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Hexafluoroacetone hydrate as a structure modifier in proteins: Characterization of a molten globule state of hen egg-white lysozyme

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Abstract

A molten globule-like state of hen egg-white lysozyme has been characterized in 25% aqueous hexafluoroacetone hydrate (HFA) by CD, fluorescence, NMR, and H/D exchange experiments. The far UV CD spectra of lysozyme in 25% HFA supports retention of native-like secondary structure while the loss of near UV CD bands are indicative of the overall collapse of the tertiary structure. The intermediate state in 25% HFA exhibits an enhanced affinity towards the hydrophobic dye, ANS, and a native-like tryptophan fluorescence quenching. 1-D NMR spectra indicates loss of native-like tertiary fold as evident from the absence of ring current–shifted ^{1}H resonances. CD, fluorescence, and NMR suggest that the transition from the native state to a molten globule state in 25% HFA is a cooperative process. A second structural transition from this compact molten globule-like state to an "open" helical state is observed at higher concentrations of HFA (≥50%). This transition is characterized by a dramatic loss of ANS binding with a concomitant increase in far UV CD bands. The thermal unfolding of the molten globule state in 25% HFA is sharply cooperative, indicating a predominant role of side-chain–side-chain interactions in the stability of the partially folded state. H/D exchange experiments yield higher protection factors for many of the backbone amide protons from the four α-helices along with the C-terminal 3_{10} helix, whereas little or no protection is observed for most of the amide protons from the triple-stranded antiparallel β-sheet domain. This equilibrium molten globule-like state of lysozyme in 25% HFA is remarkably similar to the molten globule state observed for α-lactalbumin and also with the molten globule state transiently observed in the kinetic refolding experiments of hen lysozyme. These results suggest that HFA may prove generally useful as a structure modifier in proteins.

Keywords: CD; fluorescence; hexafluoroacetone; lysozyme; molten globule states; NMR; protein folding

A detailed understanding of protein folding pathways requires structural characterization of both folded structures and stable intermediate states. While native protein structures are extremely well defined by X-ray and NMR studies, non-native intermediate states have proved less amenable to structural analysis (Dobson, 1992; Shortle, 1996). A major issue is the stabilization of non-native structures under equilibrium conditions. Modulation of protein structures by site-directed mutagenesis (Hughson et al., 1991; Chen et al., 1992; Sanz & Fersht, 1993) or by appropriate engineering of the environmental conditions (Buck et al., 1993; Kamatari et al., 1996) appear to be viable strategies for stabilization of non-native states.

Fluoroalcohols have been shown to exhibit remarkable structure stabilizing effects for peptides in aqueous solution (Nelson & Kallenbach, 1989; Bruch et al., 1992; Dyson et al., 1992; Sonnichsen et al., 1992). Enhancements of secondary structure content upon addition of fluoroalcohols have also been demonstrated in a large number of proteins (Alexandrescu et al., 1994; Buck et al., 1995; Shiraki et al., 1995). 2,2,2,-Trifluoroethanol (TFE) is the most widely investigated member of this class of structure-modulating additives. The structure-stabilizing property of fluoroalcohols has been proposed to arise from two important characteristics, namely, the hydrophobicity of the fluoroalkyl (CF3) group and the strong hydrogen bond–donating/poor hydrogen bond–accepting property of the hydroxyl (OH) group (Goodwin et al., 1996; Rajan & Balaram, 1996). Hexafluoroacetone trihydrate (HFA, hexafluoropropan-2,2-diol) (Goodman & Rosen, 1964; Longworth, 1964) is a far more potent structure inducer in peptides (Rajan et al., 1997) but has not been investigated in detail in the case of proteins. HFA (Fig. 1) is a potentially amphiphilic molecule possessing a highly

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Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; ANS, 1-anilino-8-naphthalene-sulfonate; TEF, 2,2,2-trifluoroethanol; HFA, hexafluoroacetone hydrate; GdmCl, guanidinium chloride.

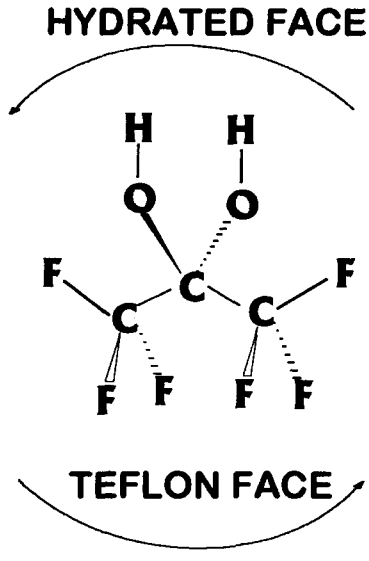

Fig. 1. Structure of hexafluoroacetone hydrate (HFA), showing the hydrophobic fluorocarbon ("teflon") face and the hydrogen bond-donating hydrated face. For properties of HFA see Murto et al. (1971).

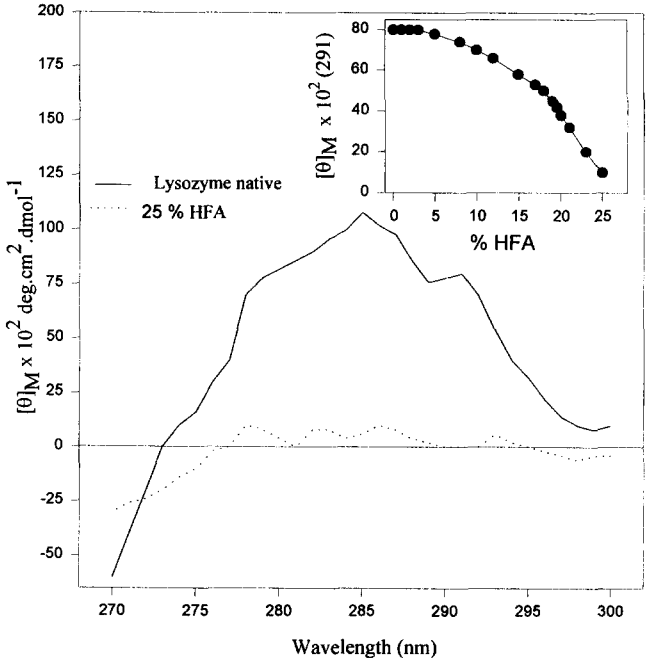

Fig. 2. Near UV CD spectra of lysozyme in the native state in aqueous solution and in 25% HFA. **Inset**: Dependence of near UV CD ellipticity at 291 nm on HFA concentrations. Protein concentration 60 μM in H_2O, pH 3.0.

hydrophobic fluorocarbon ("teflon") face and a hydrophobic hydrogen bond donating, hydrated face. These structural features have been suggested to be critically important in the mechanism of HFA-induced secondary structure stabilization in peptides (Rajan et al., 1997).

As part of a program to systematically investigate the effects of organic solvents on protein structures we have examined the structure of hen egg-white lysozyme (HEWL) in aqueous HFA. We describe the characterization of a molten globule-like state in 25% aqueous HFA. This partially folded state has the following structural features: Extensive native-like secondary structure and loss of tertiary structure as judged by far and near UV CD, respectively; exposure of hydrophobic residues as evident by hydrophobic dye (ANS) binding; absence of ring current–shifted ^{1}H NMR resonances, demonstrating loss of native-like tertiary interactions; native-like tryptophan fluorescence quenching by ionic quenchers and slow exchange of indole protons, indicative of a compact state; cooperative thermal unfolding transitions indicating the stabilization of the molten globule state by hydrophobic interactions; and persistent structure in the helical domain with the preferential unfolding of the β-sheet domain, suggested by protection factors measurement from H/D exchange experiments.

The present observations are of significance since an equilibrium non-native state with molten globule-like characteristics has not been demonstrated for hen lysozyme, although the equilibrium molten globule state of the homologous protein, α-lactalbumin, is among the best characterized (Baum et al., 1989; Kuwajima, 1989; Alexandrescu et al., 1993).

Results

Circular dichroism

Figure 2 compares the near UV CD of native lysozyme with the spectrum obtained in 25% HFA. Strong CD bands at 291 nm and 285 nm are assignable to six tryptophans, with shorter wavelength contributions arising from tyrosine residues. At 25% HFA, a dramatic diminution of the near UV CD signal is observed, suggesting a global loss of tertiary structure, resulting in disruption of the asymmetric environment of aromatic chromophores. Examination of the concentration dependence establishes a structural transition centered at ~15% HFA, which is essentially complete at 25% HFA. Interestingly, addition of HFA results in an intensification of the far UV CD bands (208 nm, 222 nm), which largely arise from the helical secondary structure (Fig. 3). This enhancement of signals may arise due to abolition of aromatic contributions to the far UV CD bands, which are positive in sign (Vuilleumier et al., 1993). The CD results suggest that in 25% HFA, lysozyme retains its secondary structure, with a global loss of tertiary structure, a feature characteristic of molten globules (Ptitsyn, 1992).

ANS binding

Figure 4A shows the effect of the addition of HFA on the fluorescence emission spectra of ANS in the presence of lysozyme. Native lysozyme binds to ANS weakly, whereas a remarkable increase in intensity with a considerable blue shift in emission maximum (478 nm) is observed in 25% HFA. Scatchard analysis of ANS binding to lysozyme in 25% HFA yields a K_d value of 6 μM, a binding stoichiometery of 1:1 (data not shown). Further addition of HFA causes a decrease in ANS fluorescence, with dramatic loss of emission intensity in 50% HFA. In the absence of protein, ANS fluorescence shows a very small enhancement with increasing concentration of HFA. Figure 4B compares the effect of HFA on the lysozyme far UV CD band intensity and the ANS fluorescence intensities. ANS fluorescence increases at low HFA concentrations with a maximum being reached at 20–25% HFA. At higher concentrations, there is a sharp fall in intensity with almost complete loss of fluorescence by 50% HFA. This may be interpreted in terms

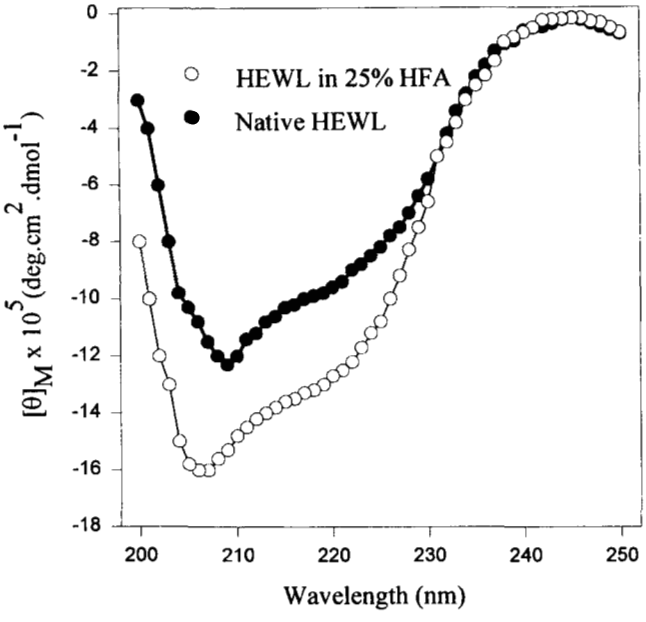

Fig. 3. Far UV CD spectra of lysozyme in the native state in aqueous solution and in 25% HFA. Protein concentration 20 μM, pH 3.0.

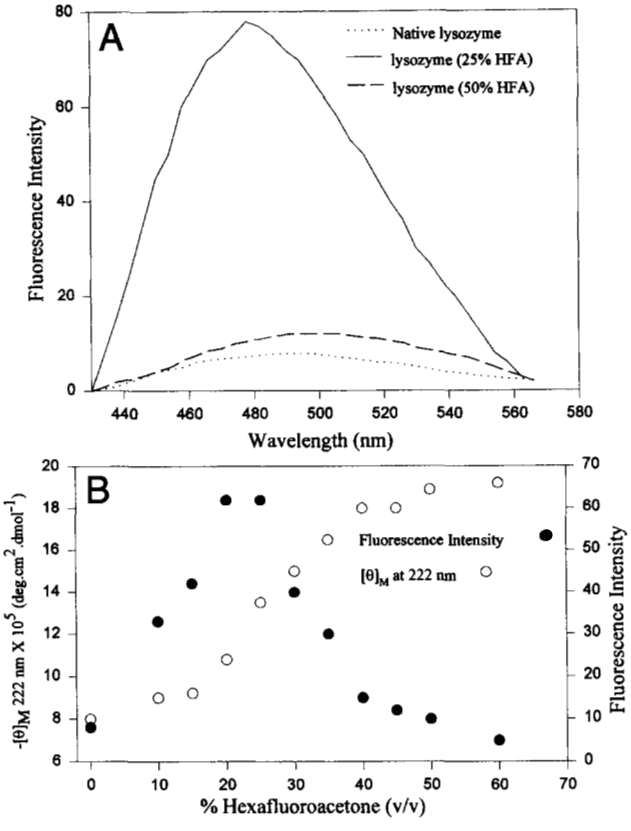

Fig. 4. A: Emission spectra of ANS in the presence of lysozyme in 0%, 25%, and 50% HFA. Protein concentration, 10 μM, ANS concentration 20 μM, pH 3.0. The excitation wavelength for ANS was 375 nm. **B:** Plot indicating changes in molar ellipticity of lysozyme at 222 nm and changes in ANS fluorescence intensity in the presence of lysozyme at different concentrations of HFA. Protein concentration was 20 μM, pH 3.0 for CD experiments. ANS concentration 20 μM and protein concentration 10 μM, pH 3.0 for fluorescence experiments.

of a loss of compactness in the molten globule state upon increasing HFA concentration, resulting in diminished ANS binding. Interestingly, the far UV CD bands show an enhancement in intensity at higher HFA concentration suggestive of stabilization of helical secondary structure. Increased secondary structure appears to coincide with the loss of ANS binding, supporting a transition from a molten globule state in 25% HFA to a non-compact state at higher HFA concentration. It is noteworthy that the molten globule state of α-lactalbumin at low pH also exhibits a loss of ANS binding with concomitant increase in helical structure at increasing concentration of TFE (Alexandrescu, et al., 1994). The non-compact state of lactalbumin in 50% TFE is presumably analogous to the state observed for lysozyme in 50% HFA. These observations suggest a strong affinity of the fluoroalcohols for the partially folded state of proteins, presumably facilitated by interaction with the exposed non-polar residues, causing a dramatic loss in hydrophobic probe binding and induction of helical structure. The TFE denatured states of many proteins have been recently investigated by Shiraki et al. (1995), who suggest population of "open" intermediate states with extensive helical structures at high concentrations of TFE.

Taken together, the observed loss of tertiary structure, persistent secondary structure, and exposure of hydrophobic residues as shown by near UV CD, far UV CD, and ANS fluorescence, respectively, strongly suggest a molten globule-like state of lysozyme in 25% HFA, which is further structurally characterized, below.

Thermal transition of lysozyme in 25% HFA

Figure 5 summarizes the temperature dependence of the near UV and far UV CD bands of lysozyme in 25% HFA. The near UV CD (270–300 nm) ellipticity is appreciable at low temperatures (<10 °C), with complete loss of ellipticity by 25 °C. The broad thermal transition centered at approximately 15 °C corresponds to a loss of tertiary structure. In sharp contrast, the far UV CD bands (200–300 nm) diminish in intensity, both at lower and higher temperature. The high temperature transition centered at approximately 55 °C corresponds to loss of secondary structure which accompanies thermal unfolding. Native lysozyme shows a sharp thermal transition at 77 °C (Dobson & Evans, 1984; Privalov, 1992). Thus, the 25% HFA state of lysozyme is appreciably less stable than the native structure. The relatively sharp high temperature transition is suggestive of cooperative unfolding of the intermediate state.

The anomalous loss of ellipticity of the far UV CD bands at low temperatures is likely to be a consequence of aromatic contributions of opposite sign in the region of 200–230 nm. The intensification of the CD bands at 270–300 nm on cooling, suggests an ordering of the internal Trp, Tyr residues, which will also result in a corresponding increase in aromatic contributions to the CD bands at lower wavelength. The possibility that the low temperature effect observed in Figure 5, may result from cold denaturation from the molten globule state (Kuroda et al., 1992; Nishii et al., 1994) may be discarded because of the opposing temperature dependence of the far and near UV CD bands. These observations emphasize the ambiguity inherent in interpretation of far UV CD data, when aromatic contributions are ignored (Chakrabartty et al., 1993, Vuilleumier et al., 1993).

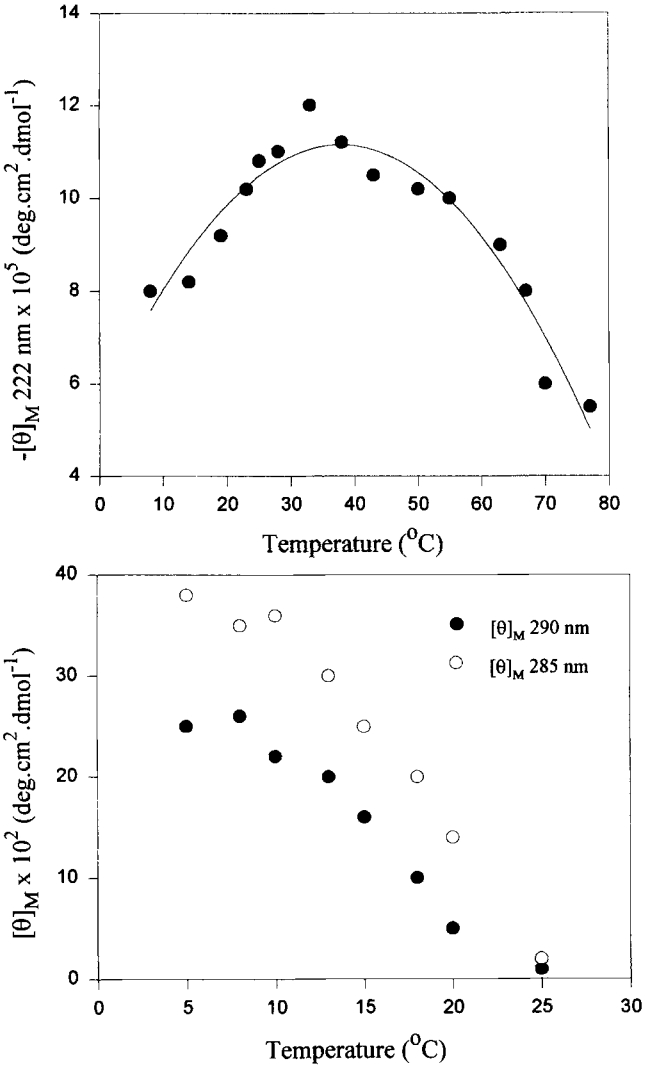

Fig. 5. Temperature dependence of far UV (top) and near CD (bottom) of lysozyme in 25% HFA, pH 3.0 monitored at 222 nm, 285 nm and 290 nm. Protein concentration 20 μM and 60 μM for far and near UV CD measurements respectively.

The cooperative thermal transition at higher temperatures from the intermediate state of lysozyme in 25% HFA, indeed suggests a predominant role of inter-side-chain interactions in determining the stability of the partially folded state. The thermal denaturation of many molten globule states are known to be cooperative, e.g., apo-myoglobin, (Nishii et al., 1994), cytochrome *c* (Kuroda et al., 1992), and retinol binding protein (Bychkova et al., 1992), suggesting an ordered organization of the non-polar residues (Freire, 1995).

Iodide quenching of tryptophan fluorescence

The quenching of tryptophan fluorescence is a useful probe of fluorophore exposure in protein structures. Figure 6 shows a modified Stern-Volmer plot of the quenching of lysozyme in its native state, in 25% HFA and 6 M GdmCl. The quenching behavior of native lysozyme and the 25% HFA state are practically identical. In native lysozyme, out of six tryptophan residues, two, W62 and

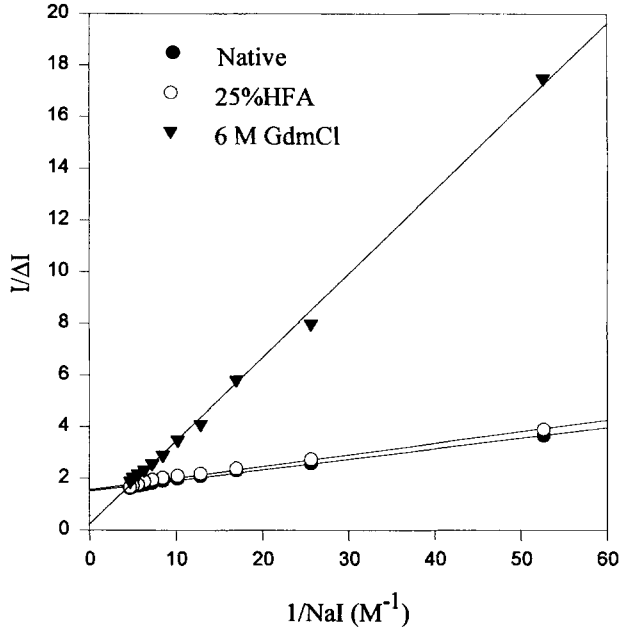

Fig. 6. Modified Stern-Volmer plot of the quenching of tryptophan fluorescence of lysozyme in three different structural states by iodide. NaI concentrations ranged from 0.019 M to 0.02 M. Protein concentration 10 μM, pH 3.0, excitation wavelength 295 nm.

W63, are completely solvent-exposed while the remaining four are buried in the hydrophobic core. These results demonstrate that the tryptophan accessibilities remain largely unaltered in the "molten globule-like" state of lysozyme in 25% HFA. In 6 M GdmCl, complete exposure of the tryptophan residues results in a dramatic enhancement of quenching efficiency in complete agreement with earlier results (Lehrer, 1971). Although disappearance of the near UV CD bands of lysozyme in 25% HFA shows disruption of specific native-like tertiary interactions, fluorescence quenching data strongly suggest that aromatic side chains of the tryptophan residues remain largely buried. This interpretation is further supported by the slow exchange of the indole protons of W28, W108, W111, and W123 in H/D exchange experiments (vide infra). It is noteworthy that iodide quenching and H/D exchange data for α-lactalbumin also suggest that the tryptophan residues are largely solvent-shielded in the low pH molten globule state (Lala & Kaul 1992; Chyan et al., 1993).

NMR studies

Several well separated "probe" resonances can easily be recognized in 1-D NMR spectra of lysozyme following earlier assignments (Redfield & Dobson, 1988). The native lysozyme structure is composed of two structural domains; a helical domain comprising of four major α-helices and a short β-domain with a triple-stranded anti-parallel β-sheet. Resonances are selected from each structural lobe (helix or sheet) to follow unfolding. Figure 7 highlights the effect of HFA on three diagnostic NMR resonances (L17 $C^{\delta}H_3$, W28 C5H, and C64 $C^{\alpha}H$). There is progressive diminution of the intensity of the probe resonances with increasing concentrations of HFA. The decrease in intensity is not a consequence of aggregation, since the line widths are found to be unaffected in titration experiments performed at lower protein concentrations.

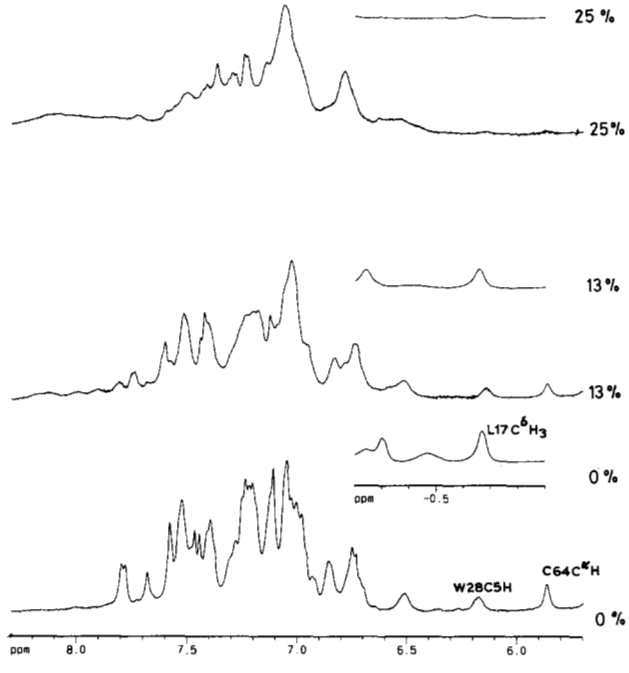

Fig. 7. Partial 1-D NMR spectra of lysozyme in D_2O, pH 3.0, showing intensity change of three selected resonances (marked) as a function of HFA concentration. Protein concentration 5 mM.

There is also no evidence of precipitation. The decrease in intensity of the probe resonances may be ascribed to the conversion of the native state to an unfolded state with slow exchange between the two states on the NMR time scale. The corresponding denatured state peaks of the marked resonances probably merge with the other resonances in the broad envelope and are consequently not observed. The unfolding transition profile monitored by three different resonances are nearly identical, suggesting that denaturation is a single cooperative event, which is virtually completed at 25% HFA with a midpoint around 13% HFA (Fig. 8).

H/D exchange

A more detailed structural characterization of the molten globule-like state of lysozyme in 25% HFA has been attempted using H/D exchange kinetics (Baldwin, 1993). This method allows identification of protons which are solvent-shielded either due to hydrogen bonding in secondary structures or by tertiary contacts with closely placed residues, since the solvent-exposed protons will be replaced by D_2O and hence not observed in ^1H spectra. Therefore, the protection of either backbone NH protons or indole NH protons can provide evidence for persistent secondary structures as well as predominant side-chain–side-chain interactions. H/D exchange has been monitored for 12 different time points ranging from 1 min to 14 h. The exchange of fifty-five amide protons and indole protons of W28, W108, W111, and W123 can be assayed under our experimental conditions. Figure 9 represents sets of 1-D spectra after different time points of exchange. All the exchangeable protons (amide protons, indole protons) show reduction in intensity with increase in time of exchange. The H15 C2H proton resonance is non-exchangeable and does not change in intensity and has been used as the internal standard. Four tryptophan indole resonances could be identified in the 1-D spectra according to previous assignments (Redfield & Dobson 1988). All of the four indole protons exchange slowly in the 25% HFA state. Particularly, W28 and W123 indole protons can be seen clearly even after 14 hours of exchange. These indole protons are among the slowest exchanging protons from the native state of lysozyme as a result of hydrogen bonding or burial inside the hydrophobic core of the protein (Radford et al., 1992a). The amide backbone protons are not resolved in 1-D spectra; however they are distinguished in 2-D COSY spectra. Four 2-D COSY spectra are shown in Figure 10 recorded after 1 min, 10 min, 5 h, and 14 h of exchange. It is apparent that many of the β-sheet protons (D52, N39, T40, N46, W63, C64, C76) exchange rapidly compared to the amide protons of helical origin. Some of the β-sheet protons which are present in the native state spectra exchange too fast to be monitored in 25% HFA. Protection factor analysis summarizes the results of exchange experiments (Fig. 11). It is pertinent to know that, even at a concentration of 25% HFA, a low concentration of the native state (~5%) is populated (Fig. 7, 8). The existence of an equilibrium between the molten globule intermediate and the native state would imply that observed protection factors will be influenced, albeit to a low extent. Since the rate of refolding from the molten globule state is likely to be significantly higher than the rate of H/D exchange, it is likely that protection factors may be slightly overestimated. However, the dramatically low protection factors for the β-sheet region suggest that this complicating feature is relatively unimportant.

Protection factors range typically from 10–100 for most molten globule states studied so far (Jeng et al., 1990; Hughson et al., 1992; Shulman et al., 1995). The protection factors for lysozyme in 25% HFA are within this range for many of the amide protons from helix A (A10, A11, M12, K13, and H15), helix B (N27, W28, V29, A32, and E35), helix C (V92, A95, V99, and K97), helix D (W108, W111, and C115), and the C-terminal 3_{10} helix (W123 and R125).

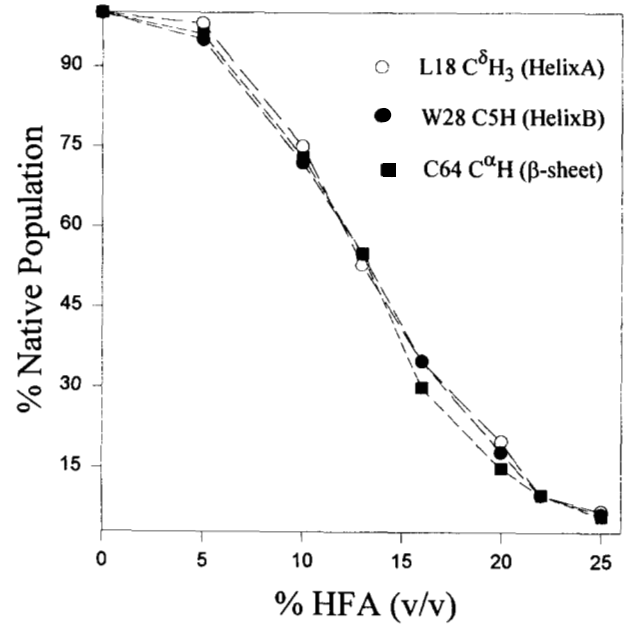

Fig. 8. Change in native state population of lysozyme as monitored from the intensity change of three selected probe resonances as a function of HFA concentration. The reduction in native state population is measured from the integration of the peak area from 1-D NMR spectra and normalized relative to the native state spectra recorded in D_2O. Protein concentration 5 mM in D_2O at pH 3.0.

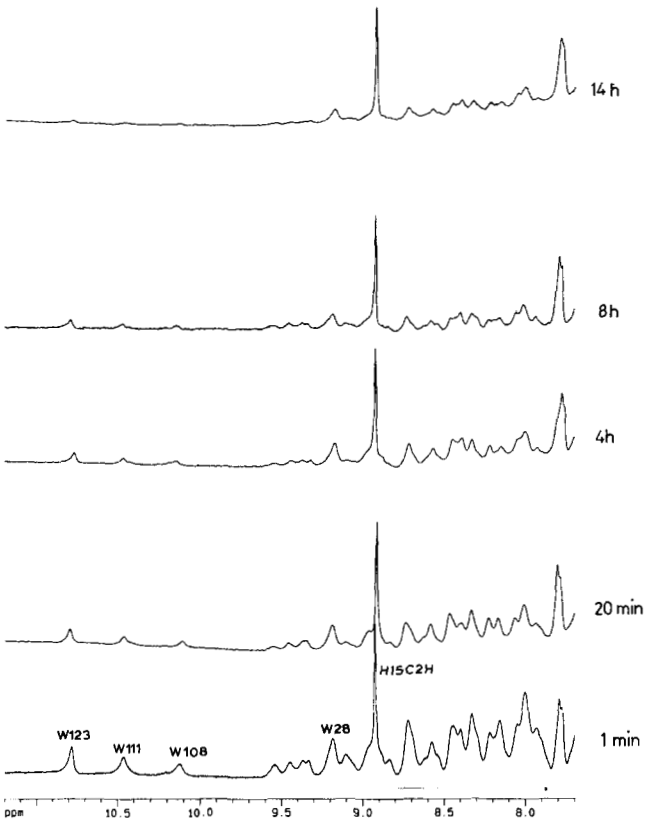

Fig. 9. The low field region of the 1-D NMR spectra of lysozyme in D_2O, pH 3.0 after refolding at different time points from 25% HFA, indicating slowly exchangeable indole resonances and amide protons. Protein concentration 5 mM.

In contrast, the triple stranded antiparallel β-sheet domain shows markedly lower protection factors (<5). This clearly demonstrates that the helical domain structure is largely preserved in the molten globule state, with unfolding of the β-sheet. Protection of the backbone amide protons of the helical domain are suggestive of native-like secondary structure, whereas protection of four side-chain indole protons (W28, W108, W111, and W123) from the helical domain are consistent with persistent tertiary interactions. In the native structure of lysozyme all the four tryptophan residues W28, W108, W111, and W123 are buried inside the helical core. The W111 indole NH proton of helix D makes a tertiary H-bond with the sidechain oxygen of N27 from helix B (Blake et al., 1965), which is an especially important probe to monitor "helix/helix docking via sidechain interactions" (Radford et al., 1992b). The significantly retarded exchange of the W111 indole proton presumably indicates survival of a native-like, weak interaction between helix B and helix D. Slow exchange of the other indole protons supports the maintenance of considerable tertiary interactions in the helical domain.

Discussion

Our results permit structural characterization of a stable intermediate state of hen lysozyme in 25% HFA. This intermediate state has the following structural features: persistent native-like secondary structures and loss of tertiary structures as evident from CD experiments; strong interaction with the hydrophobic dye ANS; absence of the "ring current"-affected ^1H resonances in NMR experiments indicating disruption of the native fold; solvent inaccessibility of the tryptophan residues, as judged by fluorescence quenching and H/D exchange suggestive of a compact structure; a cooperative thermal unfolding of the intermediate state to a further unfolded state supporting the existence of side-chain interactions in stabilizing the compact structure; protection factors analysis from H/D exchange experiments, clearly suggesting a "bipartite structure" of the intermediate state (Peng & Kim, 1994), where helical structures are largely retained with preferential loss of β-sheet structures. At higher HFA concentrations (approximately 50% (v/v)) a non-compact state with a high degree of helical structure is populated as detected by CD. A tentative mechanism for the action of HFA on lysozyme may involve initial disruption of the hydrophobic core, resulting in shifting the equilibrium in favour of the molten globule state. At higher HFA concentrations further opening of the protein structure is facilitated by solvation of exposed hydrophobic residues by the fluorocarbon face (see Fig. 1) of the solvent HFA. The high helicity in 50% HFA is fully consistent with the ability of the solvent to stabilize intramolecularly hydrogen-bonded helical conformations in isolated peptide fragments (Rajan et al., 1997).

The structural characteristics of the intermediate state of lysozyme in 25% HFA are similar to the molten globule-like states of many proteins, including the structurally homologous protein α-lactalbumin. A detailed understanding of the structures and energetics of molten globule intermediate states are of great significance, since this is a common intermediate state in the folding pathway of many proteins in vitro and in vivo (Ptitsyn, 1995). The molten globule states of α-lactalbumin and the evolutionarily related calcium-binding equine lysozyme are well studied under equilibrium conditions (Kuwajima, 1989; Morozova et al., 1995; Schulman et al., 1995). On the contrary, in the case of hen lysozyme, the molten globule-like state does not form under equilibrium-denaturing conditions explored so far. However, an intermediate state with molten globule-like properties has indeed been observed in the early stage of kinetic refolding experiments (Radford & Dobson, 1995 and references therein). This clearly suggests that molten globule-like states do exist in the folding pathway of hen lysozyme. Therefore, it is of interest to compare the equilibrium molten globule-like state of hen lysozyme in 25% HFA with its kinetic counterpart. The molten globule state, transiently detected around 50 ms has structural characteristics remarkably similar to the equilibrium molten globule in 25% HFA. The kinetic molten globule state has native-like far UV CD with an "overshoot," no near UV CD, binds to ANS, and shows native-like tryptophan fluorescence quenching by ionic quenchers (Radford et al., 1992b; Itzhaki et al., 1994). H/D pulse-labeling experiments suggest four major α-helices along with the C-terminal 3_{10} helix are protected in the molten globule state with late protection being observed from the β-sheets and N-terminal 3_{10} helix (Radford et al., 1992b). The similarities to the equilibrium molten globule state in 25% HFA are striking. Interestingly, a higher relative protection is observed for helix C in HEWL in 25% HFA as compared to the molten globule state of equine lysozyme and α-lactalbumin. The population of a small amount native state (~5%) under our experimental conditions may contribute to an overestimation of the protection factors for the more structured region of the protein. However, it is noteworthy that the presence of two alanine residues in helix C at positions 90 and 95 of HEWL may be expected to confer extra stability to the helical structure, since alanine has been

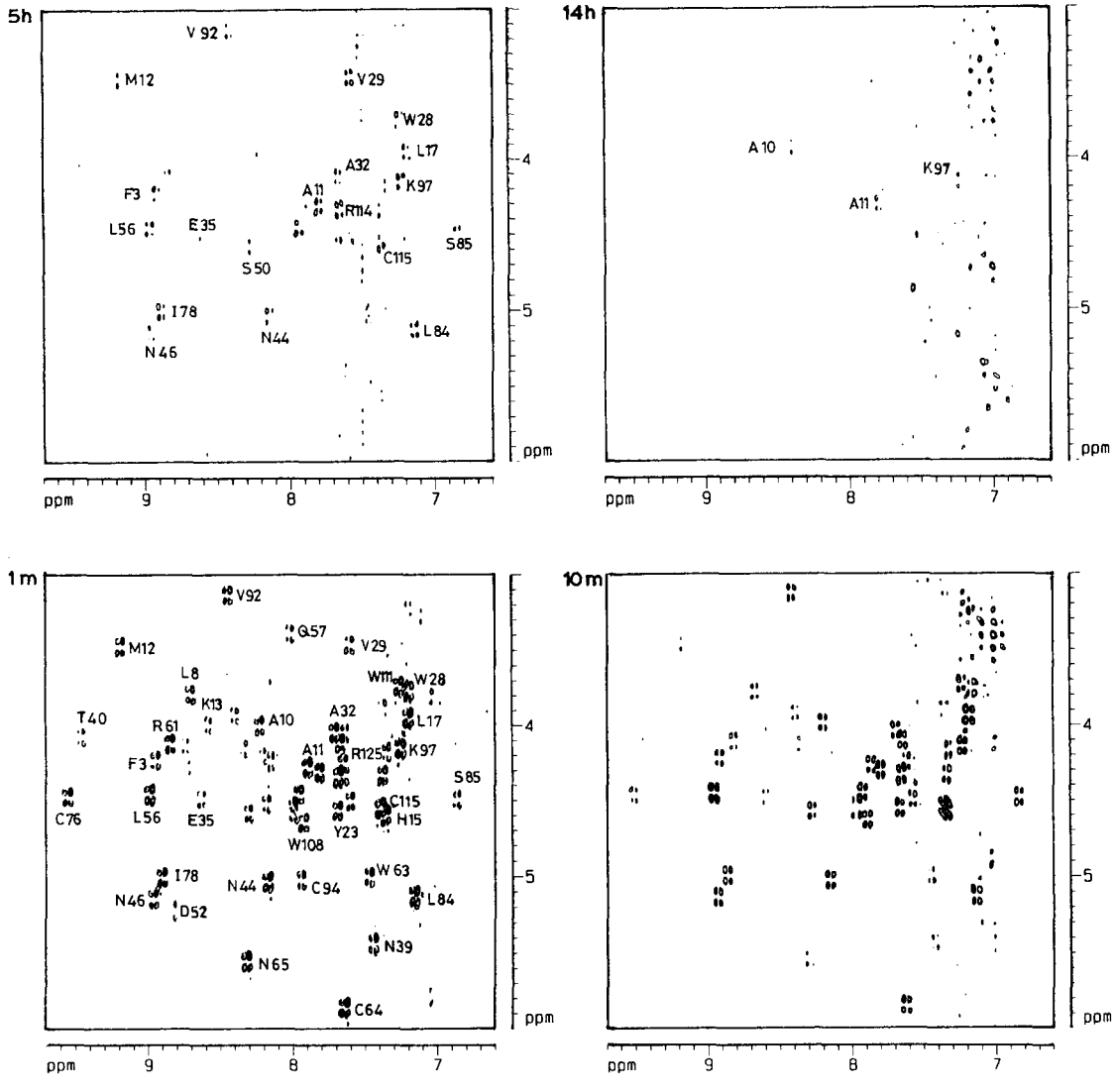

Fig. 10. The finger print region of the 2-D double quantum filtered COSY spectra of lysozyme in D_2O after refolding at four different time points. The peak assignments of some resonances are shown following Redfield and Dobson (1988). Spectra were recorded at protein concentration 5 mM, pH 3.0, 298 K.

shown to have the highest helix stabilizing propensity (O'Neil & Degrado, 1990; Chakrabartty et al., 1991). In contrast, one or both the alanines are substituted by methionine and isoleucine in α-lactalbumin and aspartic acid in equine lysozyme, all of which have a significantly lower helical propensity. It is noteworthy that NMR studies of a synthetic sequence corresponding to helix C of HEWL in aqueous TFE establish a high helical structure of this peptide in solution (Bolin et al., 1996).

Recently, aqueous organic solvents have been used in generating partially folded states of some proteins (Harding et al., 1991; Fan et al., 1993; Alexandrescu et al., 1994; Bychkova et al., 1996; Kamatari et al., 1996). A partially denatured state of hen lysozyme has been obtained in a 50% TFE–water mixture (Buck et al., 1993). A detailed structural characterization by heteronuclear NMR indicates a rather non-compact state with retention of helical structures (Buck et al., 1995, 1996). This TFE denatured state is presumably a coil-like intermediate state with very few side-chain interactions, which is distinct from the compact molten globule state as demonstrated by X-ray scattering experiments (Shiraki et al., 1995). A similar non-compact state is supported for hen lysozyme by spectral data at high HFA concentrations (>50%) in the present study. It is pertinent to note that the effect of the fluoroalcohols TFE and HFA appear to be distinctly different suggesting that specific solvent characteristics may be of importance in stabilizing diverse intermediate states. In a related study, we have characterized an ordered intermediate state of hen lysozyme in a 50% dimethylsulfoxide water mixture. The intermediate state in 50% DMSO resembles a highly ordered molten globule-like state, which has been observed in interleukin 4 (Redfield et al., 1994), apomyoglobin (Lin et al., 1994), and staphylococcal nuclease (Carra et al., 1994; Shortle, 1996). The present study, together with related reports (Dobson, 1992; Harding et al., 1991; Bychkova et al., 1996; Kamatari et al., 1996), points to the potential importance of organic solvents with contrasting structure-modulating properties in stabilizing a wide range of conformational states of proteins. The ability to characterize a range of states from disor-

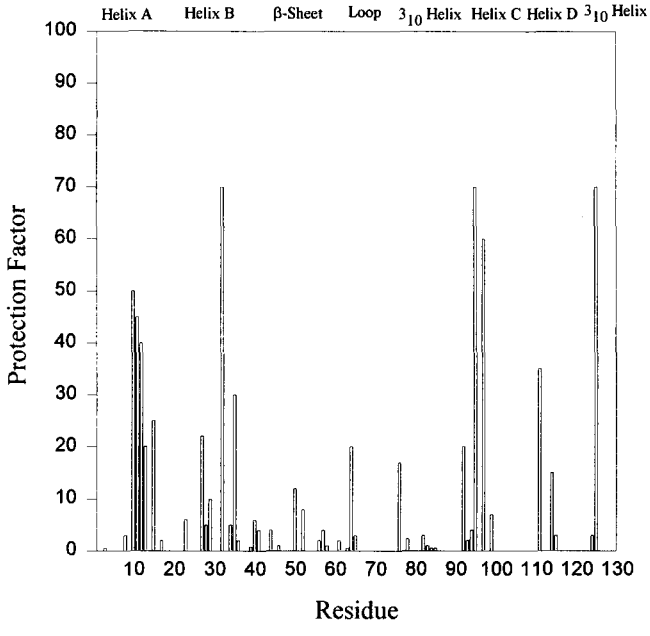

Fig. 11. Bar diagram showing distribution of protection factors of the amide protons of hen lysozyme in 25% HFA. Regions of secondary structure in the native state are marked at the top.

dered to highly ordered under equilibrium conditions will permit a detailed dissection of the folding pathway of proteins.

Materials and methods

Hen egg-white lysozyme, ANS (1-anilino-8-naphthalene sulphonate), deuterated water (D_2O) were purchased from Sigma Chemical Co. Hexafluoroacetone trihydrate was from Aldrich Chemical Co. All other chemicals were of analytical grade.

Circular Dichroism

CD spectra were recorded on a J-500 spectropolarimeter. 1 mm, 2 mm, and 5 mm quartz cells were used for far UV CD and near UV CD experiments, respectively. The concentration of lysozyme was 20 μM for far UV CD and 60 μM for near UV CD measurements. The pH of the samples were adjusted to a nominal value of 3.0 using 0.1 N HCl.

Fluorescence

Fluorescence spectra were recorded on a Hitachi 650-60 spectrofluorimeter using excitation and emission band pass of 5 nm. In studies of hydrophobic dye binding, the ANS concentration was 20 μM and the protein concentration 10 μM, at pH 3.0. HFA concentration was varied over the range of 0–60% (v/v) using a sample volume of 1 mL. The binding of ANS to the 25% HFA state of lysozyme was quantitated using a Scatchard analysis. A fixed concentration of lysozyme (20 μM) or ANS (20 μM) in 25% HFA was titrated with various concentrations of ANS or lysozyme ranging from 5 μM to 60 μM. The excitation wavelength for ANS was 375 nm. NaI quenching experiments were done by adding various aliquots of quencher from a concentrated stock. Protein concentrations were 10 μM and the tryptophan excitation wavelength was 295 nm.

^1H NMR

NMR experiments were performed on a Bruker AMX 400 spectrometer. The protein concentrations for all the experiments were 1 mM to 5 mM at pH 3.0. The probe temperature was set to 25 °C. Two dimensional double quantum filtered COSY experiments were done in a phase sensitive mode with 300 increments over 1K data points and 32 transients were collected. The data sets were zero filled to 1024 points in both dimensions prior to Fourier transformation.

Refolding experiments

Amide ^1H exchange experiments were performed with lysozyme in 25% HFA/75% D_2O at 12 different time points ranging from 1 min to 14 h, pH 3.0 at 20 °C. Refolding was initiated by eightfold dilution with D_2O, pH 3, followed by immediate lyophilization in order to retard deuterium replacement of the slow-exchanging amide protons (Buck et al., 1993); these samples were then redissolved to a final concentration of 6 mM in D_2O, pH 3.0 and phase-sensitive dQF-COSY spectra were obtained at 25 °C (Redfield & Dobson, 1988) within 4 h. Amide proton intensities were measured from absolute value of the cross-peaks using Bruker software. Intensities were scaled to COSY cross-peaks of the nonlabile aromatic 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 under identical conditions. Hydrogen exchange rates (k_{ex}) were fitted to a single-exponential decay equation. Intrinsic exchange rates (k_{int}) of the amide protons in a completely unfolded model of hen lysozyme were calculated taking into account near neighbor effects, acid, and water catalysis as recently described by Bai et al. (1993). Protection factors (k_{int}/k_{ex}) were calculated without considering the effect of cosolvents, since the effect of cosolvents on the exchange rate of NH protons are shown to be insignificant at low pH (Molday et al., 1972, Englander & Kallenbach, 1984).

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