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Intermolecular $^1H\{^{19}F\}$ NOEs in Studies of Fluoroalcohol-Induced Conformations of Peptides and Proteins

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Mixtures of fluorinated alcohols and water can selectively stabilize certain secondary structures of peptides and proteins. Such mixtures may also be of use in solubilizing hydrophobic or membrane-bound proteins. We show that intermolecular dipolar interactions between the fluorine nuclei of such solvents and the protons of a dissolved protein lead to readily detected $^1H\{^{19}F\}$ nuclear Overhauser effects. These NOEs can potentially provide information about solvent exposure of particular groups as well as indicate the formation of long-lived fluoroalcohol–solute complexes. Results obtained with HEW lysozyme in solutions containing trifluoroethanol illustrate these possibilities.

Key Words: **intermolecular NOE; lysozyme; trifluoroethanol; fluoroalcohol; TFE state.**

INTRODUCTION

Mixtures of trifluoroethanol (TFE)[2] and water were observed nearly 40 years ago to stabilize helical conformations of peptides and proteins (*1*). It has subsequently been shown that hexafluoroisopropanol and hexafluoroacetone hydrate can be even more potent in this regard. Populations of turns and various β-structures may also be enhanced by fluoroalcohols (*2*). Fluoroalcohol–water mixtures have been used to solubilize peptide aggregates (*3, 4*), although with amphipathic peptides they can induce self-association (*5, 6*).

Despite much effort, there is no single agreed-upon mechanism that accounts for the effects of TFE and other fluorinated alcohols on peptide or protein structure. Specific solvation of solutes ("solvent sorting") by fluoroalcohol/water mixtures likely is a contributor to the conformational effects these mixtures produce (*7, 8*). Competition between solvation of the solute by water and by the fluoroalcohol could lead to changes in enthalpy and entropy that favor the helical state in these mixtures. Heat capacity data underscore the importance of hydrophobic interactions that are enhanced in the presence of a fluoroalcohol (*9*). Work by Nelson and Kallenbach (*10*) suggests that electrostatic interactions may be only slightly affected by the presence of TFE, although in other systems there is evidence that TFE indeed alters electrostatic interactions (*11*). There are indications that helix stabilization arises because a fluoroalcohol strengthens hydrogen bonds within the helix backbone (*12*). Hydrogen bonding between backbone carbonyl groups and the fluoroalcohol may play a role (*13, 14*) and the propensity of fluoroalcohols to aggregate in water solutions may be an important consideration (*15*).

In the course of producing conformational effects, nuclear dipole–dipole interactions between fluorine spins of the fluorinated solvent molecules and the protons of the solute could take place through collisional encounters or through the formation of long-lived complexes between solute and the fluoroalcohol (*16*). For either situation, a solute proton–solvent fluorine intermolecular $^1H\{^{19}F\}$ NOE is reasonably expected for peptides or proteins dissolved in water–fluoroalcohol mixtures.

The first reported $^1H\{^{19}F\}$ intermolecular NOE in a biological system is from the work of Pitner *et al.* (*17*). Using cw techniques, these workers observed a selective 5% enhancement of the phenylalanine protons of gramicidin dissolved in TFE when the fluorine resonance of the solvent was saturated. Using solutions of hen egg white lysozyme in trifluoroethanol–water mixtures, we show here that smaller proton–fluoroalcohol NOEs are readily detected using pulsed field gradient methods and that such NOEs provide an avenue for the exploration of fluoroalcohol–biomolecule interactions.

METHODS

Basic theory for the contribution of intermolecular dipolar interactions to relaxation has been extended by Ayant *et al.* (*18*) to include the effects of rotational diffusion of the interacting species in addition to encounters through translational diffusion. The Ayant *et al.* model imagines a solute hydrogen located at a distance ρ_H from the center of a sphere of radius r_H. The hydrogen is relaxed by a fluorine nucleus that is present in another sphere of radius r_F with the fluorine a distance ρ_F from the center of that sphere. Both spheres tumble isotropically at characteristic rates defined by the rotational correlation times τ_H and τ_F.

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[2] Abbreviations used: CD, circular dichroism; HEW, hen egg white; NOE, nuclear Overhauser effect; TFE, trifluoroethanol; SAXS, small angle x-ray scattering.

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Presuming that the solute is present in low concentration, relaxation depends on these quantities, the concentration of solvent, and a correlation time τ, given by $\tau = (r_H + r_F)^2/(D_H + D_F)$, where $(D_H + D_F)$ is the sum of the translational diffusion coefficients for the solute and solvent, and $(r_H + r_F)$ is the closest the interacting species can approach each other. Thus, the intermolecular dipolar interaction between H and F depends on encounters between the spheres (translational diffusion) and the modulation of internuclear distances during these encounters by rotational diffusion of the spheres. In the simplest case, the fluorine and proton spins would be located at the centers of their spheres (ρ_H and $\rho_F = 0$). In that event, considerations of the van der Waals distances for each spin would suggest that $(r_H + r_F)$ would be ~2.6 A, although other choices could be made depending on the structural characteristics of the solute and solvent molecules.

It has been suggested that experimental studies of NOEs should take cognizance of the polyexponential nature of spin–lattice relaxation by observing waiting times of the order of 8 times the longest T_1 of the system before recycling the experiment (*19*). In the experiment previously proposed for $^1H\{^{19}F\}$ NOE detection (*20*), the proton magnetization is maintained near saturation so that proton T_1 relaxation is largely irrelevant to the repetition rate of the experiment. However, we find that T_1 for fluorines in fluoroalcohol–water mixtures are often in the range of 4–5 s, suggesting a long and unproductive delay for fluorine relaxation to take place before the experiment can be repeated. It is likely that in most $^1H\{^{19}F\}$ NOE experiments the mixing time will be significantly less than the fluorine T_1.We have therefore modified the previously reported experiment to include a composite 180° pulse before the start of the recovery period, as shown in Fig. 1. The phases within this composite pulse are cycled in such a way that the effect of the pulse is to leave fluorine magnetization alone if the previous experiment finished with this magnetization nearly at its equilibrium value. If the previous experiment left the fluorine magnetization inverted, the composite 180° pulse rotates it so that it is closer to its equilibrium position, thus facilitating the return to its equilibrium position. If all 180° pulses in the sequence produce their nominal rotations of the fluorine magnetization, this procedure means that a recycle delay (d_1) of approximately the sum of half the mixing time plus the acquisition time should be sufficient to return the fluorine magnetization to its equilibrium position so that the experiment can be started anew. For typical experimental conditions, this modification reduces the time of an experiment approximately 20-fold.

The concentrations of fluorine in the fluoroalcohol–water solutions used to induce secondary structures of peptides can be high—about 21 M in the case of 50% TFE/water. Depending on experimental conditions, application of the $^1H\{^{19}F\}$ NOE pulse sequence shown in Fig. 1 may be troubled by radiation damping effects during the application of the fluorine pulses or by distortions of the accumulated difference spectra that result from transient changes in sample magnetic susceptibility as the orientation of the fluorine magnetization is altered. Strategies are available for producing clean inversions in the presence of radiation damping (*21, 22*). However, for the probe configuration used in the work described here (fluorine coil exterior to the proton coil) radiation damping effects did not appear to be substantial enough to warrant taking them into account.

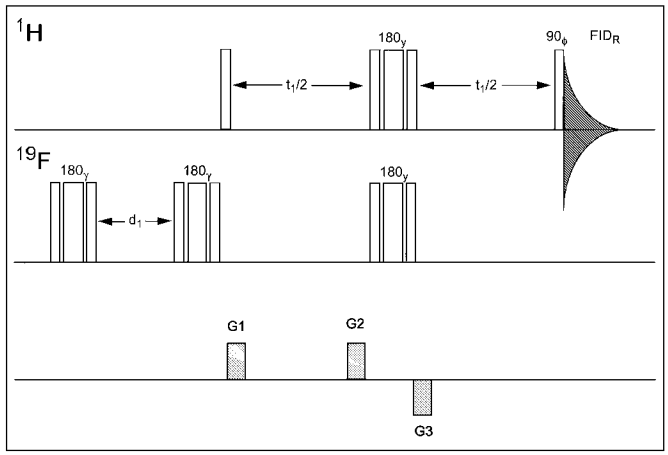

FIG. 1. A pulse sequence for collection of $^1H\{^{19}F\}$ intermolecular NOEs. All narrow bars not labeled correspond to 90x pulses. Phases are cycled according to $\gamma = (-x, y, -x, y, -x, y, -x, y)$ while $\phi = (x, x, y, y, -x, -x, -y, -y)$ and $R = (-x, x, -y, y, x, -x, y, -y)$. With fluoroalcohol-containing samples the fluorine T_1 can be relatively long compared to the mixing time + FID acquisition time; the phase of the 180γ pulses ensures that fluorine z-magnetization is returned to the positive z-axis when the previous experiment leaves it inverted. The phases ϕ and R arise from the subtraction of alternate scans to leave the NOE and standard CYCLOPS cycling of the observe pulse and receiver. Alterations of the experiment to take into account the effects of radiation damping and the effects of changes in sample susceptibility as the fluorine magnetization is shifted are indicated in the text.

The lysozyme–TFE solutions have spectral lines that are sufficiently broad that distortions of the difference spectra due to variations in the fluorine dipolar field were not observed. For samples with narrower lines where such distortions are expected, inclusion of a 90° fluorine pulse prior to or coincident with the final proton pulse of the $^1H\{^{19}F\}$ NOE experiment would be sufficient to minimize such distortions (*23*). Should fluorine T_1 relaxation be slow enough that the experiment suggested by Fig. 1 is appropriate, the first composite 180° fluorine pulse shown there, intended to realign the fluorine magnetization to its equilibrium position at the start of each scan, could be moved to be coincident with the final proton pulse. This would provide a nearly constant fluorine contribution to the sample susceptibility while still enabling a reduction of the total experiment time.

Studies by Buck *et al.* (*24*) show that there is little effect of trifluoroethanol on the tertiary structure of lysozyme, as evidenced by proton NMR and CD spectra, up to fluoroalcohol concentrations of 15% (v/v). A transition to a partially structured state of the protein is observed at concentrations higher than this, with CD observations indicating an appreciable increase in secondary structure(s) at 40% TFE, along with a loss of tertiary

structure under these conditions (*24*). Hydrogen–deuterium exchange experiments in the presence of 50% TFE show that about 25 backbone amides in regions of the protein that are helical in the native structure remain protected from deuterium exchange with solvent. Amides in β-sheet and a 3_{10}-helical region of the native structure are not protected against solvent exchange under these conditions. SAXS data indicate that the radius of gyration of the TFE-induced state of lysozyme is about 20% larger than that of the native enzyme (*25*).

RESULTS AND DISCUSSION

We have found for small molecules that the description of intermolecular dipolar interactions provided by Ayant *et al.* (*18*) leads to calculated $^1H\{^{19}F\}$ solvent–solute NOEs in reasonable agreement with experiments (D. Martinez, unpublished work). For example, the solute–solvent $^1H\{^{19}F\}$ NOEs for all protons of the amino acid derivative *N*-acetyltyrosine ethyl ester (ATEE) dissolved in TFE–water mixtures are essentially the same at a given concentration of TFE, consistent with the expected nearly equivalent exposure of these protons to interactions with the solvent molecules. For the 20% TFE sample at 25° the average slope of plots of observed NOE *versus* mixing time was $9.5\times10^{-3}\ s^{-1}$. Diffusion coefficients of ATEE and TFE in this sample were determined using the BPP-LED sequence of Wu *et al.* (*26*). Calculations done using the measured diffusion coefficients and the model of Ayant *et al.* with $\rho_H = \rho_F = 0.12$ A, $r_H = 1.32$ A, $r_F = 1.52$ A, and $\tau_F = 0.2$ ns, gave a predicted slope of $9.4\times10^{-3}\ s^{-1}$. The agreement with experiment suggests that the model of Ayant *et al.* has validity, at least for situations where the hydrogens of a molecule are fully solvent exposed in all conformations.

Calculations using experimental self-diffusion coefficients of lysozyme and trifluoroethanol (1.0×10^{-6} and $6.2\times10^{-6}\ cm^2\ s^{-1}$, respectively, in 10% TFE) and the geometrical parameters indicated earlier showed that the TFE–protein intermolecular $^1H\{^{19}F\}$ NOE would be expected to be positive under all conditions of our experiments. This qualitative conclusion was indicated even if the diffusion coefficients or radii of the spheres were in error by ±20%.

Figures 2A and 3A illustrate the results of a $^1H\{^{19}F\}$ NOE experiment with lysozyme in 10% trifluoroethanol. In 10% TFE, all observed protein–solvent NOEs are negative, ranging from −0.4 to −2.2%. (The $^1H\{^{19}F\}$ NOE on the residual protons of the CH_2/CD_2 group of the solvent is positive in all samples examined in this work.) Three tryptophan $H_\varepsilon1$ resonances in the 9.9- to 10.3-ppm range exhibit strong dipolar interactions with the trifluoromethyl groups of the solvent. Based on previous assignments of the proton spectrum of lysozyme (*27, 28*), these NOEs can be assigned to tryptophans W62, W63, and W108 as indicated. Similarly, a distinctive NOE at ca. 5.8 ppm possibly arises from HA of cysteine C64 or asparagine N59.

Some high-field methyl signals also show negative $^1H\{^{19}F\}$ NOEs in 10% TFE (Fig. 3A). Given their shifts and the previous

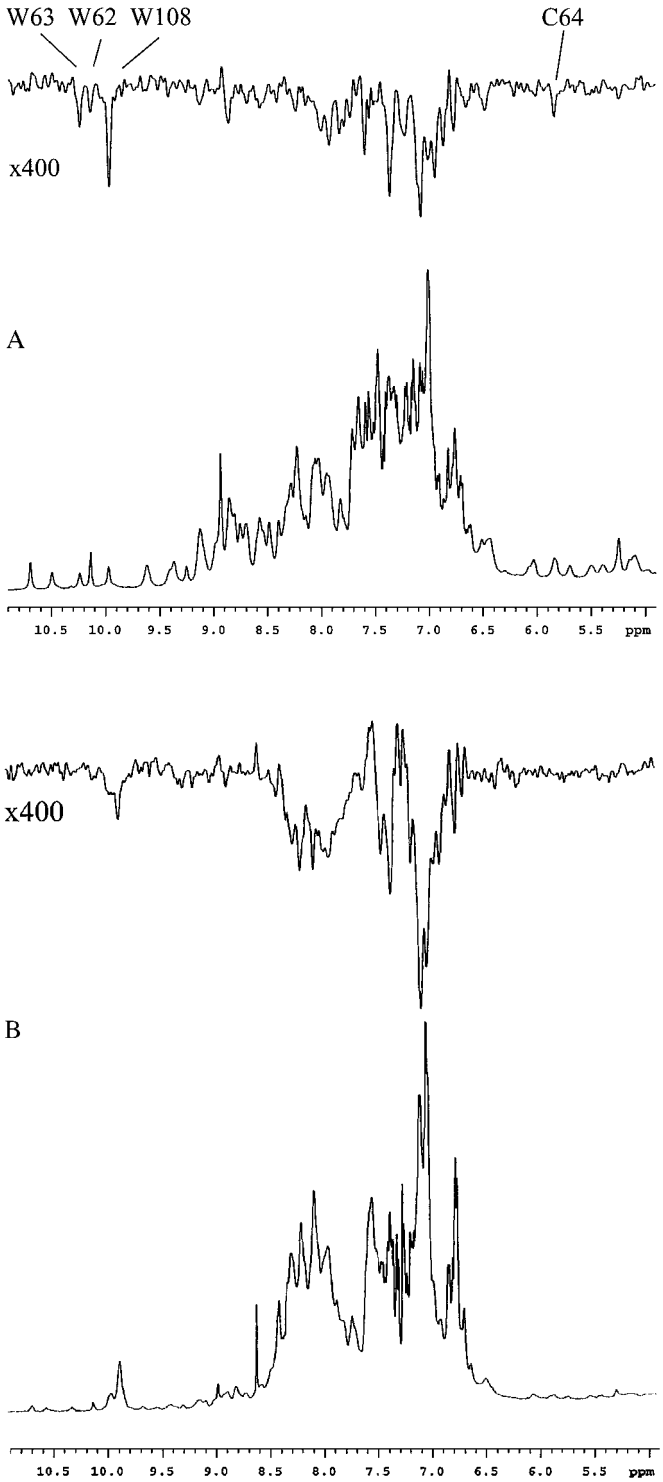

FIG. 2. Proton NMR spectra at 500 MHz of 2.5 mM HEW lysozyme in 90% H_2O/10% D_2O with added trifluoroethanol-d_3 (lower trace in each set) and $^1H\{^{19}F\}$ NOEs obtained with a mixing time of 1 s (upper trace in each set). All samples were at pH 2.0; sample temperatures were 25°C. (A) 10% TFE, (B) 20% TFE, (C) 50% TFE.

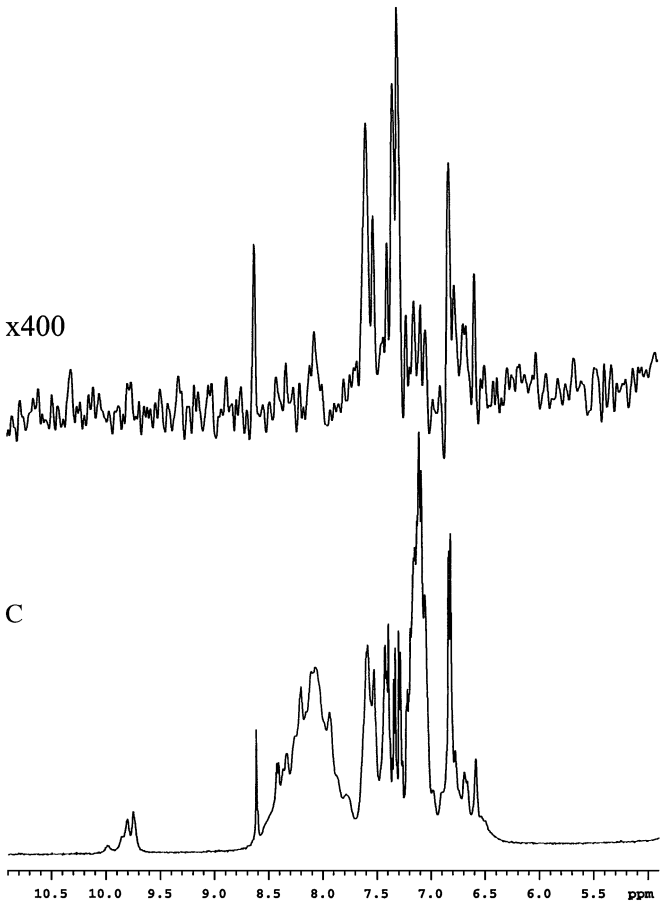

FIG. 2—*Continued*

assignments, the NOEs in the 0.0- to −0.4-ppm range can be assigned to methyl groups of isoleucine-98 (*27, 28*).

Increasing the fluoroalcohol concentration to 20% v/v leads to proton spectra of the enzyme which show signals for the native conformation as well as new signals, for example, near 8.2 and 9.8 ppm (Fig. 2B). Proton–fluorine NOEs of many of these new signals are negative but now positive NOEs are evident as well.

All of the protein is in the "TFE-state" in 50% TFE. Under these conditions essentially all negative $^1H\{^{19}F\}$ NOEs disappear, leaving small positive NOEs of ca. +0.1 to +0.5% (Figs. 2C and 3C).

Negative intermolecular $^1H\{^1H\}$ NOEs between the spins of methanol or isopropanol cosolvents and protons at the active site and other loci on the surface of lysozyme have been observed by Liepinsh and colleagues (*29, 30*). These have been interpreted in terms of site-specific interactions of these alcohols with the protein and are consonant with neutron diffraction studies which demonstrate a high occupancy binding site for ethanol (ETH 1) near residues Ile-98, Trp-63, Trp-108, Ile-58, Asn-59, and Ala-107 of crystalline lysozyme (*31*).

Proton chemical shift changes of some lysozyme signals are observed in the presence of TFE and have been attributed to site-specific binding of the fluoroalcohol to the protein surface (*24*). Consistent with the SAXS results, the rotational correlation time (τ_c) of lysozyme does not appear to change greatly in the TFE-induced state (*32*). If the lifetime of a protein–TFE complex is long relative to this τ_c, calculations show that negative $^1H\{^{19}F\}$ NOEs would be observed for lysozyme protons in the vicinity

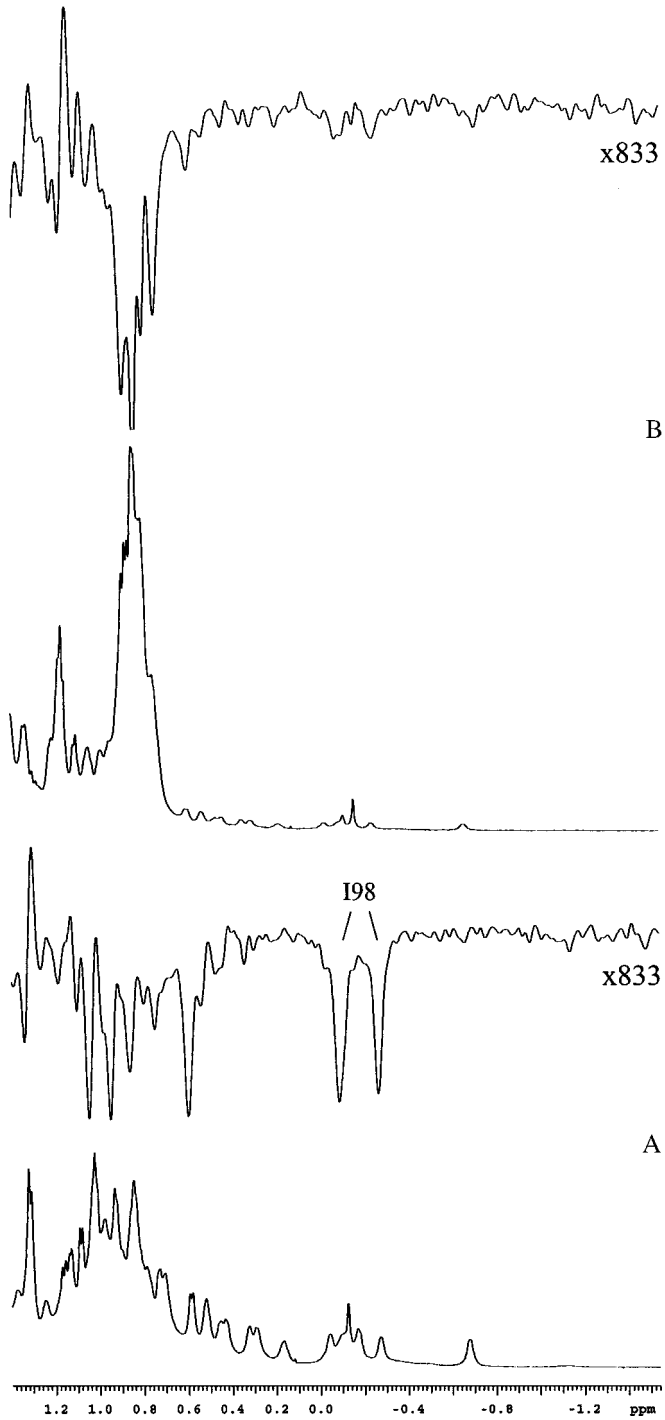

FIG. 3. The high-field regions of the spectra shown in Fig. 2. (A) 10% TFE, (B) 20% TFE, (C) 50% TFE.

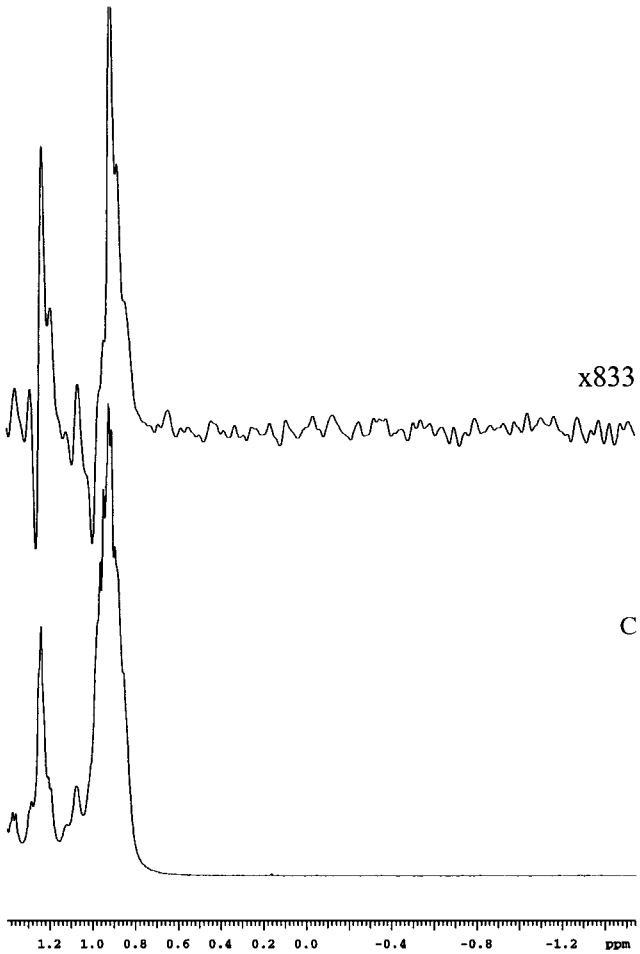

FIG. 3—*Continued*

of a protein-complexed CF_3 group(s) in either the native or the TFE-induced conformations. The negative $^1H\{^{19}F\}$ NOEs arising from the Trp-62, Trp-63, Trp-108, Cys-64, and Ile-98 side chains are thus consistent with the formation of a long-lived protein–TFE complex at an interaction site that includes these residues and is very similar to the site(s) of complex formation with methanol, ethanol, and isopropanol.

The lack of observable (positive) intermolecular NOEs that arise simply from collisions of the fluoroalcohol with solvent-exposed hydrogens in the lysozyme/10% TFE system presumably is a consequence of the low concentration of the fluoroalcohol. The side chain of Trp-62 is considerably more exposed to solvent that those of the other tryptophans which show NOEs. The smaller solute–solvent NOE observed in this case may be the result of a combination of a negative NOE generated within a long-lived complex and the positive NOE that arises by way of collisional encounters with solvent molecules.

The ability to bind TFE tightly enough to evoke negative $^1H\{^{19}F\}$ NOEs appears to be lost in the TFE-induced conformation of the enzyme. The positive $^1H\{^{19}F\}$ NOEs that are observed in 50% TFE probably are simply the result of collisions of the lysozyme structure with the TFE solvent molecules. Although assignments for the observed $^1H\{^{19}F\}$ NOEs are not yet in hand, consideration of Figs. 2C and 3C make it clear that not all protons of the TFE-induced conformation of lysozyme are equivalently exposed to solvent in this conformation. The tryptophan $H_\varepsilon 1$ for residues 62, 63, and 108 as well as a number of amide protons whose signals appear in the 7.8- to 8.6-ppm range appear to be well shielded from collisions with solvent molecules by local protein structures.

The presence of negative $H\{^{19}F\}$ NOEs with reduced magnitude in 20% TFE requires some comment. Comparisons of Figs. 2A and 2B confirm that separate signals for the native and TFE-induced state of the protein are observed under these conditions. Observations of separate sets of resonances require that the interconversion of these states be slow on a time scale defined by chemical shift differences of spins between the native and TFE states. A lifetime for these states of the order of a few milliseconds would be consistent with the observations of resolved signals for each (lifetime $\sim$1/(shift difference) $\sim$1/500 Hz $\sim$2 ms). We suggest that the appearance of negative NOEs on signals for the TFE-induced state that are observed when there are significant amounts of both conformational states present is a reflection of the interconversion of the two species. The times scale for averaging of $^1H\{^{19}F\}$ NOEs is defined by spin–lattice relaxation times, with fast exchange averaging of NOEs expected if the lifetime of a state is smaller than $\sim T_1$ (*33*). Proton T_1 values of lysozyme in these samples ranged between 0.9 and 1.6 s. To be consistent with our observations, the lifetimes of the native and TFE-induced states of lysozyme in the 20% TFE system must be longer than $\sim$2 ms but significantly shorter than $\sim$1000 ms, so fast exchange (averaged) NOEs would be present, even though separated signals are observed for each species.

CONCLUSIONS

We have demonstrated that intermolecular NOEs between solute protons and solvent fluorines, although often small, can be readily detected. Studies of a test system indicate that NOEs arising from collisional interactions as well as the formation of long-lived complexes can be diagnosed. Given the continuing interest in using fluorinated alcohols in studies of peptide and protein conformations, this experiment could be a useful adjunct to the more usual CD and intramolecular NOE experiments that are used to characterize conformation in mixed fluoroalcohol–water systems.

EXPERIMENTAL

HEW lysozyme, trifluoroethanol-d_3, and deuterium oxide were obtained from Aldrich–Sigma and used as received. Distilled deionized water was used in making samples.

Protein solutions were prepared by dissolving a defined mass of protein in a solution of 10% D_2O in H_2O. The desired volume

of deuterated trifluoroethanol was added and the pH of the sample adjusted to 2.0 by adding small amounts of 0.1 M NaOH or HCl. Sample pH values reported are meter readings and were not corrected for isotopic or cosolvent compositions. Samples were not degassed. Sample pH was measured with a Markson microcombination electrode.

Proton NMR spectra were determined at 500 MHz using a Varian Inova system and a Nalorac $^1H/^{19}F$ probe equipped with a *z*-axis gradient coil. Water signal suppression was by means of the DPFGSE method (*34*). The number of scans collected ranged from 5000 to 9000. (It should be borne in mind that only half of the collected scans lead to accumulation of the NOE, with the other half providing a correction for T_1 relaxation during the mixing time.) Diffusion coefficients were estimated using bipolar gradient pulses as described by Wu *et al.* (*26*). Gradient strengths were calibrated using the published diffusion coefficient of HOD in D_2O (*35*) or the method described by Braun *et al.* (*36*). Spin–lattice relaxation times were estimated by the inversion-recovery method.

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REFERENCES

1. M. Goodman and I. Listowsky, Conformational aspects of synthetic polypeptides VI. Hypochromic spectral studies of oligo-α-methyl-L-glutamate, *J. Am. Chem. Soc.* **84,** 3770–3771 (1962).
2. M. Buck, Trifluoroethanol and colleagues: Cosolvents come of age. Recent studies with peptides and proteins, *Q. Rev. Biophys.* **31,** 297–355 (1998).
3. S. J. Wood, B. Maleeff, T. Hart, and R. Wetzel, Physical, morphological and functional differences between pH 5.8 and 7.4 aggregates of the Alzheimer's amyloid peptide Aα, *J. Mol. Biol.* **256,** 870–877 (1996).
4. H. Zhang, K. Kaneko, J. T. Nguyen, T. L. Livshits, M. A. Baldwin, F. E. Cohen, T. L. James, and S. B. Prusiner, Conformational transitions in peptides containing two putative α-helices of the prion protein, *J. Mol. Biol.* **250,** 514–526 (1995).
5. R. G. Maroun, D. Krebs, S. El Antri, A. Deroussent, E. Lescot, F. Troalen, H. Porumb, M. E. Goldberg, and S. Fermandjian, Self-association and domains of interactions of an amphipathic helix peptide inhibitor of HIV-1 integrase assessed by analytical centrifugation and NMR experiments in trifluoroethanol/H_2O mixtures, *J. Biol. Chem.* **274,** 34174–34185 (1999).
6. C. E. MacPhee, M. A. Perugini, W. H. Sawyer, and G. J. Howlett, Trifluoroethanol induces the self-association of specific amphipathic peptides, *FEBS Lett.* **416,** 265–268 (1997).
7. M. J. Bodkin and J. M. Goodfellow, Hydrophobic solvation in aqueous trifluoroethanol solution, *Biopolymers* **39,** 43–50 (1996).
8. R. Rajan, S. K. Awasthi, S. Bhattacharjya, and P. Balaram, "Teflon-coated peptides:" Hexafluoroacetone trihydrate as a structure stabilizer for peptides, *Biopolymers* **42,** 125–128 (1997).
9. N. H. Andersen, R. B. Dyer, R. M. Fesinmeyer, F. Gai, Z. Liu, J. W. Neidigh, and H. Tong, Effect of hexafluoroisopropanol on the thermodynamics of peptide secondary structure formation, *J. Am. Chem. Soc.* **121,** 9879–9880 (1999).
10. J. W. Nelson and N. R. Kallenbach, Persistence of the α-helix stop signal in the S-peptide in trifluoroethanol solutions, *Biochemistry* **28,** 5256–5261 (1989).
11. J. K. Myers, C. N. Pace, and J. M. Scholtz, Trifluoroethanol effects of helix propensity and electrostatic interactions in the helical peptide from ribonuclease T1, *Protein Sci.* **7,** 383–388 (1998).
12. P. Luo and R. L. Baldwin, Mechanism of helix induction by trifluoroethanol: A framework for extrapolating the helix-forming properties of peptides from trifluoroethanol/water mixtures back to water, *Biochemistry* **36,** 8413–8421 (1997).
13. S. Rothemund, H. Weisshoff, M. Beyermann, E. Krause, M. Bienert, C. Mugge, B. D. Sykes, and F. D. Sonnichsen, Temperature coefficients of amide proton NMR resonance frequencies in trifluoroethanol: A monitor of intramolecular hydrogen bonds in helical peptides?, *J. Biomol. NMR* **8,** 93–97 (1996).
14. M. Plass, C. Griehl, and A. Kolbe, Association behavior of selected amino acid and oligopeptide derivatives with fluorinated alcohols, *J. Chem. Soc. Perkin Trans.* **2,** 853–856 (1995).
15. D.-P. Hong, M. Hoshino, R. Kuboi, and Y. Goto, Clustering of fluorine-substituted alcohols as a factor responsible for their marked effects on proteins and peptides, *J. Am. Chem. Soc.* **121,** 8427–8433 (1999).
16. Y. Yang, S. Barker, M. J. Chen, and K. H. Mayo, Effect of low molecular weight aliphatic alcohols and related compounds on platelet factor 4 subunit association, *J. Biol. Chem.* **268,** 9223–9229 (1993).
17. T. P. Pitner, J. D. Glickson, R. Rowan, J. Dadok, and A. A. Bothner-By, Delineations of interactions between specific solvent and solute nuclei. A NMR solvent saturation study of gramicidin S in methanol, dimethylsulfoxide, and trifluoroethanol, *J. Am. Chem. Soc.* **97,** 5917–5918 (1975).
18. Y. Ayant, E. Belorizky, P. Fries, and J. Rosset, Effet des interactions dipolaires magnetiques intermoleculaires sur la relaxation nucleaire de molecules polyatomiques dans les liquides, *J. Phys. France* **38,** 325–337 (1977).
19. S. J. Opella, D. J. Nelson, and O. Jardetsky, Dynamics of nuclear Overhauser enhancement in proton-decoupled carbon-13 nuclear magnetic resonance, *J. Chem. Phys.* **64,** 2533–2535 (1976).
20. J. T. Gerig, Gradient-enhanced proton–fluorine NOE experiments, *Magn. Reson. Chem.* **37,** 647–652 (1999).
21. J.-H. Chen, A. Jerschow, and G. Bodenhausen, Compensation for radiation damping during selective pulses in NMR spectroscopy, *Chem. Phys. Lett.* **308,** 397–402 (1999).
22. H. Bethault, H. Desvaux, G. Le Goff, and M. Petro, A simple way to properly invert intense nuclear magnetization: Application to laser-polarized xenon, *Chem. Phys. Lett.* **314,** 52–56 (1999).
23. B. Lix, F. D. Sonnichsen, and B. D. Sykes, The role of transient changes in sample susceptibility in causing apparent multiple-quantum peaks in HOESY spectra, *J. Magn. Reson. A* **121,** 83–87 (1996).
24. M. Buck, S. E. Radford, and C. M. Dobson, A partially folded state of hen egg white lysozyme in trifluoroethanol: Structural characterization and implications for protein folding, *Biochemistry* **32,** 669–678 (1993).
25. M. Hoshino, Y. Hagihara, D. Hamada, M. Kataoka, and Y. Goto, Trifluoroethanol-induced conformational transition of hen egg white lysozyme studied by small-angle X-ray scattering, *FEBS Lett.* **416,** 72–76 (1997).
26. D. Wu, A. Chen, and C. S. Johnson Jr., An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses, *J. Magn. Reson. A* **115,** 260–264 (1995).
27. C. Redfield and C. M. Dobson, Sequential ^1H NMR assignments and secondary structure of hen egg white lysozyme in solution, *Biochemistry* **27,** 122–126 (1988).
28. Y. J. Wang, T. C. Bjorndahl, and D. S. Wishart, Complete ^1H and non-carbonylic ^{13}C assignments of native hen egg-white lysozyme, *J. Biomol. NMR* **17,** 83–84 (2000).

29. E. Liepinsh and G. Otting, Organic solvent identify specific ligand binding sites on protein surfaces, *Nat. Biotechnol.* **15,** 264–268 (1997).

30. G. Otting, E. Liepinsh, B. Halle, and U. Frey, NMR identification of hydrophobic cavities with low water occupancies in protein structures using small gas molecules, *Nat. Struct. Biol.* **4,** 396–404 (1997).

31. M. S. Lehrmann, S. A. Mason, and G. J. McIntyre, Study of ethanol–lysozyme interactions using neutron diffraction, *Biochemistry* **24,** 5862–5869 (1985).

32. M. Buck, H. Schwalbe, and C. M. Dobson, Main-chain dynamics in a partially folded protein: ^{15}N NMR relaxation measurements of hen egg white lysozyme denatured in trifluoroethanol, *J. Mol. Biol.* **257,** 669–683 (1996).

33. J. T. Gerig, *in* "NMR in Drug Design" (D. J. Craik, Ed.), CRC Press, Boca Raton, FL, 1996.

34. T. L. Hwang and A. J. Shaka, Water suppression that works—Excitation sculpting using arbitrary waveforms and pulsed field gradients, *J. Magn. Reson. A* **112,** 275–279 (1995).

35. L. G. Longsworth, The mutual diffusion of light and heavy water, *J. Phys. Chem.* **64,** 1914–1917 (1960).

36. S. Braun, H.-O. Kalinowski, and S. Berger, "150 and More Basic NMR Experiments," 2nd ed. Wiley-VCH, Weinheim, 1998.