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Hydrophobic Clustering in Nonnative States of a Protein: Interpretation of Chemical Shifts in NMR Spectra of Denatured States of Lysozyme

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ABSTRACT **Chemical shifts of resonances of specific protons in the ^{1}H NMR spectrum of thermally denatured hen lysozyme have been determined by exchange correlation with assigned native state resonances in 2D NOESY spectra obtained under conditions where the two states are interconverting. There are subtle but widespread deviations of the measured shifts from the values which would be anticipated for a random coil; in the case of side chain protons these are virtually all net upfield shifts and it is shown that this may be the averaged effect of interactions with aromatic rings in a partially collapsed denatured state. In a very few cases, notably that of two sequential tryptophan residues, it is possible to interpret these effects in terms of specific, local interresidue interactions. Generally, however, there is no correlation with either native state shift perturbations or with sequence proximity to aromatic groups. Diminution of most of the residual shift perturbations on reduction of the disulfide cross-links confirms that they are not simply effects of residues adjacent in the sequence. Similar effects of chemical denaturants, with the disulfides intact, demonstrate that the shift perturbations reflect an enhanced tendency to side chain clustering in the thermally denatured state. The temperature dependences of the shift perturbations suggest that this clustering is noncooperative and is driven by small, favorable enthalpy changes. While the extent of conformational averaging is clearly much greater than that observed for a homologous protein, α-lactalbumin, in its partially folded "molten globule" state, the results clearly show that thermally denatured lysozyme differs substantially from a random coil, principally in that it is partially hydrophobically collapsed.**

Key words: protein folding, unfolded state, denatured state, hydrophobic interactions, ring current shift, magnetization transfer NMR

INTRODUCTION

The biological activities of most proteins depend upon their acquisition of a specific folded conformation under physiological conditions. Thermodynamic studies have revealed that such highly structured states are usually only marginally stable relative to denatured states which result from the cooperative breakdown of folding interactions.[1,2] Much remains to be understood about the factors which determine the stabilities of particular folded structures.[3] One reason for this is that although a great deal has been learned about the detailed structures of native proteins there is very little information about the structural properties of denatured states. The importance of this problem has been underlined recently by studies of the stability of folded structures using protein engineering; in certain cases the effects of sequence changes on the stability of the native state have been traced primarily to changes in the structure and hydration of the *denatured* state.[4] Thus there is considerable motivation for the development of experimental approaches which can provide more specific information about the conformational properties of such states.

An idealized model for a denatured protein which has often been invoked is that of a "random coil," in which only random noncovalent interactions are present at any instant, so that there are no conformational preferences beyond those imposed by local steric limitations.[5] An important feature of such a model is that a variety of physicochemical properties can be predicted in a relatively straightforward manner.[1] There is ample experimental evidence for deviation from this idealized model in real protein systems[4,6–11]; it has, however, proved to be extraordinarily difficult to characterize either the nature or the extent of residual nonrandom interactions involving specific residues; in particular the extensive conformational heterogeneity of denatured states

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precludes the use of crystallographic methods. The development of NMR methods for determining molecular conformations in solution offers a potential solution to this problem. However the dynamic properties of denatured states mean that the particular NMR methods which are applicable are quite different from those which have become established for the determination of folded protein structures.

Structural information can be derived in principle from a variety of NMR spectral parameters. In the case of denatured protein spectra the major problems encountered in this are (1) how to assign individual resonances and (2) how to interpret the various parameters knowing that they are likely to be averaged by extensive conformational fluctuations. The most readily determined parameters are chemical shifts. It is well known in the case of folded proteins that these are highly sensitive to the details of local structure. Although quantitative interpretation of shifts is more difficult than that of parameters such as nuclear Overhauser effects (NOEs) and J-coupling constants (because the net chemical shift depends on the sum of a number of interactions, which can be identified only if there is additional, independent structural information) many of the principles are understood. Through space interactions with aromatic residues, which induce so-called "ring current shifts," are known to be important, particularly for side chain proton resonances.[12] Main chain proton shifts are less well understood but it is known that through space interaction with carbonyl groups[13] and hydrogen bond strengths[14] are significant. The importance of dynamic effects on chemical shifts has also been recognized and although local fluctuations of limited magnitude appear to be of little consequence,[15] larger fluctuations can cause averaging of individual shifts which in the limit of sampling of conformational space will then approach those predicted for a random coil.

The resemblance of spectra of denatured proteins to those simulated for random coils is well known. Most of the large conformation dependent shifts which characterize spectra of folded proteins are indeed absent, consistent with the notion that these are substantially unfolded states.[16] On the other hand it has also been pointed out in several instances that small chemical shift disparities are detectable in spectra of denatured proteins where none would be expected for a true random coil, perhaps indicative of some kind of residual structure.[8,9,17,18] The problem then is to assign to specific protons resonances for which shift perturbations are apparent and to interpret these effects in structural terms.

Methods for resolution and assignment of individual resonances using 2D NMR are well established for folded globular proteins.[19] However, these methods are not straightforwardly applicable to denatured states whose spectra are relatively poorly resolved and whose structures are much less defined. A general approach to overcoming these problems is to make use of the generally well-resolved spectrum of the native state to derive information indirectly about the denatured state. For example, hydrogen exchange kinetics in the denatured state can be monitored by refolding into the native form after a certain time, then determining by NMR the extent to which individual amides had exchanged with solvent while the protein was denatured.[20,21] In order to assign resonances in the spectrum of a denatured state, magnetization transfer methods can be used to correlate them with the corresponding resonances of the folded state under conditions where the two are interconverting at rates comparable to those of nuclear spin-lattice relaxation.[9,18,21–23] Since the spectrum of the folded state can be assigned in the conventional manner this provides a means for determining the chemical shifts of individual protons in the spectrum of the denatured state. We describe in this paper the application of this latter approach to the study of reversibly thermally unfolded hen lysozyme. The feasibility of these experiments was demonstrated previously using 1D and 2D magnetization transfer methods to observe selected protons which give rise to resolved resonances.[9] Here we show how more extensive analysis of 2D NMR spectra, coupled with the recently completed assignment of virtually the entire spectrum of native lysozyme,[24] permits a substantially more detailed survey of the chemical shifts of proton resonances of this denatured state.

The extent to which limited deviations from "random coil" chemical shifts can be interpreted in conformational terms is not easy to gauge. A clearer perspective can be gained by comparison of the spectra of nonnative states under different conditions, where spectral changes may be interpretable in terms of a varying extent of residual structure—for example, this approach has been used to characterize a series of intermediates trapped on the oxidative refolding pathway of reduced bovine pancreatic inhibitor.[25] In the present study we have obtained spectra of lysozyme under a range of different denaturing conditions, where independent evidence from other physical techniques suggests that the extent of randomization may vary.[6,7,9] Although we have not been able to make detailed assignments under all these different conditions it is possible to see clear differences in the patterns of resonances in their spectra and this enables us to make some assessment of the extent to which differential levels of residual order are reflected in the chemical shifts of proton resonances.

MATERIALS AND METHODS

Hen egg-white lysozyme (three times crystallized, dialyzed, and lyophilized) was obtained from Sigma and dialyzed extensively against distilled water at low pH before use. Perdeuterated urea was prepared

by repeated lyophilization from 99.8% D_2O; perdeuterated dimethyl sulfoxide (DMSO) was obtained from Aldrich. All other chemicals used were of analytical grade.

The four disulphide cross-links of lysozyme were reduced by treatment with dithiothreitol at pH 8.5, in the absence of denaturant, as described by White.[26] The product, redissolved at pH 2.0, was purified by gel filtration on Sephadex G-75 (Pharmacia) in water at pH 2.0 and lyophilized. It was found that, provided the pH was kept low, the reduced protein was stable in solution without the need for blocking the free sulfhydryl groups.

Solutions for NMR studies were unbuffered, the pH values quoted being those measured using a glass electrode at room temperature. No corrections were made for deuterium isotope effects or the effects of added denaturants on the pH reading. The protein concentration used was generally 3.5 mM, except where concentration dependence was specifically under investigation.

NMR spectra were recorded at 500 MHz using the Bruker AM500 spectrometer of the Oxford Centre for Molecular Sciences and at 400 MHz using the AM400WB instrument of the University of Cambridge Biochemistry Department. Chemical shift values were calibrated using an internal reference of 2 mM dioxane which gives a singlet resonance at 3.74 ppm. Phase-sensitive 2D spectra were obtained using the method of time proportional phase incrementation[27] and are plotted in pure absorption mode.

RESULTS

The Spectrum of Thermally Denatured Lysozyme

Lysozyme undergoes reversible thermal unfolding with a midpoint of 75 ± 1°C under the conditions used for this study. Discrete resonances of the folded and unfolded states are observed in the ^1H spectrum since interconversion is slow compared with resolvable differences in the resonant frequencies of individual protons in the two states. On elevation of the temperature to 81°C more than 99% of the protein is in the denatured state and no resonances of the folded form can be detected. Figure 1 shows a COSY spectrum obtained under these conditions and, for comparison, the cross-peak positions which would be anticipated in the spectrum of an ideal random coil polypeptide.[28] The similarity between the two is marked, resonances of the unfolded protein clustering rather closely around the random coil positions. This contrasts strongly with spectra of the native state in which cross-peaks are widely dispersed as a result of specific interactions within the folded structure.[24]

While the broad correspondence of shifts in the experimental and "random coil" spectra is clear, closer inspection of the spectrum reveals some significant discrepancies from this general pattern; for many types of proton a spread of chemical shifts over a range of the order of 0.1 ppm is observed. There is not in general a symmetrical distribution about the random coil value, however. Instead there is a general tendency for resonances to be shifted to the high field side of this value; for example the $H^{\zeta 3}$ resonances of the six tryptophan residues are found between 6.95 and 7.05 ppm, 0.12–0.22 ppm upfield of the random coil position. This is not a uniform effect, however, as illustrated, for example, by the resonances of the H^{α} and H^{β} protons of the 10 serine residues which are all within ± 0.05 ppm of the predicted random coil shifts, giving rise to the cluster of cross peaks around (3.9, 4.5) ppm. Moreover, the disparities observed cannot simply be a consequence of the different conditions used to record spectra of the denatured protein and the small peptides from which the "random coil" shifts were derived[28]; this was verified in the case of the tryptophan protons by recording spectra of a tetrapeptide, Gly-Gly-Trp-Ala, under conditions identical to those under which the the unfolded lysozyme was studied. For this model compound the chemical shift values of the ring proton resonances were all within ±0.02 ppm of the random coil values. It may therefore be concluded that differences in chemical shifts observed for denatured lysozyme, relative to values appropriate for a random coil protein, reflect the existence of interactions which are not present in small peptides.

Resonance Assignments Through Magnetization Transfer

The significance of deviations from random coil behavior in the chemical shifts of resonances in the unfolded state can be assessed only if they can be assigned to specific protons. Attempts to achieve this using conventional sequential assignment methodology[19] were not successful because of the extensive resonance overlap and the absence of very many interresidue NOEs. Assignment could, however, be achieved by means of magnetisation transfer experiments. Figure 2 shows a contour plot of the high field region of a 2D NOESY spectrum obtained at 75°C, close to the midpoint of the thermal unfolding transition. The prominent cross peaks between 0.7 and 1.0 ppm are mainly chemical exchange correlations between the resonances of leucine, isoleucine, and valine methyl group protons of the interconverting native and denatured states. Their clustering in this relatively narrow band reflects the limited deviations of the chemical shifts of these resonances in the denatured protein spectrum from the random coil positions which are all between 0.89 and 0.96 ppm for these types of proton; in the native spectrum these peaks are scattered between −0.5 and 1.3 ppm, the large shift perturbations reflecting specific interactions in the closely packed protein interior.

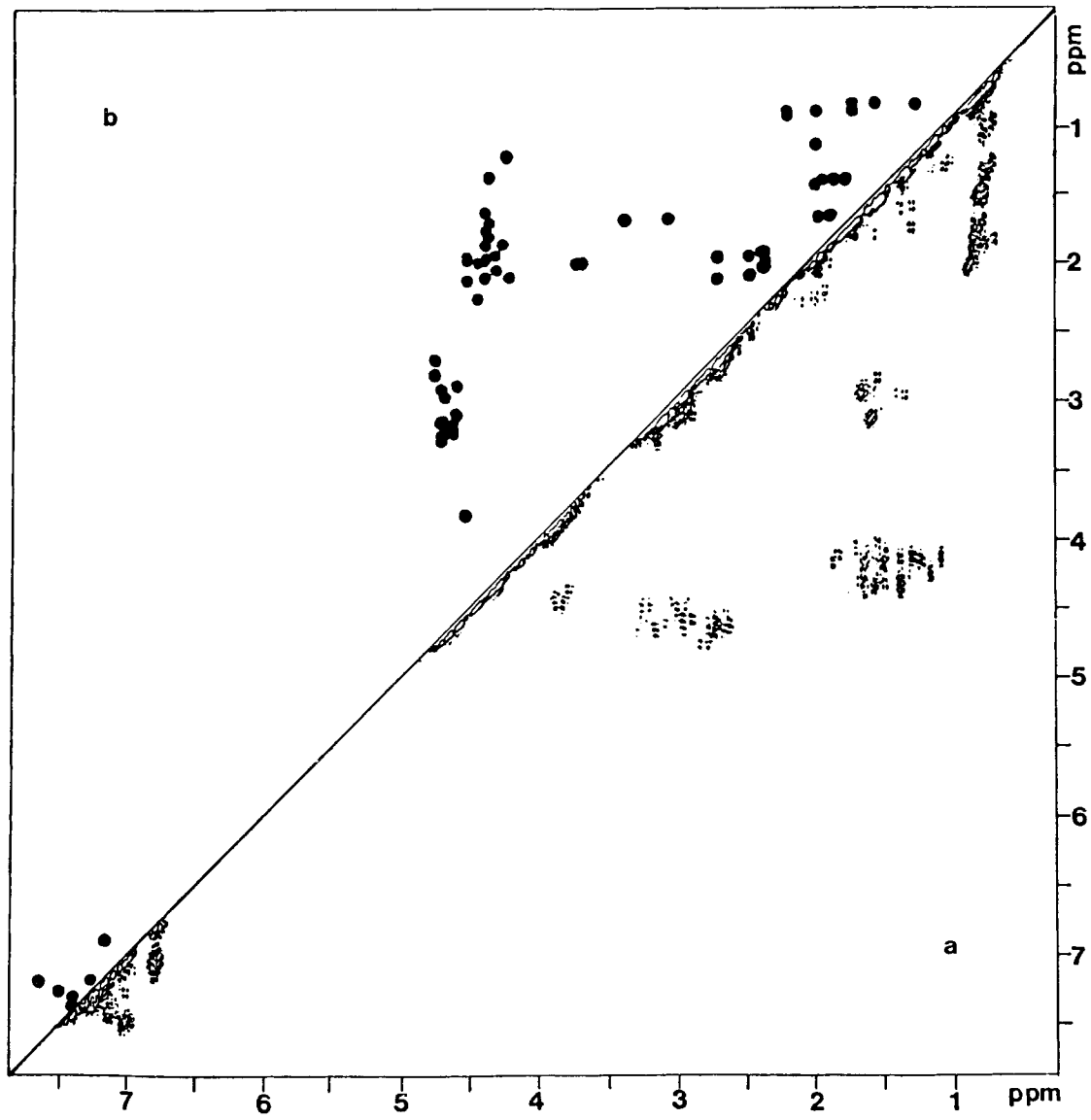

Fig. 1. **(a)** Contour plot of a phase-sensitive double quantum filtered COSY spectrum of thermally denatured hen lysozyme in D_2O, pH 3.8, 81°C. **(b)** Illustration of cross-peak positions which would be anticipated in a double quantum filtered COSY spectrum of a random coil polypeptide.[28]

Resonances of both folded and denatured states contribute to the NOESY spectrum under these conditions and the experiment detects magnetisation transfer within individual states (i.e., NOEs) as well as between the states (i.e., chemical exchange effects). This is illustrated for the Leu-17 methyl protons in Figure 2. The utility of the experiment for correlating resonances of the different states therefore depends in general on being able to discriminate between these two kinds of effect. Several approaches to this problem are possible. The simplest is to record additional spectra using identical acquisition parameters at temperatures sufficiently below and above the unfolding transition zone that in each case only one state is appreciably populated. Figure 3a shows a NOESY spectrum recorded at 65°C where virtually all the protein molecules are folded; only native NOE cross-peaks are therefore present in the spectrum. The chemical shifts of most resonances do not differ appreciably from those at 75°C, enabling the NOE effects within the native state to be identified in the spectrum recorded at 75°C, shown in Figure 3b, by comparison. By identifying NOEs within the denatured state in a similar manner it is then possible to to identify additional cross-peaks in the 75°C spectrum as arising from chemical exchange effects.

This strategy was successful in distinguishing a

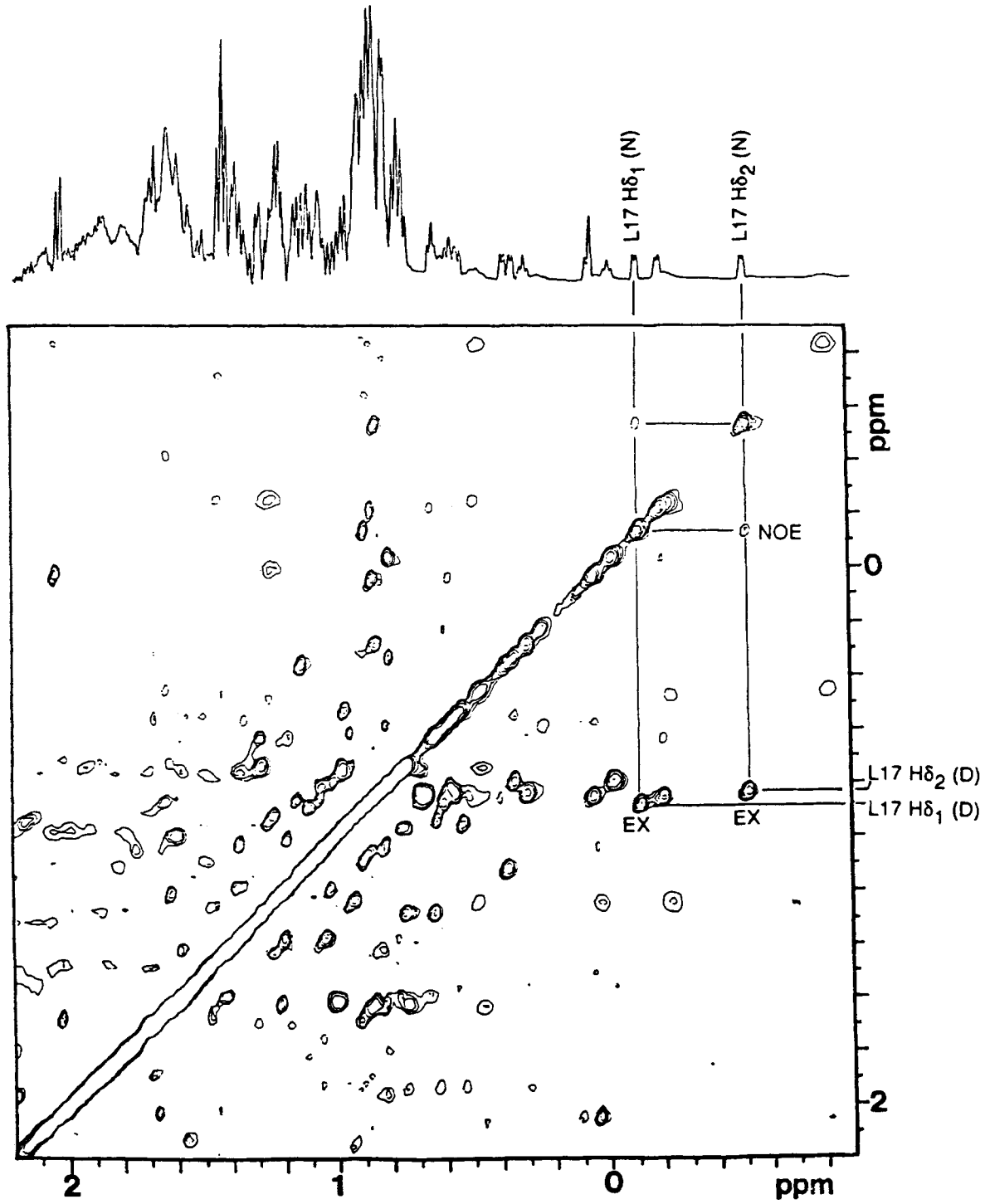

Fig. 2. High field region of a contour plot of a NOESY spectrum of hen lysozyme in D_2O, pH 3.8 at 75°C, close to the midpoint of the thermal denaturation transition. The mixing time was 250 msec and the spectrum is plotted in phase-sensitive mode. NOE and chemical exchange correlations arising from the methyl protons of Leu-17 in the native (N) and denatured (D) states are identified as examples.

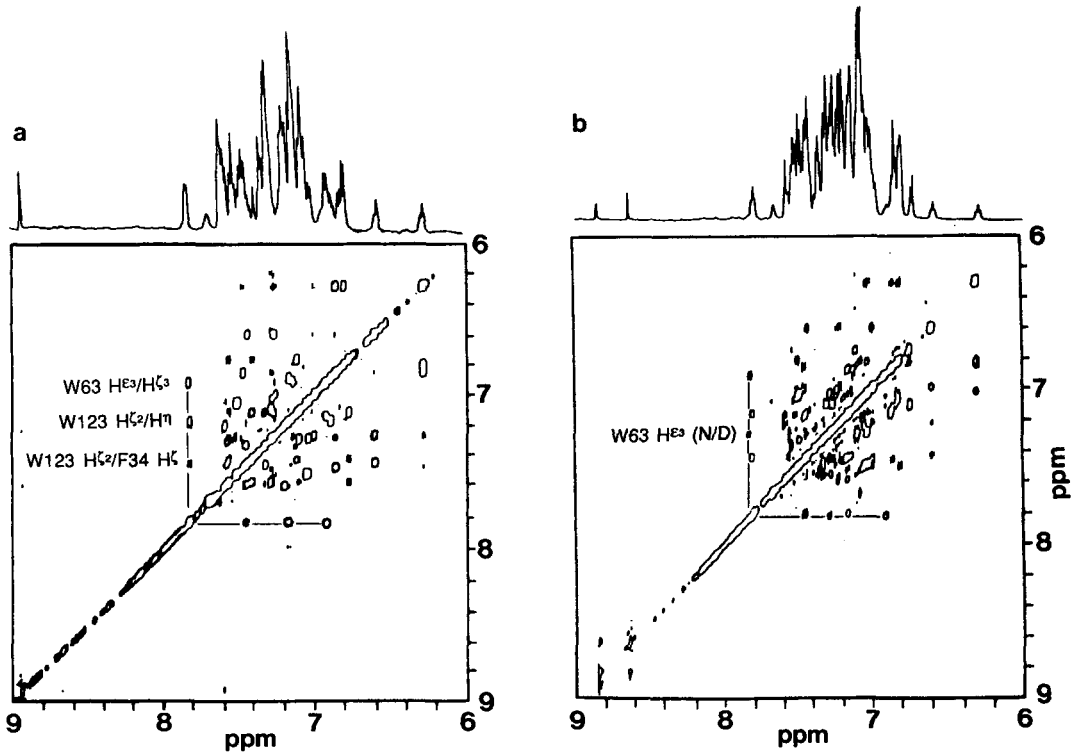

Fig. 3. **(a)** Low field region of a contour plot of a NOESY spectrum of hen lysozyme in D_2O, pH 3.8 at 65°C, where the protein is virtually all in its native state. The mixing time was 250 msec. NOE cross-peaks arising from the nearly coincident resonances of Trp-, 63 $H^{\epsilon 3}$ and Trp-123 $H^{\zeta 2}$ are indicated. **(b)** Contour plot of a NOESY spectrum obtained in an identical manner to that shown in **(a)**, except that the temperature had been raised to 75°C, close to the midpoint of denaturation. The assignment of a specific exchange cross-peak is illustrated. This peak aligns exactly with the NOE cross-peaks assigned to Trp-63 $H^{\epsilon 3}$ in the native protein, permitting its unequivocal assignment to this proton rather than to Trp-123 $H^{\zeta 2}$, whose exchange correlation is not detected at this contour level.

large number of chemical exchange correlations in the present system. Other approaches are also possible, including the use of different 2D NMR experiments such as ROESY, in which the distinction of NOE and chemical exchange effects by their different signs and TOCSY, where transferred magnetization may additionally be relayed to other scalar coupled protons, permitting further assignments to be made.[29] Provided that NOE cross-peaks can be distinguished from the exchange effects using one of these strategies, their coexistence in the spectra actually affords an invaluable means for ensuring that correct cross-assignments are made. In crowded regions of the spectrum, for example, the identification of individual exchange cross-peaks was often achieved by noting precise alignments with characteristic native state NOEs, as illustrated in Figure 3.

Chemical Shift Values of Specific Protons

The specific assignments of 65 proton resonances in the spectrum of thermally denatured lysozyme are listed in Table I. The majority of these are of protons whose *native* state resonances exhibit significant deviation from the random coil shift position; this is because these give rise to the best resolved exchange cross-peaks in the 2D spectrum. Where the chemical shift difference between resonances of the two states is small, the exchange cross peaks often cannot be resolved from the intense diagonal signal. Significant numbers of aromatic resonances (17) and methyl group resonances (19) have been assigned; in the latter case these are predominantly those which are shifted to high field in the native state spectrum. Resonances of 21 αCH protons, predominantly those shifted to low field in the native state spectrum, and 8 backbone NH resonances have also been obtained.

Figure 4 compares graphically the deviations from random coil shifts exhibited by the assigned protons in the two states of lysozyme. It is apparent that there is no clear correlation between the two sets of values. Deviations from random coil shifts cannot, therefore, be explained simply in terms of a marginal tendency to form native-like structural elements.

In the case of the αCH protons the apparent predominance of downfield shifts in the native state spectrum is, as mentioned above, a consequence of the much clearer resolution of resonances in the region of the spectrum where these are located and

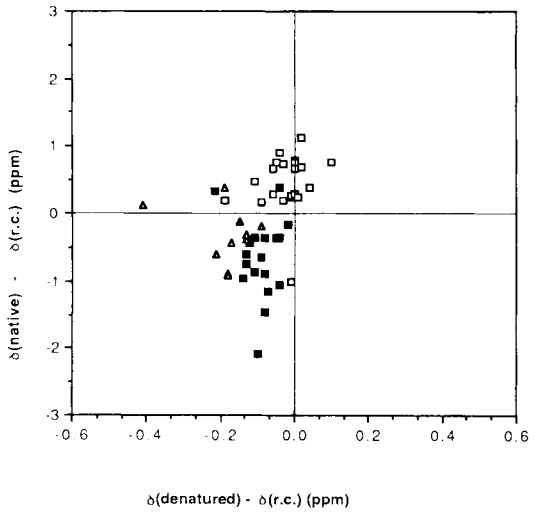

Fig. 4. Comparison of the deviations from the random coil (r.c.) chemical shift value[28] observed for individual protons in the native protein spectrum and that of the thermally denatured state. Note that the scales are very different, in order to bring out the much smaller effects typically observed in the denatured protein spectrum. The symbols denote: (■) methyl group protons; (△) aromatic ring protons; (□) main chain αCH protons.

does not reflect the overall distribution of αCH chemical shifts in the native protein spectrum. In fact, most of these downfield shifted αCH resonances arise from protons in the β-sheet region of the structure, the characteristic shift to low field arising from the proximity of the αCH protons to carbonyl groups of adjacent strands in the sheet.[13,30] In the denatured state, by contrast, it is clear that the resonances of these protons are scattered to either side of the random coil values, no obvious pattern now persisting. There is thus no indication of any residual population of the β-sheet structure in the denatured state. Too little is known, however, about the factors which determine main-chain proton shifts to permit further interpretation of these deviations from random coil behavior. The amide NH assignments which have been made in the spectrum of the denatured protein correspond to those protons whose resonances have substantial downfield shifts in the native state spectrum. In this case random coil shift values appropriate to these conditions are not available for direct comparison but comparison with the overall distribution of amide NH shifts in the 1D spectrum shows no evidence that the particular protons which have been assigned retain any marked downfield shifts in the spectrum of the denatured state.

For the methyl group resonances there is an evident bias to upfield shifts in both states of the protein and this does not arise just from an unrepresentative selection of resonances; in the native protein large deviations from the random coil shift values among side chain protons *are* much more frequently to high field than to low, regardless of the local backbone conformation. This has been noted for a variety of proteins including lysozyme.[31] In the case of denatured lysozyme it can be seen from Figure 4 that although the average size of the shift deviations is an order of magnitude smaller than in the folded state, the marked trend toward high field in the direction of these perturbations clearly persists. This suggests that the origins of deviations from random coil shifts observed in the two states are likely to be related. On the other hand there is no particular correlation between the magnitudes of individual proton shifts in the two states; indeed in a few cases the net shifts are actually in opposite directions in the different states. These points are illustrated by the three most upfield shifted resonances in the spectrum of the denatured state which do not correlate with any of the dozen or so methyl proton resonances which are shifted to high field in the native spectrum. One of these resonances has been assigned to Val-29 Hγ which has a marked *downfield* shift in the native state; the others have not been assigned, which indicates that they arise from protons not markedly shifted in the native protein spectrum.

Significant residual upfield shifts relative to the random coil position are also predominant amongst the aromatic resonances which have been assigned in the spectrum of the denatured state, again consistent with the general pattern observed in the COSY spectrum. Indeed, this tendency is even more pronounced amongst these resonances than those of methyl groups. The resonances of Trp-63 are more dramatically perturbed—in particular the $H^{\epsilon 3}$ proton lies 0.2 ppm to high field of any of the other five tryptophan $H^{\epsilon 3}$ resonances, fully 0.4 ppm upfield of the random coil position. The coupling of this proton to the adjacent $H^{\zeta 3}$ gives rise to the conspicuously isolated cross peak in the COSY spectrum at (7.00, 7.24) ppm (Fig. 1). The $H^{\delta 1}$ resonance also has a particularly marked shift, 0.1 ppm to high field of any of the other tryptophan $H^{\delta 1}$ peaks. These specific chemical shift perturbations bear no relation to those observed for Trp-63 in the native protein spectrum[24]—for example, the $H^{\delta 1}$ singlet resonance has a pronounced downfield shift in the native spectrum in contrast to to its upfield shift in that of the denatured state.

Variation of Denaturation Conditions

In order to investigate the temperature dependence of the denatured lysozyme spectrum a solution of lower pH was prepared, in which the thermal stability of the native structure is lower, permitting the denatured protein to be observed over a wider temperature range. The spectrum obtained at pH 1.8, 87°C (Fig. 5a) was found to be very similar to the spectra of the denatured state at pH 3.8, around 80°C, where the assignment studies were performed.

TABLE I. Resonance Assignments in Thermally Denatured & Native Lysozyme

Assignment	Chemical Shift (ppm)		Random Coil Shift‡ (ppm)
	Denatured State*	Native State†	
1. Methyl Group Protons			
Leucine $H\delta_1$, $H\delta_2$			
L8	0.84, 0.81	0.03, 0.57	(0.90, 0.94)
L17	0.88, 0.84	−0.13, −0.54	(0.90, 0.94)
L56	0.79, 0.84	0.33, 0.55	(0.90, 0.94)
L75	0.87	0.55	(0.90, 0.94)
Isoleucine $H\gamma_2$, $H\delta_1$			
I88	0.87, 0.83	0.55, 0.28	0.95, 0.89
I98	0.85, 0.78	−0.23, −0.03	0.95, 0.89
Valine $H\gamma$			
V29	0.74	1.28	(0.94, 0.97)
Alanine $H\beta$			
A31	1.35	1.03	1.39
A107	1.26	0.64	1.39
Threonine $H\gamma$			
T40	1.19	1.62	1.23
T43	1.21	1.07	1.23
T51	1.12	0.37	1.23
Methionine $H\epsilon$			
M12	2.01	1.70	2.13
M105	2.03	0.05	2.13
2. Aromatic Proton Resonances			
Tryptophan: $H\delta_1$			
W63	7.05	7.63	7.24
$H^{\epsilon 1}$			
W28	9.86	9.39	9.98§
W62	9.90	10.00	9.98§
W63	9.86	10.17	9.98§
W108	9.90	10.00	9.98§
W111	9.86	10.35	9.98§
W123	9.90	10.64	9.98§
$H^{\epsilon 3}$			
W28	7.47	6.77	7.65
W63	7.24	7.78	7.65
W111	7.52	7.26	7.65
$H^{\zeta 3}$			
W28	6.99	6.26	7.17
W108	6.96	6.56	7.17
W63	7.04	6.87	7.17
H^{η}			
W28	7.07	6.80	7.24
Histidine $H\epsilon$			
H15	8.60	8.80	8.70§
Tyrosine H^{δ}			
Y23	7.00	7.03	7.15
H^{ϵ}			
Y23	6.77	6.67	6.86
3. αCH Resonances			
V2	4.18	4.97	4.18
N39	4.75	5.42	4.75
T43	4.35	5.13	4.35
N44	4.75	5.03	4.75
R45	4.26	4.52	4.35
N46	4.79	5.13	4.75
D52	4.65	5.23	4.76
Y53	4.57	4.79	4.60
N59	4.71	5.67	4.75
S60	4.44	5.16	4.50
W63	4.69	4.94	4.70
C64	4.71	5.82	4.69

(*continued*)

TABLE I. Resonance Assignments in Thermally Denatured & Native Lysozyme (Continued)

Assignment	Chemical Shift (ppm)		Random Coil Shift‡ (ppm)
	Denatured State*	Native State†	
N65	4.77	5.45	4.75
D66	4.57	4.95	4.76
N74	4.74	3.73	4.75
I78	4.18	5.00	4.23
P79	4.54	5.20	4.44
L84	4.35	5.12	4.38
C94	4.63	4.97	4.69
D119	4.75	5.02	4.76
C127	4.70	4.93	4.69
4. Amide NH Resonances			
R21	7.83	8.70	
G26	7.24	9.35	
T40	7.78	9.15	
R45	7.58	8.50	
T51	8.03	8.90	
N52	7.81	8.73	
Y53	7.77	8.87	
C76	7.81	9.37	

*Assignments made at 75°C, pH3.8 by magnetisation transfer, as described.
†Chemical shifts of native state peaks at 75°C, pH 3.8. Note that these are typically marginally different from those reported in ref. 24, which were obtained at 37°C.
‡From ref. 28, except where indicated by §, in which cases the shifts were determined experimentally from spectra of small peptides recorded at pH 3.8, 75°C—this was necessary for these protons, to take account of their strong dependence on solution conditions. In the case of amide NH protons appropriate random coil shift values have not been determined for these conditions. For valine and leucine stereospecific assignments of the methyl proton resonances were not available, so their chemical shifts are shown bracketed together.

On lowering the temperature from 87 to 57°C significant changes were measurable in the spectrum (Fig. 5b). Increased deviations from the random coil shift value were evident, particularly in the envelope of leucine, isoleucine, and valine methyl proton resonances upfield of 1 ppm and in the aromatic region. The two prominent methionine H^{ϵ} singlets can also be seen to behave in a similar way in comparing Figure 5a and b; they are significantly upfield shifted at 1.99 ppm at 57°C but they shift downfield on raising the temperature, moving to 2.025 ppm (Met-105) and 2.04 ppm (Met-12) at 87°C. The fact that the two resonances move to differing extents toward the random coil position suggests that there is not a cooperative breakdown of the residual structure as the temperature is raised.

Not all resonances exhibit these temperature dependent shifts—in particular, those which are already close to the random coil position at 57°C are observed to be insensitive to further warming the solution. This is the case for the resonance envelopes arising from the majority of the alanine and threonine methyl groups, close to 1.4 and 1.2 ppm respectively, which can be seen to be virtually unaffected by temperature in comparing Figure 5a and b. For those resonances which were temperature dependent, the changes in their chemical shifts were observed to be continuous over this broad temperature range. This shows that there are small, negative enthalpy changes associated with formation of whatever conformational interactions cause the perturbations.

The spectrum was found to have no detectable concentration dependence; a spectrum obtained with a protein concentration of 0.1 mM (Fig. 5c) being essentially identical to that of the 3.5 mM solution (Fig. 5b). This shows that the chemical shift perturbations observed are unlikely to be associated with molecular association.

In order to assess the significance of the deviations from random coil shifts observed in the spectrum of thermally denatured lysozyme, spectra were obtained under other denaturing conditions, where the extent of any residual nonrandom conformational behaviour might be expected to vary. Figure 6 shows the low field region of the 1D spectrum of thermally denatured lysozyme at pH 1.8, 58°C and compares the effects of additional denaturing factors under these conditions. Figure 7 makes the same comparisons for the high field part of the spectra.

The spectra in Figures 6b and 7b show the effect on the thermally denatured protein of reducing the four disulfide cross-links. It is clear that the tendency toward net upfield shifts is diminished relative to that observed for the disulfide intact form. For example, the large envelope of leucine, isoleucine, and valine methyl proton resonances upfield of 1.0 ppm is distinctly less spread out to the high field side and the tryptophan $H^{\zeta 3}$ resonances (random coil shift = 7.17 ppm) which extend out as far as 6.95

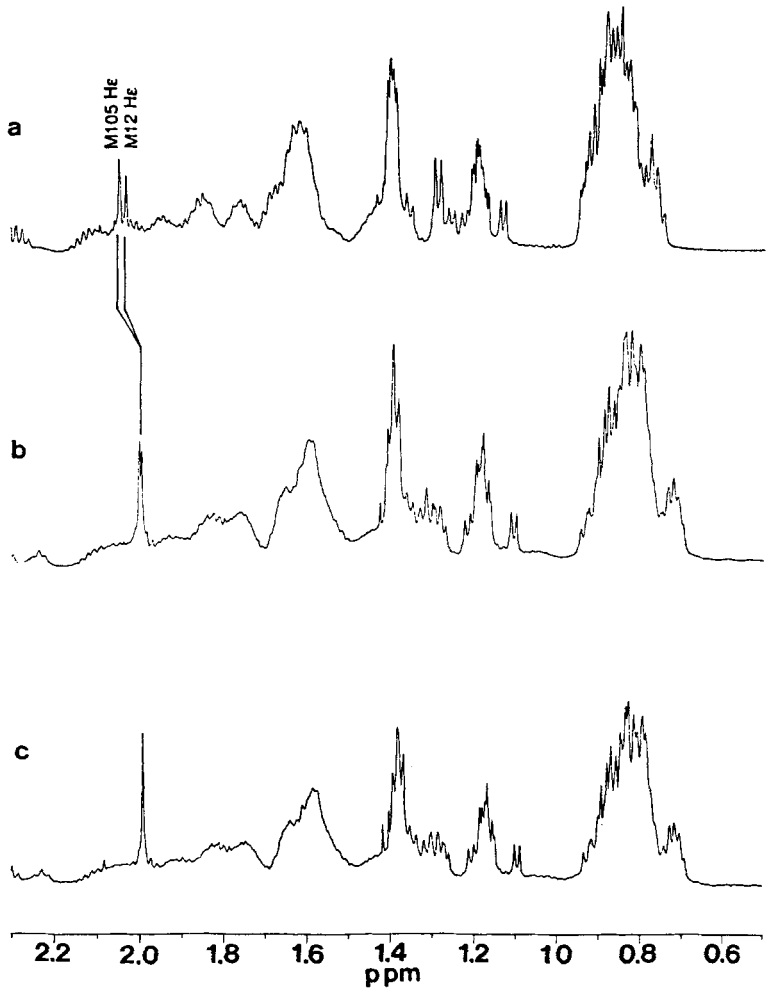

Fig. 5. Effects of temperature and concentration on the spectrum of thermally denatured lysozyme. **(a)** Low field region of the spectrum of 3.5 mM lysozyme thermally denatured at 87°C, pH 1.8 in D_2O. **(b)** As in **(a)**, the temperature having been lowered to 57°C. The temperature dependent shifts of the two methionine methyl group resonances are indicated. **(c)** As in **(b)** but with a lysozyme concentration of 0.1 mM.

ppm in the thermally unfolded form are all downfield of 7.02 ppm in the reduced protein spectrum. In each case, these are shifts in the direction of the random coil value.

As was observed with the temperature dependences, the effects of disulfide reduction on chemical shifts are not uniform. Not surprisingly, those resonances already close to the random coil position are little perturbed by disulfide reduction—this is clearly visible for the majority of alanine and threonine methyl group resonances for example. The resonance envelopes formed by these groups, clustered around 1.4 and 1.2 ppm, respectively, shift hardly at all when the disulfides are reduced, in marked contrast to the large envelope formed by the leucine, isoleucine, and valine methyl proton resonances whose overall downfield shift is obvious in the 1D spectra. Similarly, although the methyl groups of leucine residues show these significant, disulphide dependent shifts as discussed above, the H^{β} and H^{γ} methylene proton resonances (clustered around 1.6 ppm) are much less affected and move rather little when the disulfides are reduced.

Remarkably, some specifically perturbed resonances are also very little affected by removal of the disulphide cross links. For example, the isolated doublet of Thr-51 H^{γ} at 1.12 ppm in the spectrum of the thermally denatured state is actually even a little more shifted relative to the other Thr-H^{γ} protons in the spectrum of the reduced form. The large perturbations to the resonances of Trp-63 also persist in the spectrum of reduced lysozyme, as revealed in 2D spectra (not shown).

The effects of adding a chemical denaturant were also investigated. The spectra shown in Figures 6c and 7c illustrate the effects of 8 M urea on the spectrum of the thermally denatured protein. The changes in the spectrum are broadly similar to those

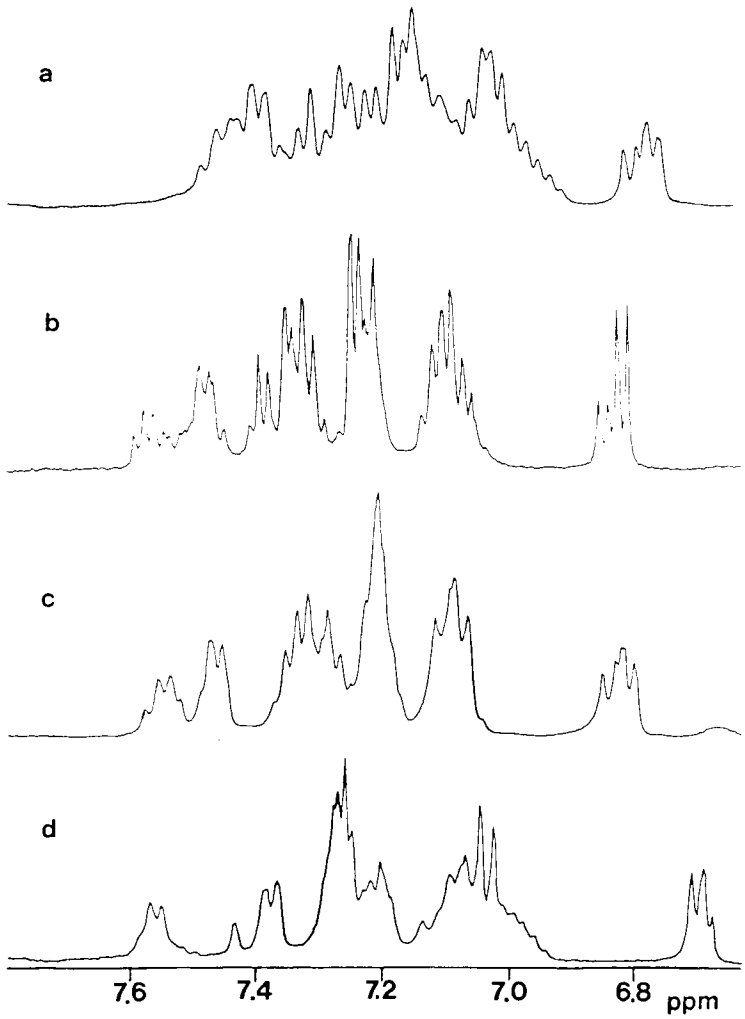

Fig. 6. Effects of varying denaturing conditions on the low field region of the 1D NMR spectrum of hen lysozyme. **(a)** Thermally denatured protein at 57°C, pH 1.8 in D_2O, as in Figure 5a. **(b)** Lysozyme with its four disulfide cross-links cleaved; other solution conditions as in **(a)**. **(c)** Lysozyme in 8 M urea-d_4 solution; other conditions as in **(a)**. **(d)** Reduced lysozyme in DMSO-d_6 solution, 57°C. Note that "random coil" shifts for some protons are significantly different in DMSO compared to aqueous solutions,[28,32] so that only the scatter of resonances rather than their actual shifts in spectrum **(d)** can immediately be compared with spectra **(a)** to **(c)**.

which resulted from disulfide reduction: a clear and rather widespread diminution of the net upfield shift which many protons experience in the thermally unfolded state. As with disulfide reduction, it is clear that some specific interactions such as the perturbations of Trp-63 and Thr-51 are not markedly affected by the denaturant.

Even with the diminution in chemical shift perturbations brought about by reduction or chemical denaturants, there remain clear disparities between the spectrum of denatured lysozyme and that which would be predicted for a true random coil. Following up our earlier study,[9] we therefore investigated the effect of a virtually nonaqueous environment to see if further disruption of residual side chain interactions is possible. Figures 6d and 7d illustrate the spectrum of disulfide-reduced lysozyme in perdeuterated DMSO. The degree of scatter of resonances of the same residue type is here lower than under any of the other conditions investigated. The random coil chemical shift values for some types of proton are different in water and DMSO solutions,[32] which somewhat complicates direct comparison between the spectra but it can be readily verified that the correspondence between chemical shifts of particular proton types in denatured lysozyme and in small peptide models is closer in DMSO than in aqueous solutions. This is illustrated in Figure 8 for the case of the tryptophan ring protons: the upfield shifts relative to the appropriate model peptide values in spectra of reduced lysozyme dissolved in DMSO are here shown to be an order of magnitude smaller than those observed for the disulfide intact denatured state in D_2O, although even here a dimin-

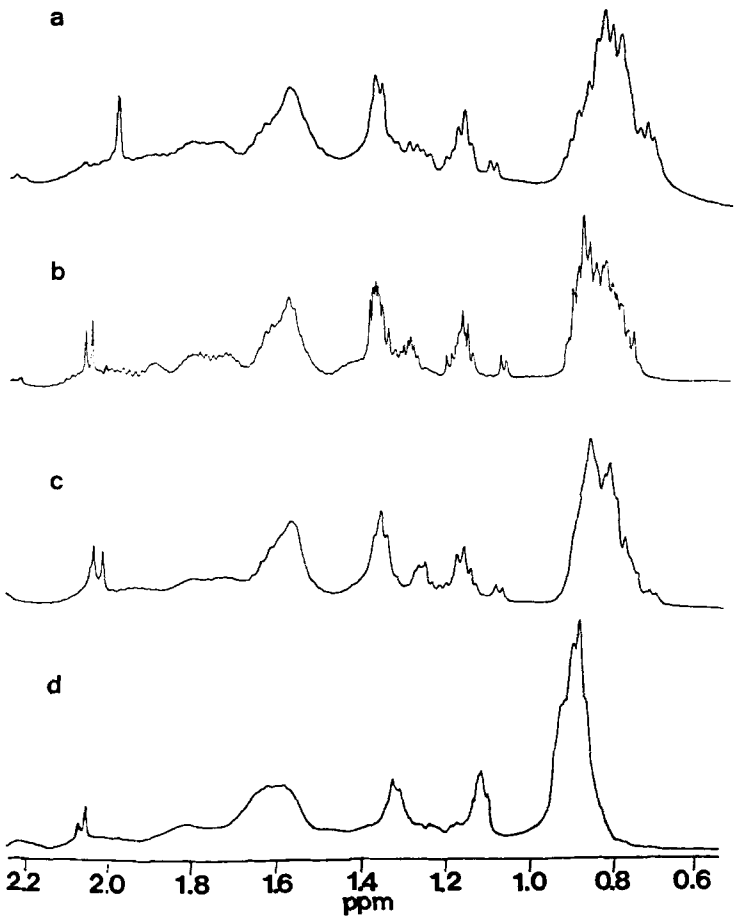

Fig. 7. High field regions of the spectra shown in Figure 6.

ished but significant upfield shift of the tryptophan $H^{\zeta 3}$ peaks is apparent. Specific perturbations including those of Thr-51 (whose H^{γ} resonance is no longer noticeably upfield shifted in Fig. 6d) and Trp-63 (whose ring proton resonances are now observed in the COSY spectrum, Fig. 8b, to be virtually coincident with those of the other tryptophans) have also evidently been disrupted in DMSO solution. Thus it appears that, at least in terms of side chain interactions, the disulfide-reduced protein dissolved in DMSO approaches a random coil more closely than any of the other denatured states investigated here.

DISCUSSION

Assignment of Denatured Protein Spectra

This study has demonstrated that chemical shifts of resonances in the ^1H NMR spectrum of a denatured protein are sensitive to side chain interactions and therefore are interesting parameters to determine for specific protons. It has been shown that 2D NMR experiments such as COSY can be used to resolve clusters of resonances corresponding to a particular proton type but that resolution and assignment of individual resonances are generally not possible using these methods. On the other hand it has been shown that detection of chemical exchange correlations between corresponding resonances of the folded and denatured states using the NOESY experiment constitutes a powerful method for determining the chemical shifts of specific protons in a largely disordered denatured state. We have now applied this approach to a number of protein systems, including α-lactalbumin, staphylococcal nuclease and urokinase.[21,23,33,34] Similar methods have been utilized in studies of thermally unfolded bovine pancreatic trypsin inhibitor.[18] The time scale of protein folding and unfolding at equilibrium is quite typically in the range 10^{-1} to 10 sec, which is appropriate for effective use of this method, suggesting that this approach is likely to be of rather general utility in probing partially disordered states of proteins.

Origins of Chemical Shift Perturbations

It has been shown in this study that significant discrepancies exist between the chemical shift values predicted for a random coil and those actually

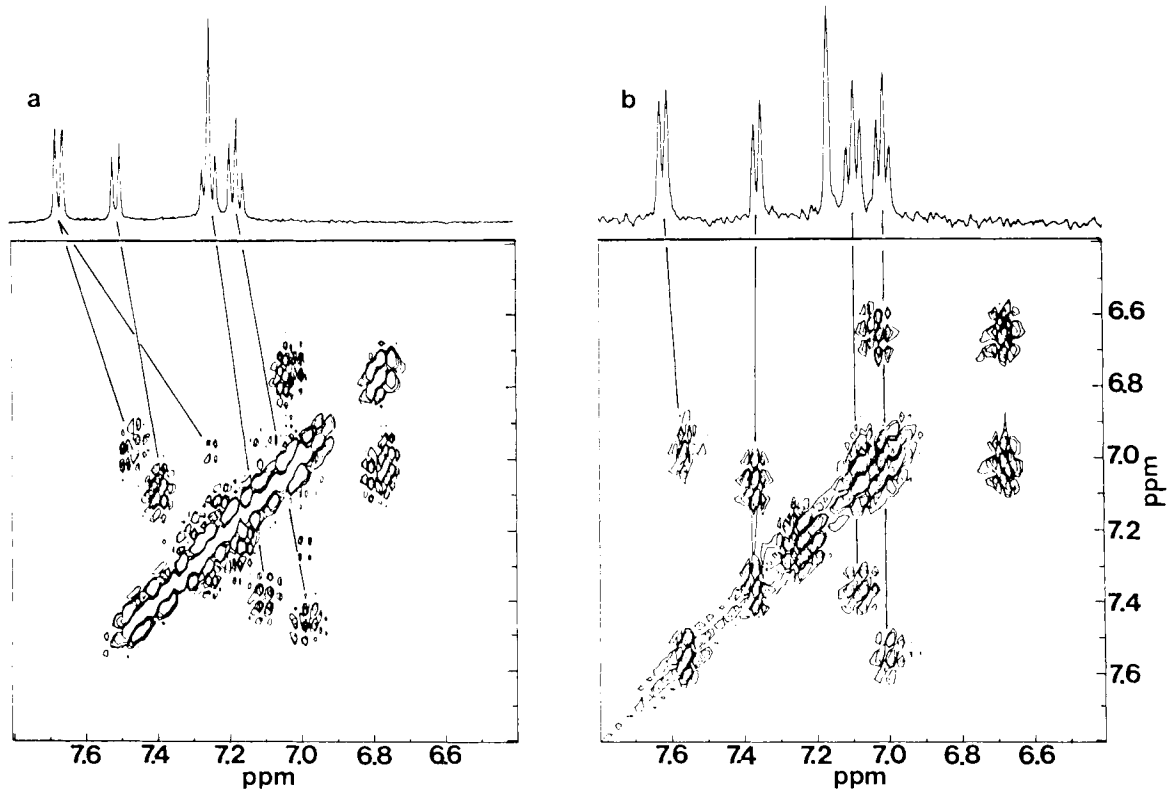

Fig. 8. **(a)** Contour plot of a COSY spectrum of thermally denatured lysozyme, pH 1.8, 57°C in D_2O. The region containing cross-peaks from aromatic protons is shown. The 1D spectrum shown above on the same scale is that of the peptide Gly-Gly-Trp-Ala; the positions of the resonances of corresponding tryptophan ring protons in the two spectra are indicated. **(b)** Comparison as in (a) of the COSY spectrum of reduced lysozyme in DMSO-d_6, 57°C and the 1D spectrum of the tetrapeptide under the same conditions.

observed in denatured states of lysozyme. This is true for both side chain and main chain protons; however in the latter case shifts are poorly understood even in folded globular proteins and the absence of any clear trend in the apparent shift perturbations observed for the denatured protein suggests that their interpretation would be far from straightforward. In the case of side chain protons a clear trend toward residual upfield shift perturbations was apparent, mirroring on a smaller scale that observed in spectra of the folded state and suggesting a possible link between the origins of these perturbations in the different states.

The chemical shifts characteristic of protons in individual amino acid residues in small peptides may be perturbed in proteins via various through-space interactions. For side chain protons the principal source of chemical shift perturbations in proteins is frequently the ring current shift, associated with through space magnetic interactions with aromatic rings.[12] This effect can be approximated as a classical dipolar interaction, varying as $(3\cos^2\theta - 1)/r^3$ (where r is distance from the centre of the ring and θ is the angle relative to a perpendicular axis passing through this center). The angular dependence is such that the average over all orientations at a given distance is zero. However, at the very short distances which give rise to large spectral effects there is greater steric hindrance to approach in the plane of the ring, where protons experience a downfield shift, than to approach perpendicular to the ring, where a net upfield shift is experienced. The observed preponderence of upfield shifts for side chain protons in native protein spectra can therefore be explained by the fact that in close packed structures protons experiencing upfield shifts are on average closer to the ring than those experiencing downfield shifts.[31]

In the denatured state of lysozyme the small net upfield shifts experienced by many protons seem likely to reflect these same features of the ring current shift, even though in this case the interaction between aromatic rings and other side chains may be much less specific than in the native structure. The net effect on the resonance of a side chain proton of nonspecific (even random) encounters with aromatic rings would be expected to be an upfield shift because the distance of closest interaction can be smaller, and the $1/r^3$ dependent shift perturbation correspondingly bigger, out of the plane of the ring,

where the direction of the shift perturbation is upfield. The magnitude of the net upfield shift would be expected to depend on the frequency of such close interactions. Interestingly, the magnitude of the average upfield shift for methyl group protons in denatured lysozyme is of the same order as the average upfield shift observed for these resonances in the spectra of a series of native proteins,[31] consistent with the idea of extensively averaged net ring current effects in the denatured protein. For example, the mean upfield shift relative to the random coil value for all methyl group resonances which have been assigned in the denatured lysozyme spectrum is 0.09 ppm. The overall average upfield shift for these types of proton in native proteins, from the data given in ref. 31, is 0.14 ppm. The somewhat smaller average shift perturbation in the denatured protein is also consistent with its being a less densely packed state.

Evidence for Residual Side Chain Interactions

Local interactions

The chemical shift of an individual proton reflects the net effect of a range of interactions averaged, in the denatured protein, by extensive conformational fluctuations. In general, therefore, it is not possible to determine the specific conformational effects which give rise to a particular shift perturbation. In a few cases, however, it is possible to make reasonable structural proposals to account for individual effects by comparison with characteristic shift perturbations observed in spectra either of the native state or of related model compounds. We focus here on two such cases in the spectrum of denatured lysozyme.

The ring protons of Trp-63 were mentioned above as showing particularly marked deviations from the random coil shift values. Closely similar perturbations to the chemical shifts of this residue have been detected in the spectrum of a peptide fragment of lysozyme, corresponding to residues 57–84, in which the two tryptophans, 62 and 63, are the only aromatic groups.[35] This strongly implies that this particular chemical shift perturbation results from well-defined local interactions between tryptophan rings adjacent in the sequence.

It is interesting to note that the chemical shifts of the Trp-63 ring protons are also strongly perturbed from the random coil value in the spectrum of native lysozyme but in a quite different manner; the $H^{\delta 1}$ singlet, for example, has a large downfield shift in the native spectrum in contrast to its upfield shift in that of the denatured state (Table II). The crystal structure of lysozyme[36] reveals that the side chains of Trp-62 and -63 are in proximity in the native conformation, as shown in Figure 9a. Ring current interactions with other groups undoubtedly also contribute to the net chemical shifts for the Trp-63 protons in the native lysozyme spectrum but calculations based on the crystal structure are broadly consistent with this disposition of the two rings in the folded structure in solution.[37] Structures have been proposed for the Trp–Trp segment in small peptides which generate ring current shifts consistent with those observed in the spectra of these peptides[38,39]; the one which is believed to be the predominant nonextended form is shown in Figure 9b. Shifts calculated from this structure bear some resemblance to those observed in the denatured lysozyme spectrum in that there are marked upfield shifts of the $H^{\delta 1}$ and $H^{\epsilon 3}$ protons (Table II). These effects are much smaller in the case of the denatured protein, suggesting substantial conformational averaging, but it nonetheless seems reasonable to suggest that this is a significantly populated local conformation in denatured lysozyme. It is clear from Figure 9b that the relative orientation of the rings is in this case quite different from that in the native structure. In both environments there appears to be a face-to-edge interaction of the two rings, which is known to be electrostatically favourable[40] and could well be a major factor stabilizing these conformations. Despite the differences in the side chain interactions it is interesting to note that the backbone conformation is not dissimilar in the two cases—this is a consequence of the interaction between the side chains in that this requires that they be *cis* relative to one another across the plane of the intervening peptide bond, placing significant steric limitations on the possible backbone torsion angles.[5] If this interpretation of the conformational preferences of the denatured form of lysozyme is valid then it suggests

TABLE II. Chemical Shifts of Ring Protons in Sequential Trp-Trp Residues

		Native lysozyme* (ppm)	Denatured lysozyme† (ppm)	Ac-Trp-TrpNH(CH_3)‡ (ppm)
W62	δ1	7.11	7.17 ± 0.02	7.32
	ε3	7.11	7.46 ± 0.04	7.58
	ζ3	7.03	6.99 ± 0.04	7.28
	η	7.19	7.10 ± 0.05	7.37
	ζ2	7.45	7.40 ± 0.03	7.62
W63	δ1	7.65	7.05	6.80
	ε3	7.76	7.24	5.80
	ζ3	6.85	7.04	6.68
	η	7.08	7.10 ± 0.05	7.10
	ζ2	7.22	7.40 ± 0.03	7.38

*At 35°C, pH 3.8; from ref. 23.
†At 75°C, pH 3.8, in cases where specific assignment by magnetisation transfer was possible. In other cases the range of values spanned by resonances of the particular proton type, determined from the COSY spectrum at 81°C is given.
‡In the peptide model, the N-terminal tryptophan residue is taken to be the equivalent of trp-62 in lysozyme, the other to be the equivalent of trp-63. The chemical shifts were determined in trifluoroethanol at 220K; from ref. 34. Under these conditions the conformer illustrated in fig. 9b is thought to be the predominant species present.

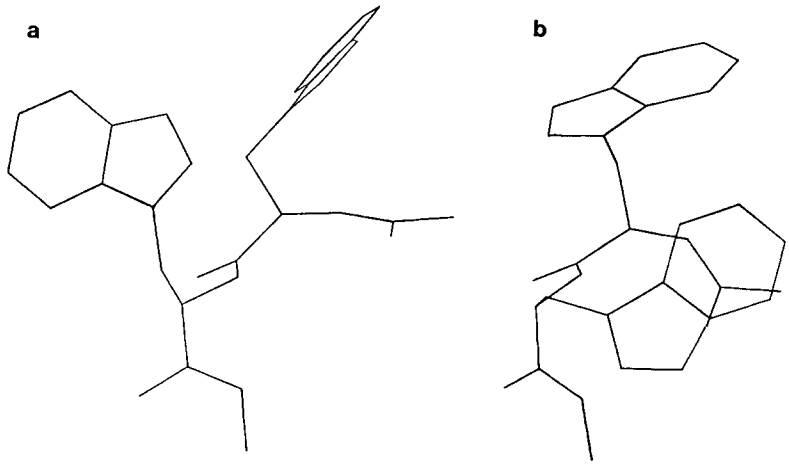

Fig. 9. **(a)** Conformation in the native structure[36] of the segment of lysozyme comprising Trp-62 and Trp-63. **(b)** Probable conformation of acetyl-Trp-Trp-NHMe in trifluoroethanol solution, as deduced by Rizzo and Jackle from energy minimization and NMR data[39]; this appears, on the basis of the chemical shifts of the ring protons, to be a significantly populated conformation of the Trp–Trp segment in aqueous denatured states of lysozyme. (The structure is drawn based on the torsion angles of the conformer denoted a_{II} in ref. 39.)

that the tendency of the chain to turn at this position, as it does in the native structure, is already to some extent inherent in the denatured form even though it is then associated with nonnative side chain interactions.

The most clearly resolved methyl group resonance in the spectrum of denatured lysozyme has been identified as that of Thr-51, which is shifted well to high field of all the other threonine methyl proton resonances. The resonance of this group also stands out in the spectrum of the native protein, as a result of a much larger upfield shift of 0.37 ppm. This perturbation in the native state can be attributed to a close hydrophobic interaction between the Thr-51 side chain and the aromatic ring of Tyr-53.[36] These residues are part of the main β-sheet structure in the folded state, in which such an interaction of next-nearest neighbor side chains is highly favorable. Tyr-53 is the only aromatic residue close in sequence to Thr-51 and it is tempting to suggest that the net upfield shift observed in the denatured state results from a similar interaction; the substantially diminished effect reflecting significant dynamic averaging in the relatively disordered denatured state. The αCH chemical shift values measured in denatured lysozyme indicate that the β-sheet as such does not persist in the denatured protein but an extended β-conformation would be expected to be a relatively favored conformation, even in a random coil.[5] The suggested interaction between Thr-51 and Tyr-53 would, therefore, be consistent with a purely local interaction of side chains, not requiring wider structural preferences in the denatured state.

The evidence of chemical shifts thus suggests in these instances that there may be a limited number of relatively specific local conformational interactions in denatured lysozyme. The relatively limited shift perturbations observed suggest, nonetheless, that even for these residues there is a substantial degree of conformational averaging. NOEs between these side chains were not detectable in NOESY spectra of the denatured protein, even though intraresidue effects were quite large. This strongly supports the conclusion that there is considerable relative motion of the residues on a rapid timescale; it also has the consequence that we have not been able to explore these specific interactions in more detail by NMR methods.

Longer range interactions

It has been observed that in the spectrum of thermally denatured bovine pancreatic trypsin inhibitor, most significant deviations from random coil chemical shift values appear to be associated with residues which have aromatic groups close by in the sequence, suggesting that such local interactions may be the dominant cause of residual shift perturbations in this species.[18] In view of the specific cases outlined above, where such local interactions appear to be implicated, the extent to which other shift perturbations in the spectrum of denatured lysozyme might be explained in similar terms was explored. Figure 10 shows the deviation from the appropriate random coil shift value as a function of the distance in the sequence from the nearest aromatic residue for assigned side chain protons in the spectrum of thermally denatured lysozyme. It is apparent that there is not a strong correlation between these parameters—indeed, some of the residues whose protons exhibit the largest shift discrepancies are remote from any aromatic residues in the sequence. This suggests that, overall, deviations from the random coil shift

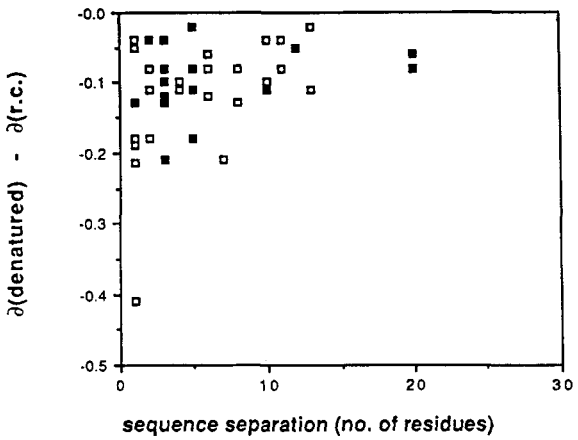

Fig. 10. Variation of the deviation from the random coil (r.c.) shift value observed for specific methyl proton resonances in the spectrum of thermally denatured lysozyme with sequence separation from (■) the nearest aromatic residue and (□) the nearest disulfide bridge.

value in the spectrum of denatured lysozyme cannot simply be ascribed to the effects of aromatic groups close in the sequence. Of course effects other than ring current shifts could be involved but it is also possible that interactions with aromatic residues remote in the sequence have significant effects on chemical shifts in the denatured state. In order to investigate this problem further and begin to assess the extent to which this might reflect a nonrandom conformational distribution it is necessary to consider the effects observed in spectra obtained under alternative denaturing conditions.

The major effect of reduction of the disulfides in denatured lysozyme was observed to be a widespread diminution of side-chain chemical shift deviations from the random coil values, relative to those observed for the thermally denatured state. This is consistent with the notion that these chemical shift perturbations result from relatively long-range (in terms of sequence) side chain interactions; when the conformational space accessible to the denatured protein is increased by removal of the cross-links the frequency of such encounters would be expected to diminish, with consequent diminution of the net chemical shift perturbations in the spectrum, as observed. Those shift perturbations which are not sensitive to disulfide cleavage are probably associated with interactions between side chains close together in the sequence, which would not be expected to be particularly dependent on cross linking. Certainly this would be true for the case of the putative interactions between Trp-62 and Trp-63 and between Thr-51 and Tyr-53 which were discussed above and which, indeed, do not appear to be significantly affected by the reduction. The fact that the majority of shift perturbations *are* in fact affected supports the idea, then, that there is a widespread incidence of interactions between side chains remote in the sequence of thermally denatured lysozyme. It is important, however, to note that these effects are not confined to residues in the immediate vicinity of the disulfides. This is also illustrated by Figure 10, which shows that there is no particular correlation between the size of deviations from the random coil shift value and the distance in the sequence from the nearest disulfide bridge.

Some increase in conformational freedom would be inevitable on removing cross-links from the protein so the differences evident in the spectrum of the reduced and thermally unfolded proteins might not, on their own, demonstrate the existence of a nonrandom conformational distribution in the latter. On the other hand the observation of very similar effects on the spectrum when urea is added, without disulfide reduction, is indicative of just this. In this case the cross-links are intact, so a reduced frequency of close interaction with aromatic residues can only be a reflection of an increased randomization of noncovalent interactions between side chains. In the case where the protein was studied in DMSO solution, with its disulfides reduced, most of the discrepancies from random coil shift values had almost disappeared, showing that under appropriate conditions the conformational distribution can be sufficiently randomized to give a spectrum approaching that predicted for a random coil. Thus, the key conclusion which can be drawn from this comparison of the spectra of different states of lysozyme is that in the thermally unfolded state there is a level of interaction between side chains considerably greater than that which is imposed by the covalent structure.

Conformational Properties of Denatured Lysozyme

The analysis of NMR spectra which we have presented suggests that there is substantial nonrandom conformational behaviour in thermally denatured lysozyme, which is subject to diminution by removal of covalent cross-links and by the action of chemical denaturants. The NMR evidence suggests that the origin of this behavior probably lies in a residual hydrophobic effect involving, particularly, the larger predominantly hydrophobic alkyl or aromatic side chains. Most of the resonances with significant, disulfide bridge-dependent shift perturbations arise from protons remote from the backbone in these long side chains. For example, among the methyl groups, there is a clear overall downfield shift in the overall envelope formed by the leucine, isoleucine, and valine methyl protons when the disulfides are reduced or urea added but rather little effect on the alanine or threonine methyl resonance envelopes (Fig. 7). Similarly, the leucine H^{δ} methyl groups exhibit much larger cross-link and denaturant dependent upfield shifts than do the H^{β} and H^{γ} protons, closer

to the backbone. Deviations in αCH chemical shifts from the random coil position were observed to be quite significant in some cases but the COSY spectra showed that by and large these were not very sensitive to disulfide cleavage, suggesting that they reflect primarily local conformational preferences. The absence of very many large, specific shifts suggests that what is being observed is probably a rather nonspecific clustering of residues in the thermally denatured form, rather than the persistence of well-defined elements of structure. It is interesting to note that this kind of behavior has been predicted for unfolded proteins on the basis of a simple lattice model for protein folding.[41] The observation that upfield shift perturbations are, on average, larger for aromatic protons than for methyl protons (Fig. 4) raises the possibility that interactions between aromatic groups may be somewhat more favorable or more orientationally specific than those between aromatic and aliphatic groups in the denatured state. It is possible that this is associated with the weak electrostatic interactions which are known to favor close interactions between aromatic rings in a perpendicular orientation in folded protein structures.[40]

Other experimental studies have also led to the conclusion that thermal denaturation leads to a less fully unfolded form of lysozyme than does the action of chemical denaturants.[6,7] In terms of backbone conformation, circular dichroism spectra appear to differ significantly from those anticipated for a random coil and a helix content of as much as 15% can been estimated for the thermally denatured protein.[42] The nature of the structure from which the disparities arise is, however, not clear. We have seen in this paper that NMR chemical shifts may also be indicative of local deviations from random coil behavior of the backbone but it has not been possible to interpret this behavior in any detail. Studies of hydrogen exchange kinetics show that there is limited protection of individual protons from solvent exchange but that this is not associated with native-like secondary structural elements.[43]

It is the presence of residual side chain interactions in the thermally denatured protein which is clearly indicated by the chemical shift analysis and this is corroborated by further experimental evidence. In particular, hydrodynamic measurements have shown that the effective radius of gyration of the thermally denatured form is less than might be anticipated for a cross-linked random coil and that it does increase upon addition of guanidinium chloride.[1,6,7] This appears to be consistent with our findings of substantially enhanced hydrophobic clustering in the absence of chemical denaturant. It should be noted, however, that on increasing the temperature the radius of gyration of the reduced protein, at least, was observed to decrease, perhaps suggesting an increasing extent of hydrophobic collapse.[42] This does not seem to accord with our observations, however, which indicate that the level of residual side chain interactions diminishes gradually as the temperature is raised. It seems likely that the extent of randomization does actually increase on raising the temperature, one consequence of this being that the chain flexibility is increased which leads to the decreased apparent radius of gyration.[42]

In drawing the conclusion that there is a significant level of residual hydrophobic interactions in denatured lysozyme it is necessary to take account of the results of calorimetric data which revealed no detectable differences in the enthalpies of thermally and chemically denatured forms[44] or in their heat capacities or that of the disulfide reduced form.[42] It is observed quite generally that exposure of hydrophobic groups to water leads to excess heat capacity in the system and it is usually accepted that this reflects increased ordering of water molecules at the interface.[1-3] Thus, it might be expected that formation of residual structure which decreased the accessible hydrophobic surface area would be detectable in these thermodynamic measurements. There are two points, however, to consider here. The first is that the differences in degree of solvent exposure of hydrophobic side chains may be relatively subtle, since a substantial increase in the level of transient association could still leave the side chains highly hydrated, on average. The large change in heat capacity on folding to the native structure is associated with burying of around 68% of the surface area of lysozyme.[45] Clearly, a very much more marginal effect is being observed in the denatured states and a correspondingly small heat capacity effect would be expected.

The other factor which needs to be considered in this is a consequence of the noncooperative character of the conformational interactions in the denatured state. If the formation of individual interactions is accompanied by a small, negative enthalpy change (as suggested by the temperature dependence) then these will break down very gradually as the temperature is raised, making a positive contribution to the heat capacity of the system. This has previously been suggested as an alternative explanation to the solvent structure effect for the high heat capacity of unfolded states[1,6] but it was dismissed on the grounds that observed heat capacities are close to those predicted for a random coil and that no heat capacity difference was detectable between unfolded states with apparently different levels of order.[42,44] We would suggest, however, that *both* effects may be operative in denatured states of lysozyme: the residual hydrophobic clustering would diminish water structuring and thus tend to lower the heat capacity but at the same time the heat lability of these clustering interactions themselves would make some compensatory heat capacity con-

tribution. Overall, therefore, in a noncooperative system the heat capacity differences between states with different levels of structure would not necessarily be expected to be very large. We therefore conclude that the calorimetric data do not preclude our suggestion that thermally denatured lysozyme exhibits a significant degree of hydrophobic clustering driven, primarily, by small favorable enthalpies of association.

Comparison With α-Lactalbumin

The persistence of a very considerable level of residual structure on thermal or acid denaturation of α-lactalbumin is well known.[10,46] Under these conditions the α-lactalbumin molecule remains very compact and its secondary structure content is estimated to be close to that of the native state; however it apparently lacks a specific tertiary structure. This species, known as the A-state, has been described as a "molten globule" by Ptitsyn and co-workers.[46] We have recently presented results of an investigation of the α-lactalbumin A-state by NMR[21] and it is of considerable interest to compare the chemical shifts of protons in this species with those of denatured lysozyme, since the two proteins are highly homologous.

Spectra of α-lactalbumin in the A-state superficially resemble that of denatured lysozyme in that deviations from random coil shifts are generally much smaller than in the native protein spectrum. However, careful comparison of the spectra shows that there is much less uniformity in shifts in the spectrum of the α-lactalbumin A-state—clustering of cross-peaks close to the random coil positions is much less clear in COSY spectra, for example. Some protons have quite marked shifts—for example, the high field cluster of methyl group protons extends as far upfield as 0.3 ppm in the A-state spectrum. Further, specific assignments of a number of protons revealed that there is a correlation between those protons which have pronounced deviations from the random coil shift in the native and A-states, where such a correlation could not be detected for denatured lysozyme. The addition of urea as a chemical denaturant also had a much more dramatic effect on chemical shifts of protons of the A-state than on denatured lysozyme; in very high urea concentrations deviations from the hypothetical random coil spectrum are similarly small for both proteins. These features support the idea that interactions in the A-state are more numerous and more ordered than in denatured lysozyme, and probably related quite closely to those in the native state, in contrast to the relatively nonspecific effects observed in denatured lysozyme. Other features of the α-lactalbumin A-state spectrum emphasize the differences between the two species, including very broad lines indicative of hindered conformational interconversions and specific NOEs between side chains; none of these is observed for denatured lysozyme. The disparity between the proteins is very clear even when spectra are obtained at the same pH and temperature. It is not merely a consequence of different solution conditions but is an intrinsic property of the denatured forms; the origin of this remarkable difference is not yet clear.

CONCLUSIONS

We can conclude from the results presented in this paper that the evidence from chemical shifts suggests that the thermally denatured form of lysozyme is somewhat collapsed as a result of residual hydrophobic interactions but that with the exception of a few cases involving residues adjacent in the sequence, this is a rather nonspecific effect. The relationship between this form and the native state is thus not a strong one in structural terms, in contrast to that of the α-lactalbumin A-state to its corresponding native state. Nonetheless, the persistence of a substantial tendency to hydrophobic clustering in the absence of well-defined elements of native-like structure is undoubtedly of great importance in understanding early events in protein folding because it means that the environment in which structure formation begins to take place may be quite different from that of a random coil. The significance of such behavior for protein folding has been questioned[47]; nonetheless, the apparent facility of the early stages of structure formation is not straightforwardly understood given the very limited tendency for isolated structural elements to form in an aqueous environment. As Dill has pointed out, an incipient collapse may be a crucial factor in facilitating folding at this stage,[41] since it has profound implications both in terms of the number of accessible conformations of the polypeptide and the stabilization of primitive structural elements.

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