Proton NMR studies of denatured lysozyme

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Evidence is presented from ^{1}H NMR studies for non-random conformational behaviour in denatured lysozyme in aqueous solution. A method is presented which permits the assignment of resonances in the ^{1}H NMR spectrum of the denatured protein by observing magnetisation transfer from resonances of the native state. The use of these experiments in characterising the denatured state and the significance of these studies for the investigation of protein folding are discussed.

^{1}H NMR *Lysozyme* *Protein denaturation* *Protein folding* *Saturation transfer*

1. INTRODUCTION

Studies of the reversible denaturation of proteins constitute a major experimental approach to the problem of determining folding pathways [1–3]. The implications of these studies can properly be appreciated only if the native and denatured states are themselves well characterised under the conditions of the experiment. Much is now understood about the structure and dynamics of native proteins but in the case of denatured states, although they are clearly more or less unfolded [1], rather little is known in detail. Proton NMR has proved to be a very powerful means of characterising native proteins in solution [4,5] and here we present results which show that it may also be useful in the study of unfolded states. We demonstrate that the spectra of one protein, hen egg-white lysozyme, in its denatured state in aqueous solution differ significantly from those characteristic of a fully unfolded polypeptide. Further we show that magnetisation transfer techniques [6–9] enable the assignment information available for the native protein to be used directly to assign resonances of the unfolded state.

2. MATERIALS AND METHODS

Hen egg-white lysozyme from Sigma was purified by dialysis at pH 3.0 and lyophilised. In most experiments labile protons were exchanged for deuterons by warming to 80°C in D_2O (pH 3.8) and then lyophilising. Deuterated (d_6) DMSO from Merck was dried over Linde 5A molecular sieves. Urea was deuterated by repeated lyophilisation from D_2O.

NMR experiments were carried out using the Brüker WH300 and home-built 470 MHz instruments of the Oxford Enzyme Group. Probe temperatures were measured using ethylene glycol [10] and are accurate to ±1°C. One-dimensional saturation transfer experiments were carried out by applying selective presaturation pulses to individual resonances. Saturation transfer was generally detected by interleaved difference spectroscopy as in [11]. Two-dimensional chemical exchange spectra were obtained using a NOESY pulse sequence with phase-shifting to suppress axial peaks [9,12].

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Abbreviation: DMSO, dimethyl sulfoxide

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3. RESULTS AND DISCUSSION

Lysozyme unfolds reversibly above 70°C in D_2O at pH 3.8 and at lower temperatures in the presence of denaturants. Exchange between folded and unfolded forms is slow on the NMR time scale, so that the unfolding is manifest in the spectrum in the appearance of new resonances, distinct from those of the native protein. In agreement with earlier studies it was observed that the intensities of native resonances change in unison as the denaturation equilibrium shifts, reflecting the highly co-operative nature of the transition [13,14].

In the spectrum of native lysozyme (fig.1a) the chemical shifts of many protons differ significantly from those observed for the same residues in small peptides. These differences, often termed secondary shifts, are mostly less than 1 ppm but a few are as large as 3 ppm [15]. In the spectrum of thermally denatured lysozyme (fig.1b) the magnitudes of such shifts are markedly smaller but they do not disappear and there persists a residual heterogeneity in the chemical shifts of resonances of the same proton type. Addition of urea causes small shifts of some resonances without making gross changes in the overall appearance of the spectrum. Addition of DMSO leads to more marked changes in many chemical shifts. In 100% DMSO the spectrum of denatured lysozyme (fig.1c) is a closer approximation to the simple spectrum which would be expected if all protons of the same residue type were equivalent. This is clearly seen in fig.1 for the $H^{\epsilon 3}$ and $H^{\zeta 2}$ doublets of the 6 tryptophan residues; in DMSO these are almost exactly coincident in both cases, giving rise to two sharp doublets at 7.34 and 7.55 ppm. In thermally denatured lysozyme these protons give rise to a cluster of peaks between 7.33 and 7.51 ppm.

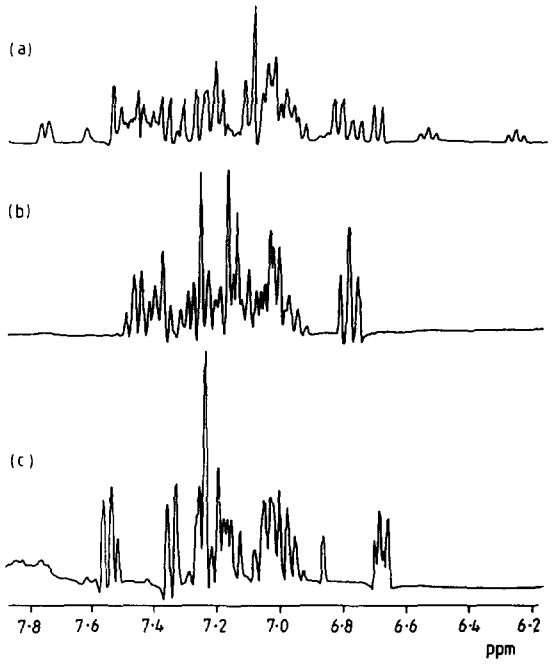

Fig.1. Low-field region of 300 MHz proton NMR spectra of lysozyme. Resolution has been enhanced by Gaussian multiplication. Labile protons have been exchanged for deuterons. (a) Native lysozyme in D_2O, pH 3.8, 65°C; (b) denatured lysozyme in D_2O, pH 3.8, 82°C; (c) denatured lysozyme in DMSO, 82°C.

The secondary shifts in native proteins arise primarily from through-space interactions with residues in close proximity [4,5]. The population of very many rapidly interconverting conformational states leads to averaging of these interactions and in the limiting case of a randomly disordered polypeptide this would result in all protons of the same residue type resonating at virtually identical frequencies. Thermally denatured lysozyme clearly deviates from this simple model, suggesting that there is a distinctly non-random population of conformers. The behaviour in DMSO suggests that this is not just a result of steric interactions between adjacent side chains or of constraints imposed by the disulphide linkages. Deviations from 'random coil' behaviour are similarly evident in ^{13}C NMR spectra of lysozyme, as noted in [16]. The proton chemical shift changes occur smoothly as a function of solvent composition as denatured lysozyme in D_2O is titrated with DMSO, indicating a gradual change in the distribution of conformers sampled by the rapidly fluctuating polypeptide, rather than a single highly co-operative transition.

To use NMR for characterising the unfolded state it is essential to observe and assign to specific protons individual resonances in the spectrum. This has been achieved by correlating the resonances with those of the native state through saturation transfer experiments under conditions where both native and denatured lysozyme are pre-

sent in equilibrium. Similar experiments have been used by other groups to correlate resonances in oxidised and reduced cytochrome *c* and in native and denatured pancreatic trypsin inhibitor [17–19]. Fig.2 shows the assignment of the two Met H^{ϵ} resonances of denatured lysozyme in this way. Selective presaturation of a native resonance leads to a decrease in the intensity of the corresponding denatured resonance, the effect being readily detectable by difference spectroscopy.

Magnetisation transfer between folded and unfolded states has also been observed using two-dimensional chemical exchange spectroscopy. In this experiment each pair of native and denatured resonances should give rise to a cross-peak in the two-dimensional spectrum. The value of this two-dimensional technique for studying exchange processes has been demonstrated in a study of electron exchange between oxidised and reduced cytochrome *c* [9,21]. Fig.3 shows cross-sections from the upfield part of the lysozyme spectrum, in which cross-peaks from methyl group protons

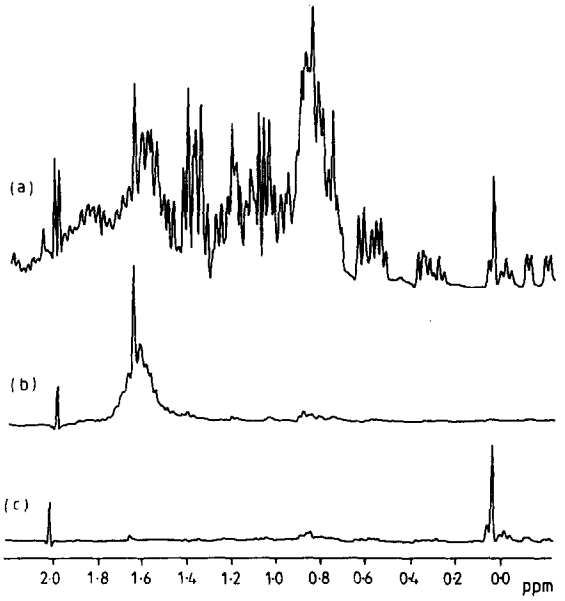

Fig.2. (a) High-field region of the 300 MHz resolution enhanced spectrum of lysozyme, pH 3.8, 77°C. In these conditions native and denatured protein are present in approximately equal proportions. (b) Saturation transfer difference spectrum obtained from presaturation of the resonance at 1.64 ppm which has been assigned to Met-12 H^{ϵ} in the native protein [20]. The singlet at 1.99 ppm in the difference spectrum is assigned to this proton in the unfolded state. (c) Difference spectrum resulting from presaturation of the Met-105 H^{ϵ} resonance at 0.03 ppm. The corresponding resonance of the denatured state lies slightly downfield of the Met-12 resonance, at 2.01 ppm.

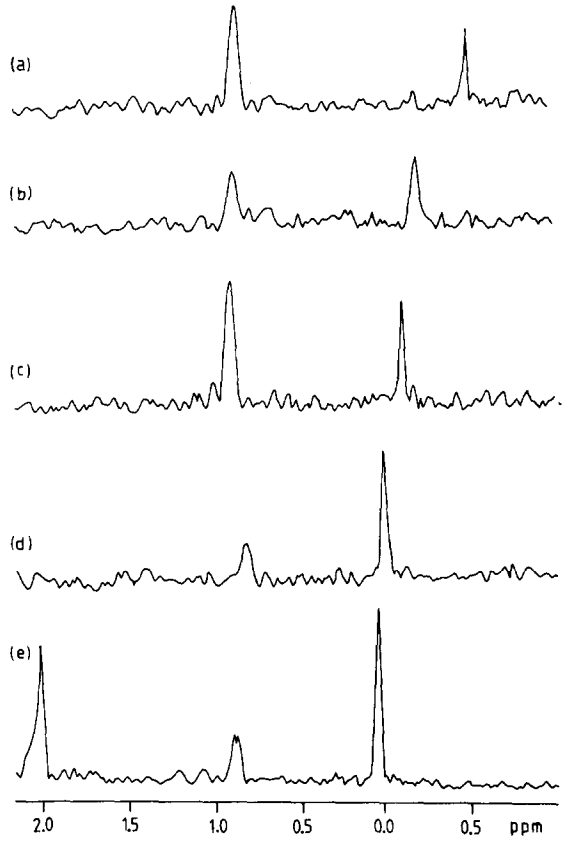

Fig.3. Cross-sections from the high-field region of a two-dimensional chemical exchange spectrum of lysozyme; pH 3.8, 77°C. In each case the peak to high field is an axial peak at the frequency of an upfield-shifted resonance of the native protein. The downfield peaks are cross-peaks at the positions of the corresponding resonances in the denatured state. These resonances have all been assigned in native lysozyme [11]: (a) Leu-17 $H^{\delta 2}$, (b) Ile-98 $H^{\gamma 2}$, (c) Leu-17 $H^{\delta 1}$, (d) Ile-98 $H^{\delta 1}$, (e) Met-105 H^{ϵ}, Leu-8 $H^{\delta 1}$. The spectrum was obtained at 470 MHz with a mixing time of 1.0 s. The time domain data comprised 512 points in the t_1 dimension and 4096 points in the t_2 dimension. The t_1 dimension was zero-filled twice prior to Fourier transformation. Resolution was enhanced by application of a phase-shifted sine-bell function in both dimensions and a symmetrisation routine was applied to help suppress artifacts.

which are shifted to high field in the folded state are clearly resolved. Individual responses of the unfolded protein can be identified by these means even in conditions where much of the protein is native. Hence it is possible to study the unfolded protein under conditions where it actually can refold.

It has been shown that the hydrogen-exchange kinetics of individual labile protons in the vicinity of the denaturation transition depend on the rates of folding and unfolding and on the rate of hydrogen exchange from the denatured state [14]. As well as permitting qualitative observation of individual resonances of the denatured protein, the saturation transfer experiments can be used to determine independently the rates of folding and unfolding at equilibrium by, for example, observing the time development of the effects [6,7]. A combination of these approaches should thus permit further characterisation of denatured lysozyme by comparison of proton labilities in various parts of the molecule (in preparation).

It is of interest to consider the significance of these experiments for protein folding. The early stages of this process probably involve the formation of localised elements of folded structure which may subsequently combine leading to more extensive folding [3,22]. The probability of such nucleation events is clearly liable to be influenced by inherent conformational preferences of the unfolded protein. In particular, if parts of the chain have a tendency to form elements of native-like secondary structure, these are likely to act as folding nuclei.

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