highly complex process involving numerous weak co-operative non-covalent interactions.[2] Despite the complexity of the problem, many small proteins (< 100 residues) fold on a timescale of only a few milliseconds.[3] Current models suggest that folding proceeds through a hierarchical process[4] in which the lower limit to the kinetics of folding is determined by local structural events such as the formation of α-helices, β-turns and β-hairpins that nucleate the collapse and folding of the polypeptide chain.[5] Observations on the folding of these structures with submillisecond half-lives have necessitated new methods of detection.[5]

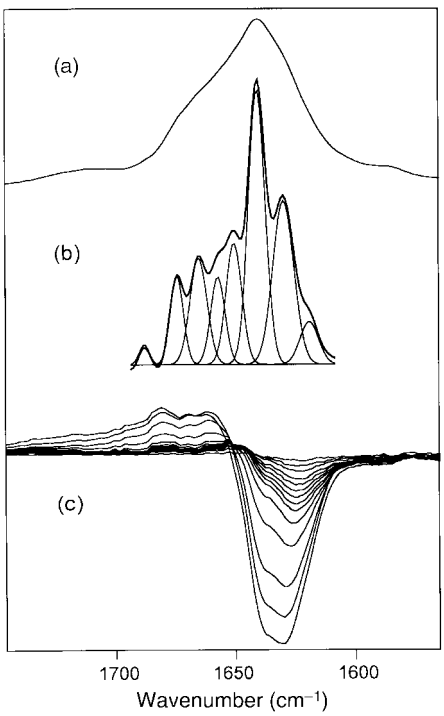

Fig. 1 (a) Conventional and (b) deconvoluted FTIR spectra of bovine ubiquitin at 25 °C pH 1.0 showing 8 principal bands.[18] (c) FTIR difference spectra, showing the change in the absorption envelope with temperature over the range 278 to 363 K. Data were collected using a Nicolet Nexus 670 FTIR spectrometer equipped with MCT detector.

Infrared spectroscopy has emerged as an important technique for studying protein structure and fast folding kinetics.[6,7] The position of the amide stretching vibration (1610–1680 cm^{-1}) has been shown to be strongly correlated with protein secondary structure because of its sensitivity to hydrogen bonding, dipole–dipole interactions and the geometry of the peptide backbone.[8,9] The possibility of detecting by IR both native, as well as transient non-native structures, on the protein folding pathway has already been demonstrated.[10] The application of vibrational spectroscopy to the study of rapid kinetic processes in chemical systems is well documented,[1] and is now being applied to biological molecules.[6,10–12] Here we examine the steady state and time-resolved folding/unfolding of a small protein by IR spectroscopy, and demonstrate rapid formation of protein secondary structure on the timescale of a few milliseconds.

We have used bovine ubiquitin (a small protein of 76 residues, free of disulfide bridges) as a model system). Ubiquitin consists of both α-helix and β-sheet, forming a highly stable, compact structure that has been shown to fold in a two-state process.[13] We have been investigating the mechanism of folding by examining the conformational propensity of protein fragments for evidence of possible nucleation sites for folding of the native structure. In particular, the N-terminal β-hairpin sequence in isolation shows evidence for a small population of native-like structure in water,[14] while hairpin analogues, with mutated β-turn sequences, have demonstrated both native and non-native conformational features.[15] The kinetics of folding of these isolated elements of secondary structure is of current interest in the context of understanding the mechanism of folding of the native protein.

Here we probe the structure of both the native and unfolded states of bovine ubiquitin, and the unfolding transition, at pD 1.0 in D_2O solution by FTIR. NMR spectra of the native protein at pH 1.0 and 7.0 are essentially identical, indicating that there are no significant changes in the structure. However, at low pH, the reduced T_m allows the thermal unfolding transition to be investigated. In Fig. 1, we illustrate conventional (a) and deconvolved (b) steady state FTIR spectra of native folded ubiquitin at 25 °C (after NH → ND exchange). Fitting the deconvolved absorption envelope enables us to identify eight principal bands which have been assigned according to literature precedent,[8–10] and on the basis of features identified in the X-ray structure.[16] The absorption profile changes significantly with temperature as the protein unfolds, resulting in a reduction of the β-sheet band at 1629 cm^{-1}. The appearance of weaker peaks in the range 1660–1680 cm^{-1} has been attributed to disordered structure or turn-type conformations.[8–10] IR difference spectra, showing the change in the absorption envelope with temperature, are shown in Fig. 1c. Monitoring thermal unfolding from the change in integrated intensity of the principal β-sheet band observed at 1629 cm^{-1} at 25 °C, demonstrates a reversible cooperative sigmoidal melting process with a T_m of ~65 °C at pH 1.0. Parallel NMR melting

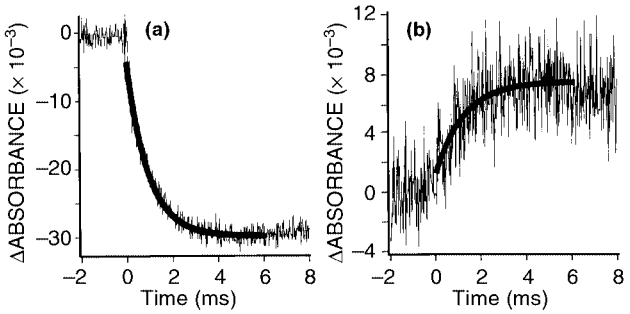

Fig. 2 Time-resolved IR spectra showing the decay/growth of the signals due to disappearance of protein secondary structure at 1635.5 cm^{-1} (a), and appearance of random coil at 1667 cm^{-1} (b) monitored as the protein unfolds following the T-jump. The line of best-fit is shown, assuming a single exponential process.

experiments under identical conditions give a very similar T_m value.

We have investigated the kinetics of folding by IR close to the mid-point of the thermal unfolding transition where the change in absorption is most temperature-sensitive. Using a 10 nanosecond-duration 1.9 μm pulse from a Raman-shifted Nd:YAG laser, we have generated effectively an instantaneous temperature-jump of ~8 °C, initiated at 67 °C. Changes in the IR spectrum, corresponding to transient perturbation of the equilibrium between folded and unfolded states, were used to measure fast folding kinetics outside the normal time-range accessible using rapid mixing techniques.[6] The transient IR spectrum obtained 4 ms after the T-jump is in good agreement with the IR difference spectrum obtained from the steady state spectra taken at 67 and 75 °C. The T-jump was calibrated from the change in absorbance of the D_2O band at 1620 cm^{-1}, providing an internal thermometer. The time-resolved change in the IR absorbance was monitored at ~1640 and ~1670 cm^{-1}, corresponding to the disappearance of bands due to ordered secondary structure, and appearance of the weak band due to random coil structure, respectively (Fig. 2). Subtraction of the solvent absorption, collected in an identical T-jump experiment in D_2O alone, reveals an exponential decay/growth curve at these wavelengths,which we are readily able to fit to a single rate process consistent with a two-state folding model (Fig. 2a, b). Despite the much lower signal-to-noise ratio at 1670 cm^{-1}, both kinetic traces yield the same observed rate constant $k_{obs} \approx 1000\ s^{-1}$. Assuming that k_{obs} is the sum of the folding and unfolding rate constants, $k_{obs} = k_F + k_U$,[5,13] and that the equilibrium constant at a given temperature is given by $K_{eq} = k_F/k_U$, then we are able to estimate a rate of folding at 75 °C (at the T_{max} of the T-jump) of ~400 s^{-1}.

We have demonstrated from equilibrium FTIR measurements of ubiquitin that the various elements of secondary structure result in a unique fingerprint. We have demonstrated that the relaxation kinetics for folding/unfolding of β-sheet structure provides a convenient handle for monitoring fast dynamic processes. Comparison with results from fluorescence and amide NH exchange experiments identified some common features.[13,17] Both methods have identified a major folding phase with a half-life of 5–10 ms at 25 °C. However, fluorescence-detected kinetics also show very rapid hydrophobic collapse (< 2 ms) within the dead-time of the stopped-flow experiment.[13] This early collapsed structure does not protect amide NHs against exchange, the latter occurring on a longer timescale (~10 ms). In the folding/unfolding studies presented in this work, we have monitored by IR spectroscopy the rapid formation of protein secondary structure with a half-life of 2–3 ms ($1/k_F$) at 75 °C. Despite the difference in folding temperature between this and earlier studies,[13,17] the rate of formation of secondary structure is in broad agreement, as far as comparisons are possible between these complementary techniques. While previous fluorescence measurements have detected very early burial of hydrophobic residues, we show that time-resolved IR, as with amide NH exchange, monitors the formation of hydrogen bonded secondary structure during a slower folding phase on the timescale of a few milliseconds.

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