A Partially Folded State of Hen Egg White Lysozyme in Trifluoroethanol: Structural Characterization and Implications for Protein Folding†

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ABSTRACT: The effect of 2,2,2-trifluoroethanol (TFE) on the solution conformation of hen egg white lysozyme has been investigated using circular dichroism (CD) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Addition of TFE to lysozyme at pH 2.0, 27 °C, up to a concentration of 15% (v/v) induces only slight changes in the NMR spectrum. However, above this concentration a cooperative transition to a new but partially structured state of the protein is observed. This state shows no structural cooperativity against further denaturation and is characterized by an ellipticity in the far-UV CD greater than that of the native protein. Near-UV CD intensity is dramatically reduced compared with that of the native state, and ^{1}H NMR studies indicate that side-chain interactions are substantially averaged in this denatured state. Solvent proton/deuterium exchange rates for 66 amide hydrogens were measured site-specifically by a combination of amide trapping experiments and 2D ^{1}H NMR. Significant protection from exchange occurs for about 25 backbone amides, the majority of which are located in regions of the protein that are helical in the native enzyme. By contrast, amides located in a second region of the native protein which contains a β-sheet and one 3_{10}-helix as well as a long loop show little protection. This pattern of protection resembles that found in the stable molten globule state of α-lactalbumin and in an early kinetic intermediate detected in the refolding of hen lysozyme.

The characterization of conformational transitions and folding intermediates is central to the study of protein folding (Creighton, 1990; Kim & Baldwin, 1990), but the detailed description of partially folded proteins is often difficult due to the high cooperativity of folding. Using mild denaturing conditions, however, partially folded states have been found for a number of proteins that are stable at equilibrium. The nature and degree of structure observed in these states can vary widely; some are largely disordered while others exhibit substantial conformational preferences (Christensen & Pain, 1991; Dill & Shortle, 1991; Dobson, 1992). A major objective is, therefore, to characterize these stable partially folded states and to relate their structures to those inferred for transient folding intermediates from kinetic studies.

Studies of lysozyme and the homologous protein α-lactalbumin have been particularly important in this regard. A stable, compact molten globule state is observed during equilibrium denaturation of α-lactalbumin; by contrast, the unfolding transition of lysozyme is a highly cooperative event in which only the native and highly unfolded states are stable at equilibrium (Dolgikh et al., 1981; Evans & Dobson, 1984; Baum et al., 1989). Despite this, stopped-flow circular dichroism (CD)[1] experiments have suggested that similar intermediates are populated during kinetic refolding of the two proteins. In addition, NMR studies on hydrogen exchange protection have shown that the stable molten globule state of α-lactalbumin resembles an intermediate populated transiently during the refolding of hen lysozyme (Kuwajima et al., 1985; Miranker et al., 1991; Radford et al., 1992b). There is, therefore, considerable incentive to try to generate stable partially folded states of lysozyme at equilibrium using different denaturing conditions.

A second strategy used to overcome the cooperativity of protein folding is to study protein fragments (Wright et al., 1988) and peptide models (Staley & Kim, 1990). Characterization of the conformation of such peptides in aqueous solutions is, however, often complicated by the dynamic behavior of these molecules (Dyson & Wright, 1991). Consequently, structure-stabilizing cosolvents, principally trifluoroethanol, are commonly employed to stabilize peptide conformations in solution (Sanford et al., 1991; Bruch et al., 1991). Recently it has been shown in several peptide systems that TFE does not induce secondary structure indiscriminantly, but regions of the polypeptide chain that are helical in the native state are stabilized preferentially (Nelson & Kallenbach, 1989; Lehrman et al., 1990; Segawa et al., 1991; Sonnichsen et al., 1992; Dyson et al., 1992). Little work has, however, been carried out on the effect of TFE on the conformation of intact proteins. Whether TFE stabilizes native-like structures or alters or induces novel structures, therefore, is a fundamentally important question.

In light of these issues, we have studied the effect of TFE on the structure of intact hen lysozyme in its native and denatured states. Evidence from CD for the denaturation of this protein to a partially structured state in aqueous TFE solution has previously been reported (Galat, 1985). Here we report NMR and CD evidence on the nature of the equilibrium denaturation transition of hen lysozyme in TFE and a detailed characterization of the structural features of its partially denatured state.

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[1] Abbreviations: TFE, 2,2,2-trifluoroethanol; NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser spectroscopy; NOE, nuclear Overhauser effect; COSY, J-correlated spectroscopy; CD, circular dichroism.

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MATERIALS AND METHODS

Materials. Hen egg white lysozyme and the tetrapeptides Gly-Gly-Tyr-Arg and Ala-Gly-Ser-Glu were obtained from the Sigma Chemical Co. Lysozyme was dialyzed extensively at pH 2.0, lyophilized, and stored at −20 °C; 99% 2,2,2-TFE-d_3 was purchased from Cen Saclay (France). The exchangeable deuterium on TFE-d_3 was exchanged by distillation from H_2O to allow for a steady deuterium lock on TFE in 2D ^1H NMR experiments. TFE-h_3 was purchased from Aldrich.

Circular Dichroism (CD). CD measurements were made on a Jasco J-720 spectropolarimeter. A 1-cm cell was used throughout. The concentration of lysozyme samples was typically 2–4 μM for far-UV and 20–40 μM for near-UV CD studies. The pH of each sample was adjusted to 2.0 after addition of TFE or urea. Molar ellipticities, θ (in degrees centimeter squared per decimoles of residues), are reported.

^1H NMR Spectroscopy. ^1H NMR spectra were acquired at 27 °C on 500- and 600-MHz Bruker AM spectrometers belonging to the Oxford Centre for Molecular Sciences. One-dimensional NMR spectra were acquired over 8K complex points using a sweep width of 7246.4 and 6024.2 Hz, respectively. NOESY spectra were acquired in the phase-sensitive mode using a mixing time of 200 ms; 1K increments of 4K complex data points were collected. The F1 dimension was zero-filled twice, and resolution enhancement with double-exponential window functions was applied in both dimensions. Protein concentrations were typically 2.5 mM. The pH was adjusted for individual samples in TFE and is uncorrected for isotope and TFE effects.

Amide Hydrogen Exchange. (a) Bulk Exchange Experiments. The lyophilized enzyme (final concentration 2.5 mM) was dissolved in 50% TFE-d_3/D_2O and the pH rapidly adjusted to 2.0. 1D NMR spectra were acquired immediately at 27 °C at intervals of 4.5 min over a period of 12 h. At chosen time points, the protein was refolded by 6-fold dilution into 20 mM deuterated sodium acetate buffer in D_2O, pH 3.8. Spectra were acquired immediately and 4 h after the dilution. Amide hydrogen intensities were integrated between 8.80 and 7.75 ppm and were normalized to the area of the 59 aromatic hydrogens (7.75–6.60 ppm) acquired for the same sample, but in which all of the amide hydrogens had been exchanged with deuterons by heating the sample to 58 °C for 15 min.

(b) Selective Bulk Exchange of Rapidly Exchanging Amide Hydrogens. Lysozyme was fully deuterated by dissolution at pH 2.0 in 99.9% D_2O and heating to 80 °C for two periods of 20 min. The protein solution was cooled and lyophilized. Deuterated lysozyme was then dissolved at 2.5 mM in H_2O, pH 2.0, 27 °C, for 2 h. During this procedure, selective exchange of amide deuterons that are weakly protected in the native state occurs for solvent hydrogens. Following a second lyophilization step to remove H_2O, protein samples were dissolved in either D_2O or 50% TFE-d_3/D_2O at pH 2.0, 27 °C, and exchange of amide hydrogens was monitored as described above. In this case, spectra were acquired at intervals of 1.5 min.

(c) Site-Specific Measurement of Exchange Rates from the TFE State. Hydrogen exchange of lysozyme in 50% (v/v) TFE-d_3/D_2O was allowed to proceed for various lengths of time (3–810 min) at pH 2.0, 27 °C, as previously described for the bulk exchange experiments. After various periods of exchange, the protein was rapidly refolded by 6-fold dilution into D_2O, pH 3.0, to the native state, and the protein solution was immediately lyophilized. Samples were redissolved, to a final concentration of 5 mM, in 20 mM deuterated sodium acetate buffer in D_2O, pH 3.8, and phase-sensitive COSY spectra were acquired at 35 °C (Redfield, 1988). A total of 256 increments over 2K complex points and 16 transients were collected; the total acquisition time was 3.5 h. Amide proton intensities were taken as the sum of the absolute values of the four phase-sensitive components and were scaled to the C3,5H–C2,6H cross-peaks of the nonlabile protons of Tyr-23 and Tyr-53. Amide proton occupancies were normalized to those measured for a sample of hen lysozyme freshly dissolved in D_2O at pH 3.8 and 35 °C acquired under identical conditions. Hydrogen exchange rates (k_{ex}) were fitted as single-exponential decays. Intrinsic exchange rates (k_{int}) predicted for amides in an unstructured polypeptide chain were calculated taking into account near-neighbor inductive effects on acid and water catalysis (Molday et al., 1972; Jeng & Englander, 1991). Protection factors (k_{int}/k_{ex}) uncorrected for TFE cosolvent effects are reported together with exchange half-lives.

(d) Hydrogen Exchange in Tetrapeptides in 50% (v/v) TFE-d_3. The effect of TFE upon intrinsic exchange rates of unprotected amides was estimated on the basis of two unstructured tetrapeptides, Gly-Gly-Tyr-Arg and Ala-Gly-Ser-Glu. Peptides were lyophilized from H_2O or 50% TFE-d_3/H_2O at pH 2.0. Each peptide was then dissolved (8 mg/mL) at pH 2.0 in D_2O or 50% TFE-d_3/D_2O and exchange was monitored by recording 1D NMR spectra at intervals of 1 min at 27 °C. The decreases in amide resonance intensities were fitted to single-exponential decays.

RESULTS

Perturbations Induced by TFE in the Native State of Hen Lysozyme. Figure 1 shows near- and far-UV CD spectra of lysozyme in different concentrations of TFE. In the presence of 15% (v/v) TFE, the far-UV CD spectrum of the native enzyme is unperturbed, indicating that this concentration of TFE apparently has little effect on the secondary structure of the protein. Small intensity changes in the near-UV CD are observed under these conditions and are consistent with association of alcohol molecules with the native enzyme (Kurimatsu et al., 1972). In accordance with this, analysis of 1D and 2D ^1H NMR spectra shows that 15% TFE-d_3/H_2O (v/v) induces little change in chemical shift (<0.08 ppm) for the majority of resonances (Figure 2). For a limited set of resonances, however, significant changes in chemical shift (>0.08 ppm) are observed. Of particular interest are the resonances of the indole NH hydrogens of Trp-63, Trp-108, and Trp-111 as well as the amide resonance of Asn-66 which experience changes in chemical shift similar to those observed in the presence of $(GlcNAc)_3$ or urea (Lumb & Dobson, 1992). These shifts can be attributed to the binding of TFE molecules to the active site of the native enzyme. Other shifts are consistent with TFE molecules binding at surface sites, many of which have been previously identified as binding sites for ethanol molecules (Lehmann et al., 1986). Taken together, these results indicate that the structure of the native enzyme is not significantly affected by the change of solution conditions at low TFE concentrations.

Nature of the Denaturation Transition of Lysozyme Induced by TFE. Figure 3a shows the ellipticity at 222 nm as a function of the concentration of TFE. At concentrations of TFE exceeding 15% (v/v), a substantial increase in ellipticity at 222 nm is seen, consistent with cooperative restructuring of the protein molecules to a new state, which is characterized by a negative ellipticity much greater than that of the native enzyme. The midpoint of this transition occurs at about 20%

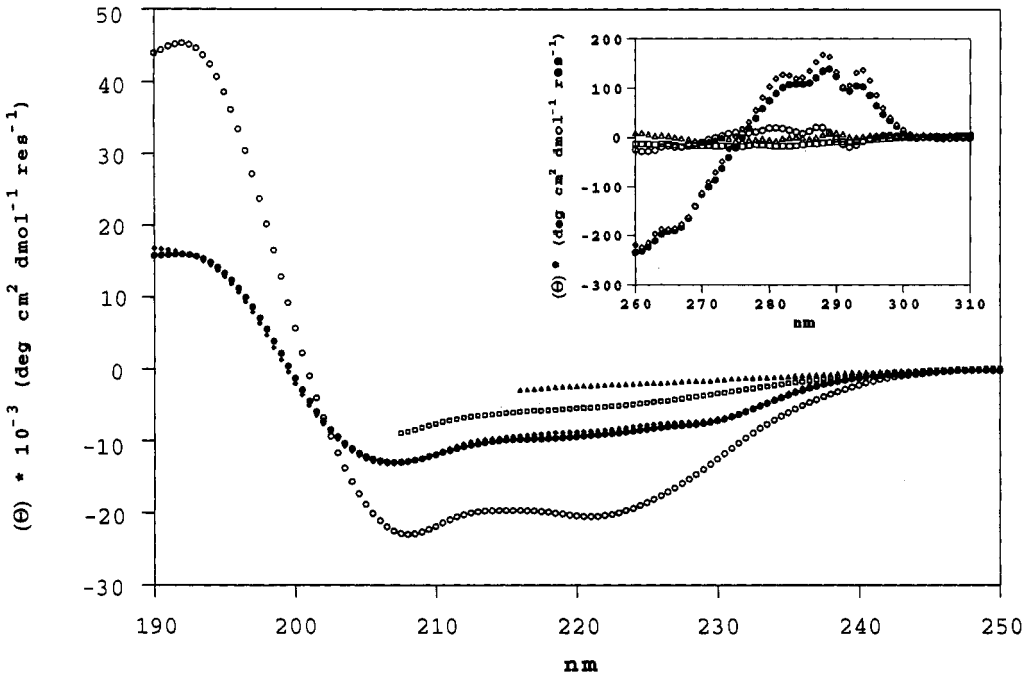

FIGURE 1: Far- and (inset) near-UV CD spectra of lysozyme in the native state in aqueous solution (●), the native state in 12.5% TFE (v/v) (◇), the TFE state (50% v/v) (○), and the thermally (65 °C) (□) and urea (8 M) (△) denatured states.

(v/v) TFE. A further, more gradual, increase of ellipticity is observed as the concentration of TFE is then raised further to about 80% (v/v). In the near-UV CD, a transition at about 20% (v/v) TFE is also observed but in this case involves a substantial loss of ellipticity.

Dramatic changes are also observed in the 1D ^{1}H NMR spectrum as the concentration of TFE is raised above 15% (v/v) (Figure 2). Resonances characteristic of denatured protein appear with a concomitant decrease of native peak intensities. For example, two resonances for the C2H proton of the single histidine residue (His-15) are observed: one at the chemical shift of the native protein and the other at a near-random-coil chemical shift. No appreciable line broadening is evident in the spectrum; the resonances of the native and denatured states are in slow exchange. Peak intensities of the resonances of the native and denatured protein, therefore, can be related directly to the population of these states. Figure 4 shows the change in intensity of several resolved resonances in the 1D ^{1}H NMR spectrum over the transition region of 15–25% (v/v) TFE. The resonances followed arise from residues in different parts of the protein structure; their intensity change is coincident with each other and with the changes in near- and far-UV CD, indicating that denaturation is, to a good approximation, a simultaneous and global event. Dilution of TFE to concentrations below 20% (v/v) in similar experiments demonstrated that the transition is fully reversible under these conditions.

Characterization of the TFE State of Hen Lysozyme by CD and NMR. A concentration of 50% (v/v) TFE-d_3 at pH 2.0 and 27 °C was chosen for structural studies on the TFE denatured state, on the basis of the observation that the conformational transition is virtually complete at this concentration of TFE as judged by CD and NMR spectroscopy. This state is referred to as the "TFE state". The significant increase of negative ellipticity in the far-UV CD at 222 nm during the denaturation transition is in sharp contrast to the decrease in signal intensity observed during denaturation of lysozyme by urea (Figure 3a). The substantial increase in ellipticity in the TFE state is quite remarkable; in 50% (v/v) TFE, an ellipticity more than twice that in the native enzyme is achieved. By contrast, the intensity of the near-UV CD spectrum is substantially reduced relative to that of the native enzyme (Figure 1, inset). A near-linear decrease in ellipticity is observed in the far-UV CD on heating the TFE state (Figure 3b). This again contrasts with the highly cooperative transition observed for thermal denaturation of hen lysozyme at pH 2.0 in H_2O solution (Radford et al., 1991). The cooperativity of structural stabilization in the TFE state, therefore, is substantially reduced, giving rise to a broad melting curve over the range of temperatures studied. In accord with this, the 1D ^{1}H NMR spectrum of lysozyme in the TFE state (Figure 2) is characterized by the absence of significant chemical shift dispersion and substantial resonance overlap. Resonances for five of the six tryptophan indole hydrogens are, however, resolved in the 1D ^{1}H NMR spectrum, and resonances arising from the ring protons of one tryptophan and a second aromatic residue are significantly perturbed from random-coil values (Figure 2).

The 2D NOESY spectrum of the TFE state shows a significant number of NOE's, which may arise from intra- as well as inter-residue through-space interactions (Figure 5). Assignment of these resonances to specific residues could not be made by conventional methods, however, because of the poor chemical shift dispersion in the spectrum. It is, nonetheless, informative to compare this spectrum with those obtained for the native state (Redfield & Dobson, 1988) and the denatured protein in 8 M urea (Figure 5). By comparison with spectra of the native state, NOE's in the spectrum of the TFE state are fewer in number, much more poorly dispersed, and of lower intensity. A substantial number of NOE's are also seen in the spectrum of the enzyme denatured in 8 M urea. In this case, the resonances are even less well dispersed and are weaker in intensity than seen in the spectrum of the TFE state. The chemical shifts and line widths of resonances in the spectrum of the TFE state are sensitive to temperature and to TFE concentrations exceeding 50% v/v. There is no significant effect of protein concentration on these parameters. Line widths, particularly those arising from indole and amide protons, increase with decreasing temperature or increasing TFE concentration. This suggests that temperature and TFE

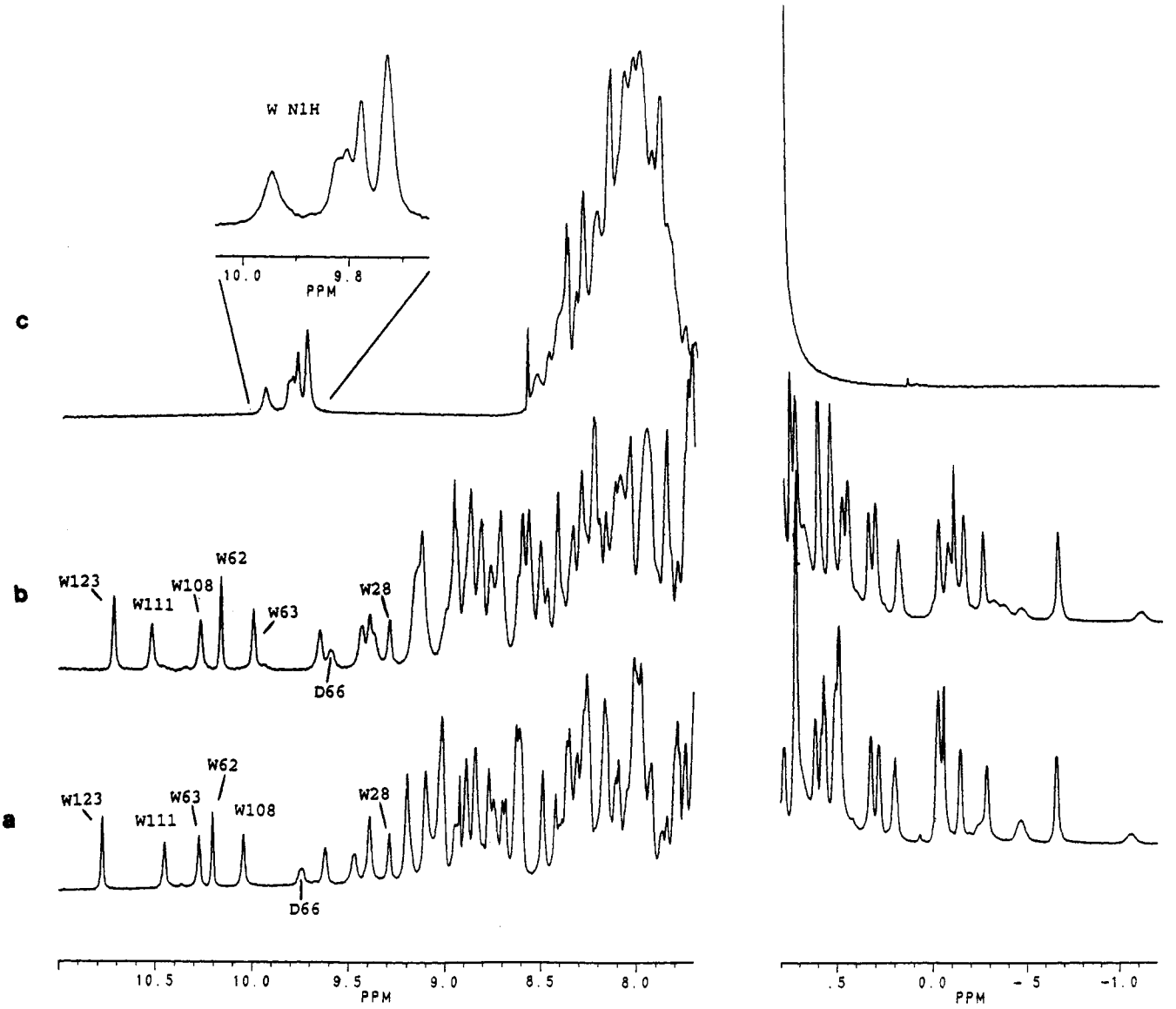

FIGURE 2: Amide and methyl regions of 1D ^{1}H NMR spectra of hen lysozyme at pH 2.0, 27 °C. (a) Native state, 90% H_2O/10% D_2O; (b) TFE-bound state, 12.5% TFE-d_3/H_2O (v/v); (c) 50% TFE-d_3/H_2O (v/v). The small peak near 0 ppm in (c) arises from an impurity. The tryptophan indole NH resonances are shown for the TFE state as an expanded inset.

concentration may effect a change in the dynamics or the compactness of the TFE state.

Characterization of the TFE State of Hen Lysozyme by Hydrogen Exchange Methods. The exchange time course of all amides from the TFE state of hen lysozyme is shown in Figure 6a. The profile is clearly multiphasic, suggesting that some amides exchange significantly more slowly than others. Integration of the amide intensity in the 1D ^{1}H NMR spectra gives an estimate of the sum total of amide hydrogens that remain with full or partial intensity. After 9 min, an intensity corresponding to approximately 84 of the original 127 amides remains. Under the conditions of our experiments (pH 2.0, 27 °C), the half-life for exchange of amides in unstructured model peptides is predicted to be about 3–4 min (Molday et al., 1972; Jeng & Englander, 1991), and, calculated for a random coil of the length and sequence of hen lysozyme, an amide intensity corresponding to 32 protons would be expected to remain after this time. Exchange of some or all amides, therefore, is much slower than expected. A similar exchange profile was obtained when the concentration of lysozyme was diluted 5-fold, confirming that the slowly exchanging amides in the TFE state do not arise as a result of intermolecular association or aggregation.

To examine the effect of TFE on the intrinsic rate of hydrogen exchange, exchange in two unstructured tetrapeptides (Gly-Gly-Tyr-Arg and Ala-Gly-Ser-Glu) in D_2O and in 50% (v/v) TFE-d_3/D_2O was measured at pH 2.0, 27 °C. For these two peptides, the rate of exchange of individual amides in 50% TFE (v/v) is within a factor of 3 of that found in D_2O. Change in the intrinsic exchange rates is, therefore, an unlikely explanation for the considerable retardation of exchange rates observed in the TFE state.

To examine whether amides protected in the TFE state are also protected in the native state of hen lysozyme, the TFE denatured protein was rapidly refolded after different periods of exchange by 6-fold dilution into 20 mM deuterated sodium acetate buffer in D_2O, pH 3.8. Figure 7 shows the 1D ^{1}H NMR spectrum before and after this solvent jump after 2 h of exchange. It is apparent that the great majority (>80%) of the amide intensity is transferred into the native state. Little exchange occurred subsequently over a period of 4 h from the native conformation, indicating that the majority of the amides concerned are also strongly protected from exchange in the

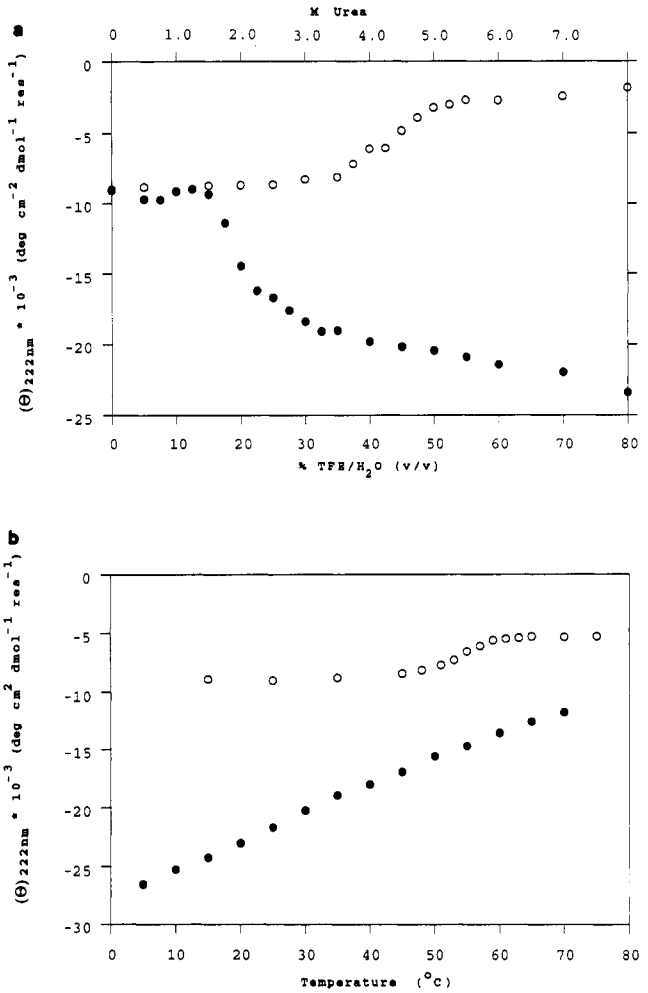

FIGURE 3: Molar residue ellipticity at 222 nm monitoring denaturation of hen lysozyme induced (a) by urea (○) and TFE (●) at pH 2.0, 27 °C, and (b) by temperature in the presence (●) and absence (○) of 50% TFE v/v, pH 2.0, 27 °C.

native protein. It is, therefore, possible to identify the amides which are protected from exchange in the TFE state by jumping the conditions to those favoring rapid refolding to the native conformation and examining the amide cross-peak intensities in phase-sensitive COSY spectra. In this manner, the exchange of individual amide hydrogens in the TFE state can be measured using the known assignments of the native protein (Redfield & Dobson, 1988).

Amide intensities for 66 of the 126 amide hydrogens in hen lysozyme were measured at 9 time points ranging from 3 to 810 min of exchange in the TFE state. The resulting exchange profiles were fitted to single-exponential decays, and the half-lives of exchange are shown and compared with those predicted for amides in unstructured model peptides in Figure 8. Substantial protection from exchange (up to 200-fold) is observed for amides in the TFE state. Such large protection factors cannot be accounted for by the effect of TFE on the intrinsic rates of hydrogen exchange. In accord with this, 17 amides that are highly protected in the native state exchange at rates close to their intrinsic exchange rates in the TFE state. In addition, while the exchange rates for amides of seven asparagine residues could be measured, only two of these were significantly retarded in the TFE state. Similarly, rates of exchange for 7 of the 12 alanine residues could be measured, but only 5 were significantly protected in the TFE state. Most significant in this regard, however, is the observation that amides located in several continuous stretches

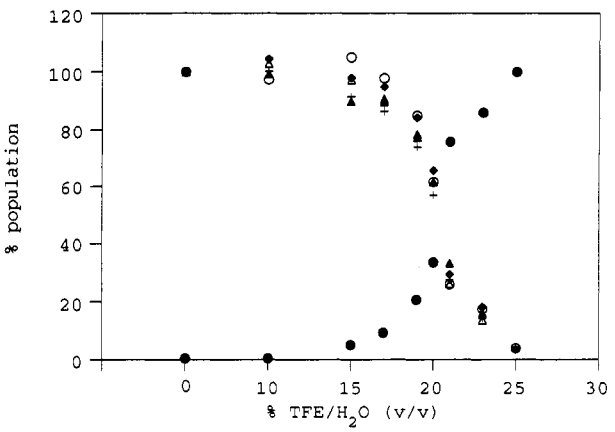

FIGURE 4: Intensity of selected resonances in the 1D ^{1}H NMR spectrum of lysozyme as a function of the concentration of TFE: (△) γCH_3 of Leu-17 (A-helix); (♦) C5H of Trp-28 (B-helix); (+) C5H of Trp-108 (D-helix); (▲) ϵCH_3 of Met-105 (hydrophobic box). The location of these residues in the native structure is given in parenthesis. There is no significant change in line width of these resonances over this range of TFE concentration. Data are also given for the C2H resonance of His-15 in the native (○) and denatured (●) states as populations of the respective state. These values are expressed relative to the peak intensity of the same resonance in the native protein in 100% D_2O or, in the case of His-15 in the denatured state, to the intensity of this resonance when lysozyme is fully TFE-denatured (30% TFE v/v). Errors in estimation of peak intensities are ±5%.

of the polypeptide sequence are protected from exchange. This suggests that these form regions with persistent structure in the TFE state.

The protection factors for amides in the TFE state fall qualitatively into two groups (Figure 9). Significantly protected sites (protection factor exceeding approximately 35) comprise amides in the four α-helices and the short 3_{10}-helix at the C-terminus of the protein and four additional amides (Val-56, Ile-58, Asn-59, and Ile-78). With the exception of the latter four, these amides are all located in the α-domain in the native protein (Figure 9). By contrast, the majority of amides which are protected only weakly lie in the central portion of the polypeptide chain which comprises a triple-stranded β-sheet, a 3_{10}-helix, and a long loop, or are located in loop regions which link helical elements in the α-domain.

Exchange rates calculated for the 66 individual amide hydrogens may be used to examine whether there is protection of amides in the TFE state in regions not protected in the native enzyme. A bulk exchange profile may be constructed by summation of the exchange rates of the individual amides followed. This profile considers only the exchange of the 66 amides which could be measured in the TFE state because they are protected in the native state. At times greater than 100 min, this curve is essentially coincident with that obtained for all 126 amides measured from the bulk exchange experiment in Figure 6a. Thus, amides most highly protected in the TFE state are also protected in the native protein. However, at shorter periods of time (<100 min), there is a significant divergence of the two curves. This could arise from amides that are protected in the TFE state and are only weakly protected or are unprotected in the native protein. The exchange of these amides was monitored selectively in a lysozyme sample in which all of the amides which are significantly protected from exchange in the native state had been selectively deuterated. The exchange profile of the remaining amide hydrogens is shown in Figure 6b, and is compared with the exchange profile calculated for an equivalent number of amides in a random polypeptide of length

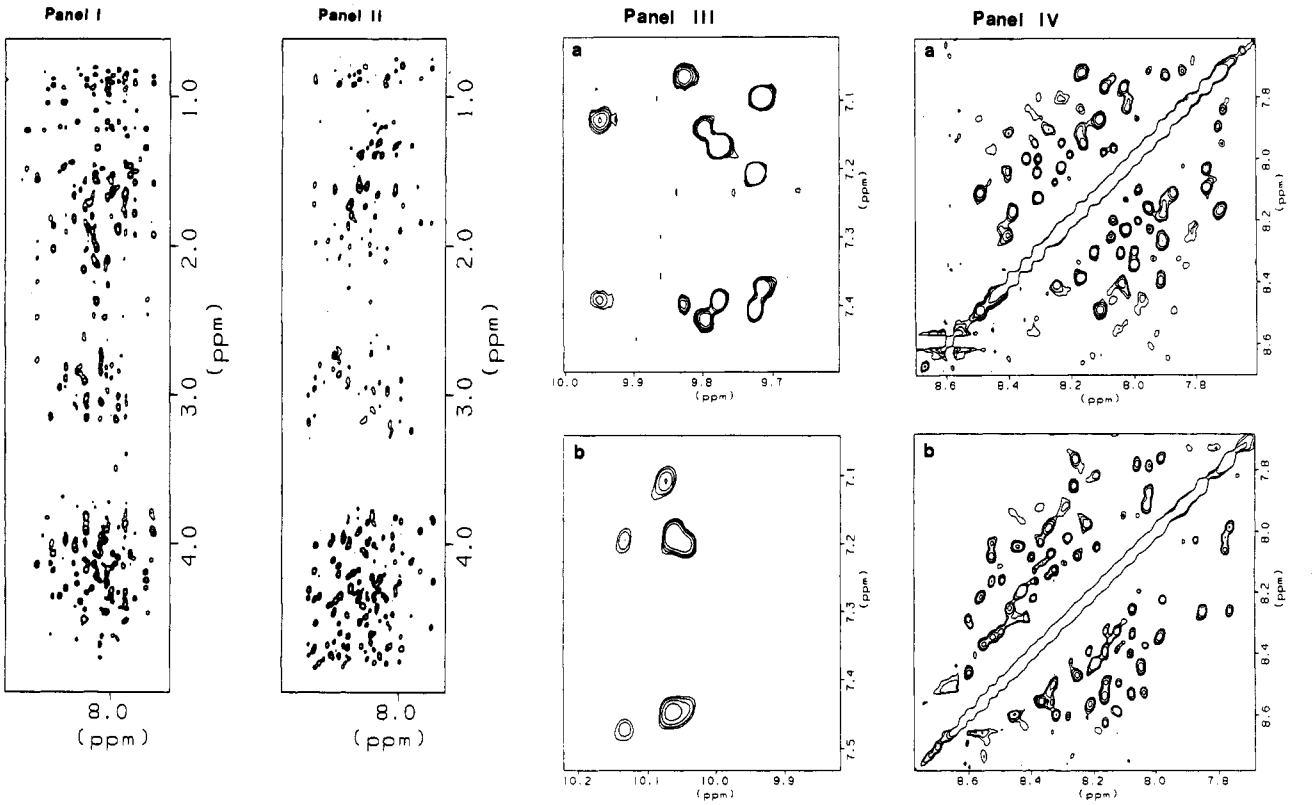

FIGURE 5: NH–C$^{\alpha}$H and NH–side-chain regions of the 2D NOESY spectrum (mixing time 200 ms) of hen lysozyme in 50% TFE-d_2-OH/H_2O (v/v) (panel I) and 8 M urea (panel II) at 27 °C, pH 2.0. The tryptophan indole to Trp C2H/C7H and NH–NH regions of the spectra in 50% (v/v) TFE (a) and 8 M urea (b) are shown in panels III and IV, respectively.

and sequence composition of hen lysozyme. While several amides which are weakly protected or are unprotected in the native state are also weakly or moderately protected in the TFE state, an upper limit of 2–5 can be placed on the sum total of amides that are significantly protected only in the TFE state. Addition of this profile to that calculated for amides protected in both the TFE state and the native protein results in a profile essentially identical to the measured bulk exchange.

The rate of exchange of tryptophan indole hydrogens in the TFE state is also rapid relative to the rates in the native enzyme (Wedin et al., 1982). Even at 10 °C in 50% TFE (v/v), these protons are fully exchanged within 3 min. The rate of exchange for tryptophan indole protons in unstructured peptides under these conditions is about 200 min^{-1} (Nakanishi et al., 1978). Ignoring possible effects of TFE on intrinsic rates, the protection factor of these residues in the TFE state cannot exceed about 300.

DISCUSSION

Denaturation in TFE. Far- and near-UV CD and analysis of 2D ^{1}H NMR spectra have shown that the conformation of native lysozyme is not significantly perturbed by concentrations of TFE up to about 15% (v/v) at pH 2.0, 27 °C (Figures 1, 2, and 3a). Small changes in the near-UV CD and in the ^{1}H NMR chemical shift may be attributed to TFE molecules associating with the protein surface and the active site (Kuramitsu et al., 1972; Lehrman et al., 1986; Lumb & Dobson, 1992). Predenaturation of the protein surface and local multistate unfolding in TFE proposed by Galat (1985) were not seen under the conditions of our experiments. A major unfolding transition, however, occurs between 15 and 30% (v/v) TFE. This transition is cooperative, but increasing the concentration of TFE above 30% (v/v) causes a further increase in ellipticity in the far-UV CD. Disruption of the secondary structure in the TFE state of hen lysozyme on increasing the temperature is characterized by a broad approximately linear transition, suggesting that the TFE state may represent an ensemble of related conformational substates in dynamic equilibrium with each other.

Secondary Structure in the TFE State. The far-UV CD spectrum of the TFE state of hen lysozyme is characterized by a negative ellipticity at 222 nm of more than twice that of the native enzyme (Figure 1). Ellipticity at this wavelength is thought to reflect predominantly the α-helical structural content of proteins although aromatic residues and disulfide bonds could also contribute significantly to the far-UV CD spectrum (Manning & Woody, 1989; Siligardi et al., 1992). Several possibilities for this increase of negative ellipticity may be considered. The first possibility is that there is a significant positive contribution to the circular dichroism at 222 nm from aromatic residues or disulfide bonds in the native state of hen lysozyme; loss of such tertiary structure in the TFE state thus could cause an apparent increase in ellipticity at his wavelength. Alternatively, the α-helical content of the protein may increase in the presence of TFE either by extension of existing α-helices, by induction of new α-helices, or by an increase in the regularity of the helices in the presence of reduced tertiary interactions (Manning et al., 1988). In the native enzyme, approximately 44 of the 129 residues are involved in α-helical structures as judged by the magnitude of the ellipticity at 222 nm (Greenfield & Fasman, 1969). To account for the large increase in negative ellipticity in TFE simply in terms of additonal helix, a further 36 residues of the polypeptide chain would have to be accommodated in α-helical structure. It is not clear how this could occur, although TFE could induce α-helical structure in the native 3_{10}-helices, the β-sheet, or the loop regions, particularly the long loop including

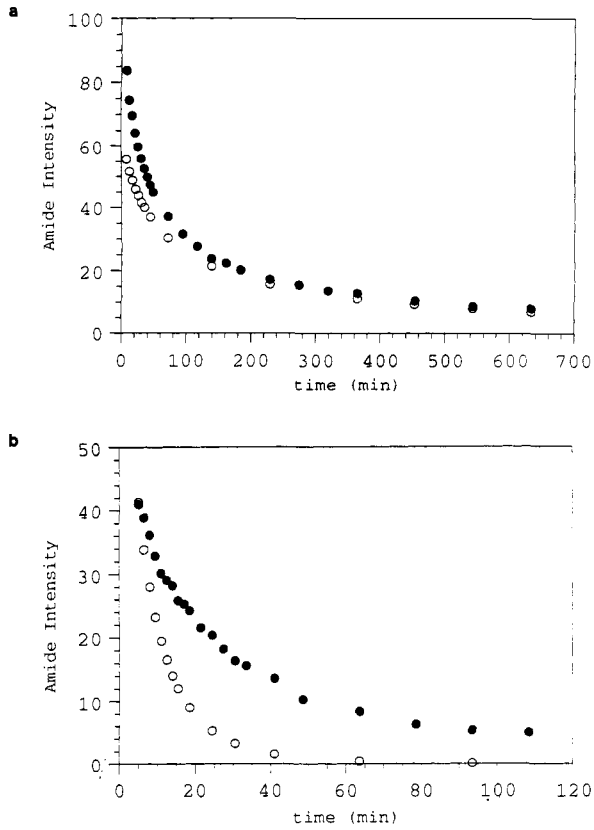

FIGURE 6: (a) (●) Bulk hydrogen exchange from the TFE state and (○) bulk exchange time course of the 66 amides measured individually. (b) (●) Selective bulk exchange of rapidly exchanging amide hydrogens in the TFE state and (○) exchange time course computed for the 127 amide hydrogens of hen lysozyme exchanging at their intrinsic rates. The exchange of amide hydrogens into 50% TFE/D_2O (v/v), pH 2.0, 27 °C, was measured in each experiment. Errors in the integration of peak intensities are <±10%.

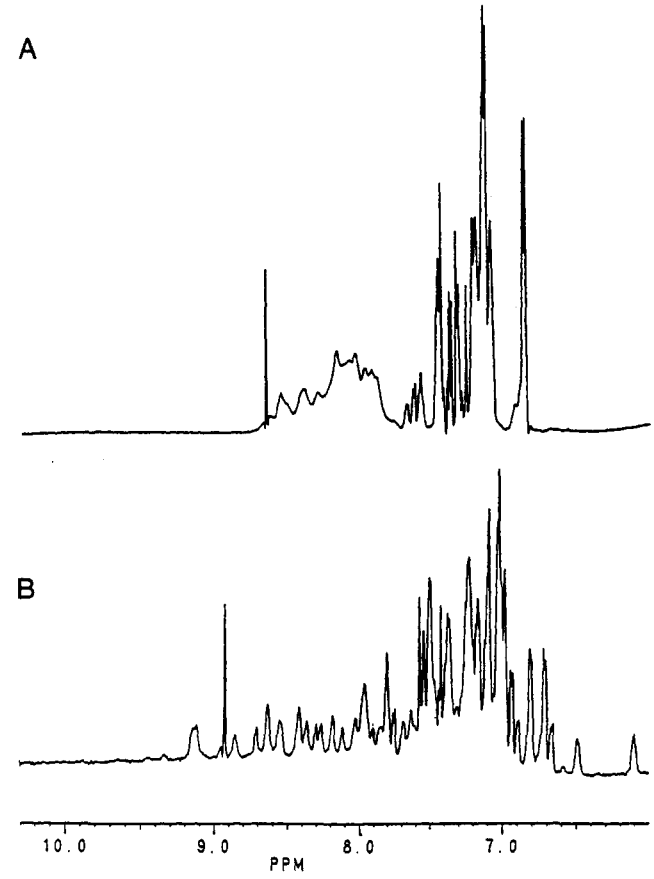

FIGURE 7: (A) Amides remaining in the 1D ^1H NMR spectrum after 2 h in the presence of 50% TFE-d_3/D_2O (v/v), pH 2.0. (B) Amide intensity transferred to the native-state spectrum after refolding of sample (A).

residues 61–78. Interestingly, in human α-lactalbumin, the molten globule state also shows an increase (of about 40%) in negative ellipticity relative to that of the native state (Ewbank & Creighton, 1991), although in the molten globule states of several other proteins an ellipticity equal to or less than that of the native state is observed (Dolgikh et al., 1981; Hughson et al., 1990; Jeng & Englander, 1991).

Results similar to those described here for the TFE state of hen lysozyme have also been observed for other proteins in organic solvents (Harding et al., 1991; J. Baum, personal communication; P. A. Evans, D. N. Woolfson, and D. H. Williams, personal communication). Indeed, several all β-proteins have been shown to adopt α-helical conformations in alcohols (Dufour & Haertle, 1991; Jackson & Mantsch, 1992), and it has been suggested that a peptide corresponding to the β-sheet region of lysozyme may adopt α-helical structure in TFE solution (Segawa et al., 1991). This would support the possibility that at least some additional α-helical structure may be induced in lysozyme in the presence of TFE.

Through-space information from nuclear Overhauser effects (NOE's) is extremely useful for identifying the presence of secondary structure in native proteins. In the case of the TFE state of lysozyme, however, the substantial chemical shift overlap in the ^1H NMR spectrum precluded ready assignment of resonances to individual residues. In addition, assignments could not be made using magnetization transfer techniques (Evans et al., 1991) since conditions under which the native and TFE states are interconverting on an appropriate time scale could not be found. Despite this, it is interesting to investigate the number and type of NOE's observed in the NOESY spectrum of the TFE state and to compare these with spectra of other conformational states of lysozyme obtained under similar conditions (Figure 5). About 30 strong NH–NH NOE's are observed in the spectrum of the TFE state. This compares with 66 NOE's of this type observed in the spectrum of the native protein. Considering the extent of helix inferred by CD, this number is much less than expected. However, NOE's may overlap or be weak or missing from the spectrum of the TFE state, for example, because of its high degree of conformational averaging. Furthermore, the presence of a substantial number of NH–NH NOE's cannot, in isolation, be taken as evidence for α-helical structure (Dyson & Wright, 1991). Indeed, a similar number of NH–NH NOE's are also observed in the spectrum of the protein denatured in 8 M urea (Figure 5), a state that appears substantially unfolded when examined by far- and near-UV CD (Figure 1). In the latter case, however, the NOE's are less well dispersed and of lower intensity than in the spectrum of the TFE state and presumably arise from very localized conformational preferences in the denatured protein. This is supported by the reduced number and intensity of NH–side-chain NOE's observed in the spectrum of the urea-denatured protein (Figure 5). The present NMR studies cannot provide a detailed description of structure in the TFE state; they are, however, consistent with the presence of at least some residual structure in this denatured state of lysozyme.

A third technique widely used to detect persistent secondary structure in peptides and partially folded or native proteins involves the measurement of the rate of amide hydrogen exchange (Roder, 1989). Amide hydrogens involved in

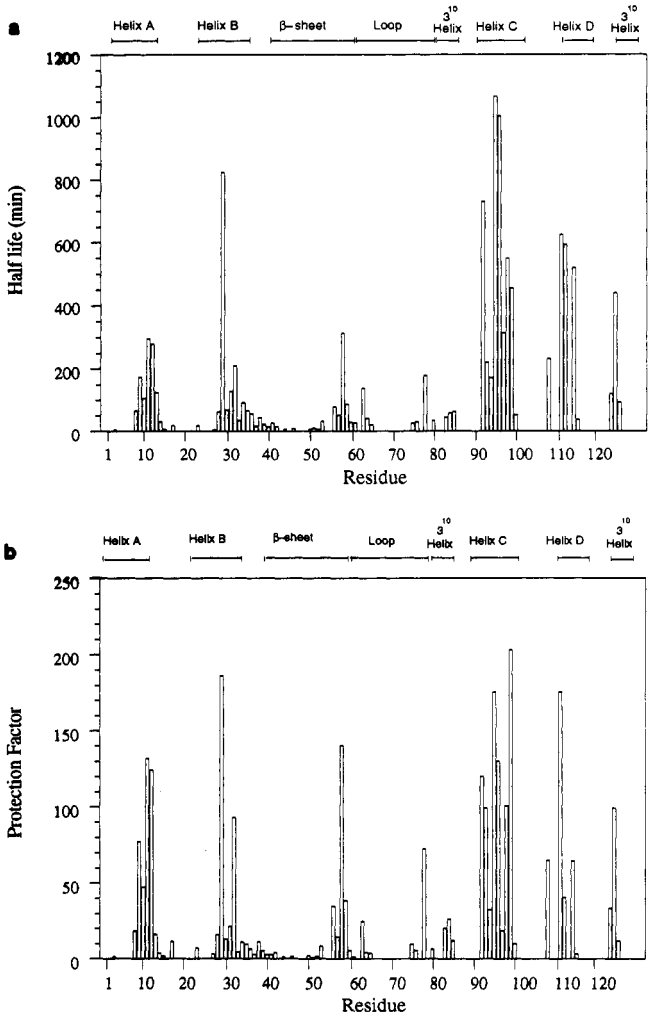

FIGURE 8: (a) Half-lives of amides protected in the TFE state of hen lysozyme versus the protein sequence. (b) Protection factors (uncorrected for TFE effects) of amides protected in the TFE state. Regions of secondary structure in the native state are shown.

secondary structure or deeply buried within the protein core show relatively slow exchange with solvent deuterons (Woodward, 1982; Englander & Kallenbach, 1984). The profile of bulk exchange measured for all of the amides in the TFE state resembles closely that calculated from the measured exchange rates of the 66 amide hydrogens for which site-specific rates of exchange could be determined (Figure 6a). This clearly demonstrates that the large majority of amides strongly protected from exchange in the TFE state are also protected from exchange in the native enzyme. It follows that any novel structure in the TFE state (as viewed by far-UV CD) does not significantly protect amides from exchange. By contrast, about 25 amides are significantly protected in both the native and the TFE states. Interestingly, the β-sheet of lysozyme shows little protection from exchange in the TFE state despite the fact that some structure is stabilized by TFE in a peptide fragment corresponding to this region in the native enzyme (Segawa et al., 1991). Clearly, any non-native structure in this region of the protein in the TFE state is not sufficiently persistent to be detected by the hydrogen exchange methods used here.

The largest protection factors of amides in the TFE state are about 200. These values are much smaller than those for the same amides in the native protein, which range from 10^5 to 10^7 (Radford et al., 1992a). They also contrast with the exchange behavior observed for amides in the acid-denatured state of CM^{6-127}lysozyme and for lysozyme denatured ther-

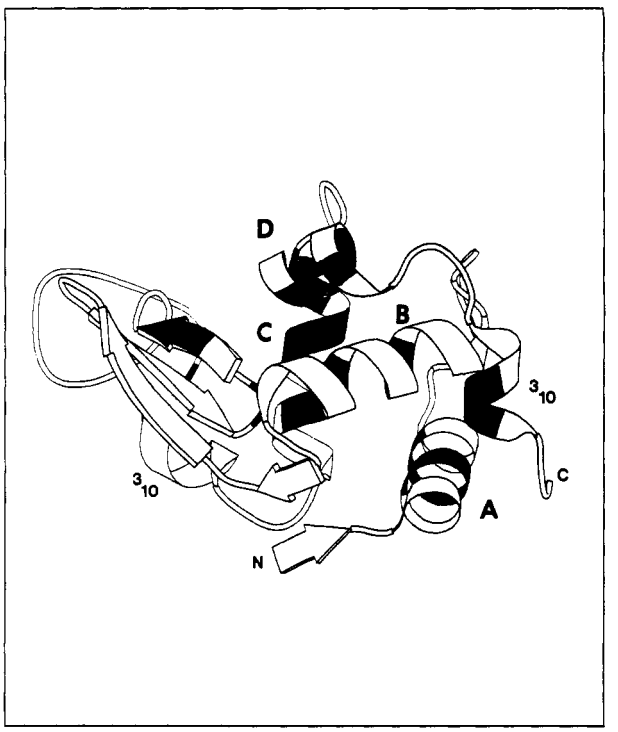

FIGURE 9: Schematic representation of the conformation of native lysozyme. Regions of the polypeptide chain with amide protection factors exceeding 35 in the TFE state are shaded. This figure was drawn using the program MolScript (Kraulis, 1991).

mally or by 8 M urea; in these cases, the exchange rates for the majority of amides resemble closely those expected for an unstructured polypeptide (Radford et al., 1992a; Buck, unpublished data). The magnitude of protection factors in the TFE state is similar to those reported for the molten globule states of several proteins (Baum et al., 1989; Hughson et al., 1990; Jeng et al., 1991; C.-L. Chyan, C. Wormald, C. M. Dobson, P. A. Evans, and J. Baum, unpublished results). The pattern of protection in the TFE state resembles, at least in outline, that of the molten globule state of α-lactalbumin (Baum et al., 1989; C.-L. Chyan, C. Wormald, C. M. Dobson, P. A. Evans, and J. Baum, unpublished results) and a transient intermediate detected in the refolding of hen lysozyme (Miranker et al., 1991; Radford et al., 1992b). Thus, the β-sheet region of lysozyme and the 3_{10}-helix involving residues 80–85 are not appreciably protected in any of these three states. Several similarities and differences between these states may also be noted. The four α-helices and the C-terminal 3_{10}-helix are all highly protected in the transient intermediate, but the B-helix is only weakly protected in the TFE state. Interestingly, little α-helical structure is found in a peptide corresponding to the hydrophobic B-helix of lysozyme in TFE (Segawa et al., 1991). This suggests that TFE stabilizes α-helical structure only weakly in hydrophobic amino acid sequences (Lehrman et al., 1990; Segawa et al., 1991). In accord with this, a small cluster of residues involving Trp-63, Cys-64, and Ile-78, which form a hydrophobic patch on the surface of the native lysozyme molecule, is protected in the refolding intermediate, but only one of these residues (Ile-78) is protected in the TFE state. Three residues in the β-sheet domain, Val-56, Ile-58, and Asn-59, are protected in the TFE state alone. These amides are in close proximity and could suggest that this region of the protein may be structured in some manner in the TFE state. Alternatively, the apparent protection in this region may arise from limitations in the Molday factors reported for these residues (Robertson & Baldwin, 1991), especially in TFE solutions.

Side-Chain Ordering in the TFE State and Its Role in Stabilizing Secondary Structure. The presence of significant residual chemical shift dispersion in the ^{1}H NMR spectrum of the TFE state suggests that a number of side-chain interactions persist in this state. Specifically, resonances of one tryptophan and possibly one other aromatic residue as well as resonances of several aliphatic side chains are significantly perturbed from random-coil chemical shift values. Dispersion of several resonances has been observed in spectra of lysozyme denatured thermally and by urea (Evans et al., 1991) although the number of resonances with significant chemical shift perturbation and the magnitude of the induced shifts are smaller in the latter two states. The presence of a large number of intense NH–side-chain and NH–aromatic NOE's in the spectrum of the TFE state suggests that there are a number of long-range interactions involving side chains in this state. However, at least the majority of the structured regions in the TFE state must lack specific and persistent tertiary interactions involving aromatic side chains which would give rise to an intense near-UV CD spectrum. The nonpersistent nature of side-chain interactions in the TFE state is supported by the observation that the protection factors of tryptophan indole protons and of amides located in regular elements of secondary structure are less than about 300. This is in contrast to the more substantial protection of these protons observed in the native protein and for one tryptophan and several amides residues in an early kinetic refolding intermediate of lysozyme (Radford et al., 1992a,b). This suggests that side-chain interactions in the TFE state are fewer in number and are less persistent than those in the native protein and the kinetic folding intermediate.

The role of side-chain interactions in stabilizing structure against hydrogen exchange is difficult to define. In the native protein, side-chain interactions may influence the rate of amide exchange by burial of amides in the protein interior, by distortion of secondary structure in hydrophobic regions, or by local tertiary restraints on dynamics (Goodmann & Kim, 1991; Radford et al., 1992). In linear peptide systems, favorable side-chain interactions are presumed to slow hydrogen exchange by stabilizing secondary structure directly (Rohl et al., 1992). The situation in partially folded conformations of intact proteins is likely to be complicated and may be intermediate between these two extremes.

The four α-helices in the TFE state are not protected to equal extents; the C- and D-helices are protected more strongly than the A- and B-helices in this denatured state. By contrast, in the native protein, amides in the A- and B-helices have some of the highest protection factors observed (Radford et al., 1992a). Interestingly, little structure is induced by TFE in a 32-residue peptide fragment of lysozyme corresponding to the C-, D-, and C-terminal 3_{10}-helices in the native enzyme (Segawa et al., 1991). This suggests that long-range interactions may contribute to the stability of this region of the polypeptide chain in the TFE state. In accord with this, a periodicity in the magnitude of protection is observed for amides in the C-helix in the TFE state. Thus, amides of residues Ser-91, Cys-94, Lys-97, and Ser-98 exchange relatively rapidly compared with the remaining residues in the sequence 91–100. A similar, but clearer, periodicity is seen in the C-helix in the native protein and is attributed to the amphipathic nature of the helix and docking of this helix against the hydrophobic core (Radford et al., 1992a). Retention of this pattern, although reduced in magnitude in the TFE state, suggests that the amphipathic nature of this helix may have been preserved in this state. However, limitations in the application of Molday factors to hydrophobic residues could also contribute to the observed pattern of exchange (Robertson & Baldwin, 1991). Taken together, these observations suggest that side-chain interactions persist in the TFE state but neither their extent, their location nor their dynamic behavior can be inferred from the present experiments.

Implications for Protein Folding. It is now clear that during the refolding of proteins from denaturing media, secondary structure, particularly helical structure, is formed rapidly, often within the first few milliseconds of folding (Kuwajima, 1989; Roder, 1989; Dill & Shortle, 1991; Radford et al., 1992b). During refolding, collapse of the protein structure may be concomitant with, or may precede, such secondary structure formation. The environment produced by this collapse is likely to be hydrophobic in nature and may help to generate or stabilize secondary structure. If the interactions generated during the collapse are relatively nonspecific, it is possible that the secondary structure formed in mixed organic/aqueous solvents may be related to that formed during the early stages of folding. If this is the case, the present finding that the most persistent regions of structure in the TFE state are a subset of those of the native structure could well be of significance in understanding protein folding. In the case of lysozyme, the majority of amides protected in TFE are in segments of the protein which are helical in the native state. This is consistent with studies of peptide fragments of proteins which show that helical structure is stabilized preferentially in regions helical in the native protein (Segawa et al., 1991; Dyson & Wright, 1992). The experimental strategy used here, however, does not enable us to determine directly whether the persistent structure in the TFE state is also helical, but, particularly in light of the far-UV CD data, it is certainly consistent with this supposition.

One intriguing question raised in this work is the origin of ellipticity in the far-UV region generated in TFE. In the case of lysozyme, if new helices are formed they are clearly relatively unstable and do not protect the amides concerned from hydrogen exchange. This observation could be of relevance for folding because it suggests how pathways of folding might originate. For example, if native-like secondary structure is preferentially stabilized in nonaqueous environments, then there will be a higher probability that following hydrophobic collapse native-like higher structural elements or domains rather than non-native structures will form. In this manner, folding could be directed toward native rather than non-native structures. This does not exclude the possibility, however, that in some proteins formation or non-native structure could be significant in folding; it is possible, for example, that helical structure formed initially might subsequently be transformed into β-sheet structure later in the folding process. It is interesting in this regard that the denatured state of several predominantly-β proteins in organic solvents contains significant α-helical structure (Baum et al., personal communication; Woolfson et al., personal communication; Dufour & Haertle, 1991; Jackson & Mantsch, 1992).

Support for the notion that denatured states such as that described here may be of significance in understanding protein folding comes from the relationship of the pattern of hydrogen exchange protection observed in the TFE state to that observed for an intermediate sampled during kinetic refolding of lysozyme from GuHCl. Furthermore, the pattern of exchange is also similar to that found in the stable partly folded (molten globule) state of the homologous protein α-lactalbumin formed at low pH, that has been related to transient intermediates of

both proteins (Kuwajima et al., 1985; Radford et al., 1992b). All of these states have highly protected amides centered in regions that form α-helices in the native state, but none in regions that form the β-sheet, an observation that has led to the idea of "folding domains" within the protein structure (Miranker et al., 1991; Radford et al., 1992b). The degree of protection from exchange (>500-fold) is significantly greater in the kinetic intermediate of lysozyme than in the TFE state of this protein or in the acid-denatured state of α-lactalbumin (Baum et al., 1989; C.-L. Chyan, C. Wormald, C. M. Dobson, P. A. Evans, and J. Baum, unpublished results). We believe, however, that this may well be due to the short lifetime of the former, leaving insufficient time for major conformational fluctuations that can expose amides and give rise to exchange prior to trapping in the folded state of the protein.

Although much remains to be learned about proteins denatured in TFE, the finding in the present study that addition of this cosolvent stabilizes predominantly native-like secondary structure in a partially folded state of an intact protein is of considerable significance. It provides support, for example, for the idea that studies of peptide fragments of proteins in TFE (Segawa et al., 1991; Dyson et al., 1992) can have direct relevance to understanding the structure and stability of protein folding intermediates. It also gives rise to the possibility that TFE might be of practical use in providing a favorable environment for the renaturation of proteins from their aggregated states; this is presently under investigation in our laboratory. Finally, in a more general context, the present results support the proposition that an increasingly wide range of stable denatured states can be generated and that their detailed study and comparison of their properties with those of peptide fragments and transient kinetic intermediates should be of considerable value in efforts to understand mechanisms of protein folding on a structural level.

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