The Fluorous Effect in Proteins: Properties of α_4F_6, a 4-α-Helix Bundle Protein with a Fluorocarbon Core†

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ABSTRACT: To test the prediction that extensively fluorinated (fluorous) proteins should be more stable and exhibit novel self-segregating behavior, the properties of the de novo designed model 4-α-helix bundle protein, α_4F_6, in which the hydrophobic core is packed entirely with the extensively fluorinated amino acid L-5,5,5,5′,5′,5′-hexafluoroleucine, have been compared with its nonfluorinated counterpart, α_4H, in which the core is packed with leucine. α_4F_6 exhibits much greater resistance to proteolysis by either chymotrypsin or trypsin than α_4H and resists unfolding by organic solvents far better than α_4H. Whereas increasing concentrations of ethanol or 2-propanol cause the helices of the α_4H tetramer first to dissociate into monomeric helices and then to completely unfold, these solvents have little effect on the structure of α_4F_6. In contrast, increasing the concentrations of the fluorinated alcohol trifluoroethanol promotes dissociation of both α_4H and α_4F_6 to monomeric helices, whereas the secondary structure of both peptides remains intact. ^{19}F NMR experiments indicate that the two peptides can form mixed α-helical α_4F_6:α_4H bundles and thus do not exhibit the self-segregating behavior predicted by the fluorous effect. We conclude that the properties of α_4F_6 are best explained by the more hydrophobic nature of the hexafluoroleucine side chain, rather than the low solubility of fluorocarbons in hydrocarbon solvents that forms the basis of the fluorous effect.

Fluorocarbons differ significantly from hydrocarbons in their physicochemical properties because a carbon−fluorine bond possesses the opposite dipole moment from a carbon−hydrogen bond and is also stronger and less polarizable (*1*). Extensively fluorinated molecules therefore tend to be chemically inert and extremely hydrophobic. These properties have proved useful in the design of anesthetics, refrigerants, chemically resistant polymers, and drug delivery agents. Extensively fluorinated molecules tend to be poorly soluble in most organic solvents but dissolve in fluorocarbon solvents, a phenomenon referred to as the "fluorous effect" (*2–5*). This property has been exploited to facilitate the purification of organic compounds by tagging them with perfluorocarbon "tails" that allow molecules to be selectively extracted from reaction mixtures into fluorocarbon solvents (*2, 4*).

Although the phase separation of fluorocarbon−hydrocarbon mixtures is often ascribed to "fluorophilic" or "fluorous" interactions between fluorocarbon molecules, this is not strictly correct. The phenomenon arises because the cohesive dispersion forces between hydrocarbon molecules are greater than between fluorocarbon molecules, because hydrocarbons are more polarizable, and thus fluorocarbons are excluded from the hydrocarbons. More generally, the mutual solubility (or immiscibility) of a mixture of two nonpolar solvents is related to the difference in the solubility parameter, δ, which is defined as

$$\delta = (\Delta E^V/V)^{1/2}$$

where ΔE^V is the energy of vaporization and V is the molal volume of the pure liquid at a given temperature (*6, 7*). As the difference in δ between the two solvents increases, the heat of mixing becomes more unfavorable until they are no longer miscible. As discussed by Scott (*6*), fluorocarbons have low δ values both because they have low boiling points and larger molal volumes than hydrocarbons.

There have recently been a number of studies to investigate whether the interesting material properties associated with simple fluorocarbons can be engineered into proteins by incorporating extensively fluorinated analogues of hydrophobic amino acids into their structures (*5, 8–10*). So far, most studies have focused on synthesizing fluorous analogues of peptides designed to adopt α-helical coiled-coil structures (*11–17*). Fluorinated analogues of leucine and valine have been introduced at "a" and "d" positions of the canonical coiled-coil heptad repeat to produce proteins with extensively fluorinated hydrophobic cores. Such proteins have been shown to be significantly more stable toward unfolding by heat and chemical denaturants (*11–15, 18–21*). There is also evidence that fluorinated side chains, incorporated at the hydrophobic interface between helices, can mediate the specific self-association of α-helical peptides. In particular, self-segregating behavior was observed in peptides designed to form a parallel coiled-coil dimer (*12*); in one peptide, **H**, six leucines were incorporated at the "a" and "d" positions, while in the other peptide, **F**, hFLeu[1] was substituted at these positions. The preference for the peptides to form **HH** and **FF** homodimers over **HF** heterodimers was estimated to be

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[1] Abbreviations: hFLeu, L-5,5,5,5′,5′,5′-hexafluoroleucine; CD, circular dichroism; TFA, trifluoroacetic acid; TFE, trifluoroethanol; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol.

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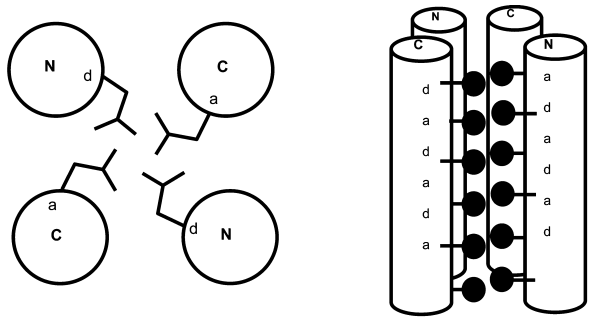

	abcdefg	abcdefg	abcdefg a
α_4H	Ac-GN ADE**L**YKE	**L**ED**L**QER	**L**RK**L**RKK **L**RSG $CONH_2$
α_4F_6	Ac-GN ADE**X**YKE	**X**ED**X**QER	**X**RK**X**RKK **X**RSG $CONH_2$

FIGURE 1: Diagram illustrating the hydrophobic core packing of the *de novo* designed antiparallel four-helix bundle proteins α_4H and α_4F_6. Left: View down the helical axis of the bundle illustrating the packing of leucine or hFLeu side chains for alternating "a" and "d" positions in the core. Right: Side view illustrating how the leucine or hFLeu residues (solid balls) from successive "a" and "d" positions pack to form layers through the core of the protein. The sequences of α_4H and α_4F_6 are shown below the diagrams (X = hFLeu).

about 26:1, as assessed by disulfide cross-linking. Other studies have examined the self-association of membrane-spanning α-helical peptides (*17, 22*); the α-helix propensities and spatial demands of various fluorinated amino acids (*23, 24*) have also been investigated.

We have described previously the design, synthesis, and characterization of a series of fluorinated antiparallel 4-α-helix bundle proteins designated α_4F_n (where $n = 2$, 4, 6) (*20, 21*). In the parent protein, α_4H, all six layers of the hydrophobic core were packed with leucine at the "a" and "d" positions of the canonical heptad repeat, as illustrated in Figure 1. In the fluorinated series of proteins either two, four, or six layers of the hydrophobic core were substituted with (*S*)-5,5,5,5′,5′,5′-hexafluoroleucine (hFLeu) at the "a" and "d" positions to introduce progressively more trifluoromethyl groups into the hydrophobic core of the protein; in the most extensively fluorinated protein, α_4F_6, a total of 48 trifluoromethyl groups packed all six layers of the helical bundle. Our design proved to be structurally robust, as all of the fluorine-substituted proteins were well folded and retained the intended 4-helix bundle structure. Moreover, we found that increasing the number of hFLeu residues increased $\Delta G_{\text{unfolding}}$ for the peptides in an almost linear fashion with a per-residue stabilization of $\Delta\Delta G \sim -0.3$ kcal/mol per residue, suggesting that fluorination could be used to fine-tune the stability of proteins.

Following upon our initial characterization of these proteins, we have now undertaken a further comparison of α_4F_6 and α_4H to determine the effect of fluorination on the biological and chemical stability of a protein. We have investigated how fluorination alters the stability of the protein toward degradation by proteases. We have compared the stability of the proteins toward unfolding by fluorinated and nonfluorinated solvents. Lastly, we have used ^{19}F NMR to test the prediction that the self-segregating properties of fluorocarbons should result in α_4F_6 and the α_4H proteins preferentially forming as homotetramers as opposed to mixed tetramers.

EXPERIMENTAL PROCEDURES

Materials. L-5,5,5,5′,5′,5′-Hexafluoroleucine was synthesized as described previously (*25*) and converted to fmoc- or t-Boc-protected derivatives by standard procedures. The sequences of the peptides α_4F_6 and α_4H used in this study are shown in Figure 1. These were synthesized by automated fmoc procedures (α_4H) or manual t-Boc procedures (α_4F_6) as described previously (*20, 21*). Trypsin and chymotrypsin from bovine pancreas were purchased from Boehringer Mannheim. Methanol, ethanol, 2-propanol, and trifluoroethanol (TFE) were purchased from Fisher Chemical Co. and were of HPLC grade.

Proteolysis Experiments. Stock solutions of peptides at a concentration of 1 mM in Milli-Q grade water were prepared and stored at −20 °C. A 0.5 mg/mL solution of tryptophan was prepared and stored at 4 °C. Solutions of chymotrypsin and trypsin were prepared at a concentration of 1 mg/mL in 1 mM HCl just prior to use. Proteolytic digestions were performed at 25 °C by mixing together 10 μL of peptide stock solution, 10 μL of 200 mM Tris-HCl buffer, pH 7.8, containing 20 mM $CaCl_2$, and 0.5 μL of tryptophan solution (as an internal standard). Digestion reactions were initiated by the addition of protease, 0.5 μL, to give a ratio of 80:1 (w/w) peptide to protease. At various times 5 μL of the reaction mixture was removed and quenched using an equal volume of 1 M HCl. Samples were stored at −20 °C until analysis by HPLC. All digests were repeated three times. Control reactions containing all reagents except protease were performed, and no degradation of the parent peptide was observed.

The products of proteolytic digestion were analyzed by reverse-phase HPLC using a C_{18} column. Samples were diluted to ∼40 μL with 5% acetonitrile and 0.15% acetic acid for injection onto the column and eluted with a linear gradient of 5−90% acetonitrile, containing 0.15% acetic acid. The relative amount of peptide remaining undigested at a given time was determined from the peak area relative to that of the internal standard tryptophan peak.

Solvent-Induced Unfolding of Peptides. Circular dichroism (CD) spectra of peptides (20 μM) were recorded with an Aviv 62DS spectropolarimeter at 25 °C. Mean residue ellipticities, Θ_M, were calculated using eq 1

$$\Theta_M = \Theta_{obs}/10lcn \quad (1)$$

where Θ_{obs} is the ellipticity measured in millidegrees, c is the molar concentration, l is the cell path length in centimeters, and n is the number of residues in the protein.

The hydrophobicity of the solvent–water mixtures (log P_{mix}) was calculated assuming that the hydrophobicity varied as a linear function of solvent composition according to eq 2

$$\log P_{mix} = X_{solvent} \log P_{solvent} + (1 - X_{solvent}) \log P_{water} \quad (2)$$

where X is the mole fraction of each solvent and log P values were obtained from previously reported values (*26, 27*).

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman XLA analytical ultracentrifuge equipped with scanning UV−visible optics (*28*). The initial peptide concentration was 200 μM in 10 mM potassium phosphate buffer, pH 7.0, containing various mole fractions of ethanol or TFE. The temperature was 293

K. The samples were centrifuged at 35000, 37500, 40000, 42500, and 45000 rpm and were judged to have obtained equilibrium when successive radial scans were indistinguishable. The data were fitted to either a monomer−tetramer equilibrium, assuming a monomer molecular weight of 3300 for α_4H and 3947 for α_4F_6, or to a single species using the Ultrascan software package (B. Demeler, University of Texas Health Science Center at San Antonio; www.ultrascan.uthscsa.edu). Partial specific volumes were calculated using the method of Cohn and Edsall (*29*): the partial specific volume of α_4H was calculated as 0.74 $cm^3\ g^{-1}$; the partial specific volume of α_4F_6 was calculated as 0.66 $cm^3\ g^{-1}$.

^{19}F NMR Spectra. ^{19}F NMR spectra were recorded using a Varian 400 MHz NMR spectrometer equipped with a ^{19}F probe. Peptide samples (0.5−2.0 mM) were prepared in 10% D_2O in a final volume of 0.5 mL and adjusted to pH 7.0 with NaOH unless otherwise noted. Spectra were recorded at 25 °C and were referenced to trifluoroacetate ion = 0 ppm.

RESULTS

Stability of Peptides toward Proteases. Previous studies of fluorous peptides have focused almost exclusively on the effect of fluorination on the physicochemical properties of the peptides. Relatively little is known about how fluorous residues may alter the interactions of proteins with other biological molecules. To help to answer this question, we investigated how the incorporation of hFLeu changes the susceptibility of the protein to proteolysis. Small proteins and peptides are often subject to degradation by proteases, either because they are unstructured or because their structures are sufficiently dynamic that transient unfolding renders them substrates for proteases. We have previously shown that fluorination results in a significant increase in ΔG_{unfold} and less dynamic structure for α_4F_6 compared with α_4H (*20*). We reasoned that this might confer resistance toward proteases, a useful property that could potentially be exploited to increase the effectiveness of various bioactive peptides that are subject to proteolysis.

We examined the stability of α_4H and α_4F_6 toward digestion by trypsin and chymotrypsin because both peptides contain a number of potential cut sites for each protease. Proteolytic digestions were set up at 25 °C using an 80:1 ratio (w/w) of peptide to protease, and the time course of the disappearance of the parent peptide was monitored by HPLC. As shown in Figure 2, α_4H was much more susceptible to proteolysis by either trypsin or chymotrypsin than α_4F_6. Whereas α_4H was almost completely digested by trypsin after 15 min, less than 50% of α_4F_6 had been digested after 110 min. Similarly, whereas over 90% of the α_4H peptide was degraded by chymotrypsin after 110 min, α_4F_6 is essentially impervious to digestion by chymotrypsin under the same conditions.

Stability of Peptides toward Solvent Denaturation. One of the more unusual properties of fluorocarbon solvents is their tendency to self-segregate into separate phases due to their low solubility in hydrocarbon solvents; for example, hexane and perfluorohexane are immiscible. As we have discussed previously (*3*), this property predicts that, by comparison with natural proteins, fluorous proteins should be resistant to denaturation by organic solvents. The rationale for this prediction is that fluorous amino acid side chains should be less soluble than hydrocarbon side chains in hydrocarbon solvent molecules. On the other hand, a fluorous protein should be more prone to denaturation by fluorinated solvents as the fluorous side chains would be expected to partition into the fluorinated solvent molecules.

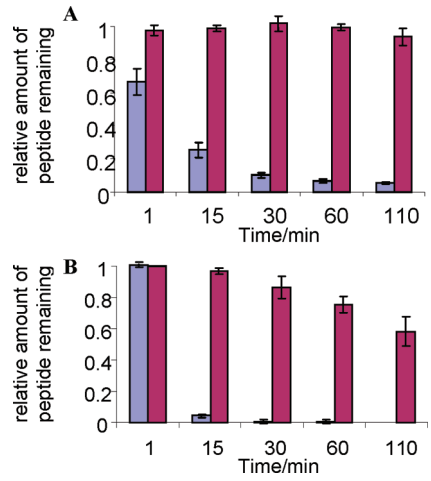

FIGURE 2: Proteolytic digestion of α_4H and α_4F_6. (A) Time course of digestion of α_4H (gray bars) and α_4F_6 (maroon bars) by chymotrypsin. (B) Time course of digestion of α_4H (gray bars) and α_4F_6 (maroon bars) by trypsin. The relative amount of intact peptide remaining at a given time was determined by reverse-phase HPLC.

To test this hypothesis, we have examined the solvent-induced unfolding of α_4F_6 and α_4H by water-miscible fluorocarbon and hydrocarbon organic solvents that vary in their hydrophobicity. We first investigated the unfolding of both peptides in response to increasing concentrations of the methanol, ethanol, and 2-propanol, which form a series of chemically similar but increasingly more hydrophobic water-miscible solvents. Peptide unfolding was monitored by following the increase in ellipticity at 222 nm due to the loss of α-helical structure in buffered solutions containing 20 μM peptide and increasing mole fractions of organic solvent. The results are shown in Figure 3 in which relative ellipticity of the each peptide is plotted against both mole fraction of alcohol and log *P* calculated for the solvent mixture.

The unfolding of α_4H follows a trend in which methanol, ethanol, and 2-propanol are increasingly effective denaturants. Methanol has little effect on the helicity of the peptide, whereas increasing concentrations of ethanol initially result in a slight increase in helicity before the peptide begins to unfold. 2-Propanol is the most effective denaturant, with the peptide becoming almost completely unfolded at high 2-propanol concentrations. In marked contrast, none of the alcohols were able to effectively unfold α_4F_6, and the peptide retained ~80% or greater helicity, as judged by ellipticity at 222 nm, under all solvent conditions. Indeed, at high concentrations of ethanol or 2-propanol the helicity of the peptide begins to increase slightly.

The fluorous effect predicts that the peptides should demonstrate the opposite behavior in fluorinated solvents, and therefore we examined the effect of trifluoroethanol (TFE), as shown in Figure 3D. As expected, TFE did not denature α_4H. Instead, there is an initial sharp increase in helicity with increasing mole fraction of solvent, which levels off after about $X_{TFE} = 0.15$. This is in accord with the well-

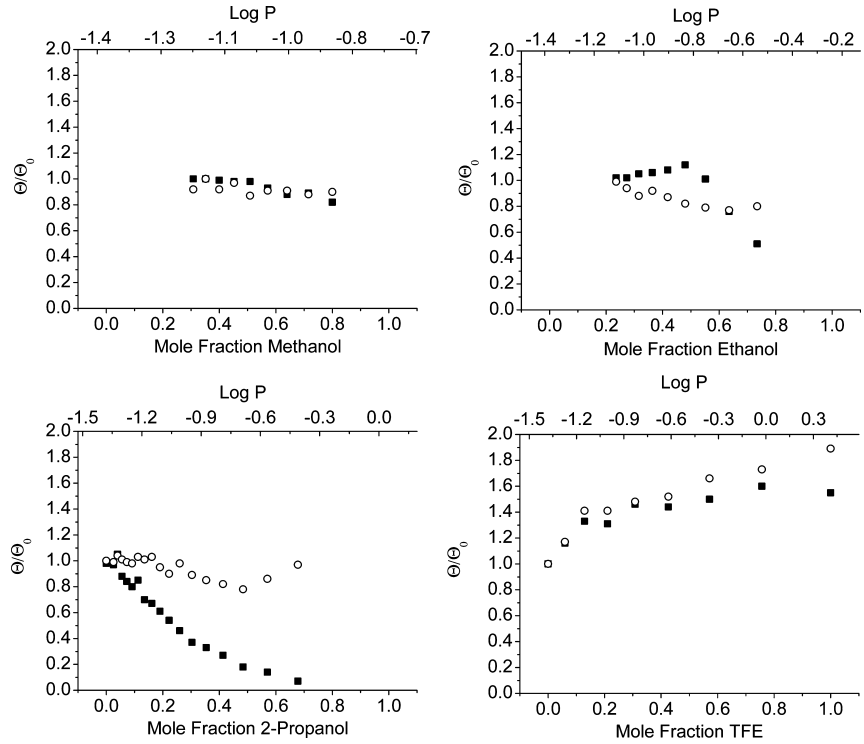

FIGURE 3: Helicity of α_4H and α_4F_6 in different alcohol−water mixtures. The relative helicity of α_4H (■) and α_4F_6 (○), defined as Θ_X/Θ_{water}, is plotted as a function of the mole fraction of alcohol (lower scale) and log *P* for the alcohol−water mixture (upper scale) for (top left) water–methanol mixtures, (top right) water−ethanol mixtures, (bottom left) water−2-propanol mixtures, and (bottom right) water−TFE mixtures.

known property of fluorinated alcohols to increase the helix content of partially structured peptides (*30–32*). However, contrary to predictions, TFE does not appear to denature α_4F_6, as judged by CD spectroscopy (Figure 3D); instead, it promotes an increase in helicity similar to that observed for α_4H.

Analytical Ultracentrifugation. CD spectroscopy is primarily sensitive to changes in secondary structure, but it is possible that the solvents may disrupt the tertiary structure of the 4-helix bundle while maintaining the helical conformation of the individual peptides. To investigate this possibility, we used sedimentation equilibrium analytical ultracentrifugation to examine the effect of these solvents on the apparent molecular weight of the proteins. We have shown previously that in the absence of organic solvents both α_4H and α_4F_6 form well-structured tetramers (*21*).

The sedimentation of the peptides was investigated in the presence of various concentrations of either ethanol or TFE. In 25% (v/v) ethanol, ($X_{ethanol} = 0.13$) the average apparent molecular weights of α_4H ($M_{r\,app} = 11000 \pm 1000$) and α_4F_6 ($M_{r\,app}$ 14000 ± 1000) are consistent with both peptides remaining predominantly tetrameric. However, at 50% (v/v) ethanol ($X_{ethanol} = 0.31$) the molecular weight of α_4H ($M_{r\,app} = 4500 \pm 500$) indicates that it is nearly completely dissociated into monomers, although from the CD spectrum it still appears to be helical. In contrast, the α_4F_6 peptide remains predominantly tetrameric in 50% ethanol ($M_{r\,app} = 12400 \pm 1000$), consistent with the prediction that hydrocarbon alcohols would not be effective denaturants of the fluorocarbon core of α_4F_6.

Initial centrifugation experiments in the presence of TFE showed a progressive decrease in the apparent molecular weight of both peptides with increasing mole fraction of TFE,

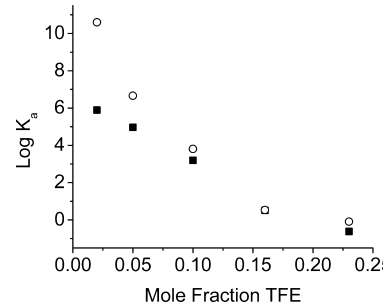

FIGURE 4: Plots of log K_a for α_4H (■) and α_4F_6 (○) as a function of the mole fraction of TFE. K_a was determined from analytical ultracentrifugation experiments as described in the text, assuming an equilibrium between monomeric and tetrameric peptide species.

suggesting that TFE was promoting dissociation of the peptides. Therefore, a more extensive series of sedimentation measurements were conducted at various concentrations of TFE ranging from 5% to 40% (v/v) ($X_{TFE} = 0.03-0.23$). Association constants were calculated from fits to the sedimentation traces obtained at multiple speeds assuming the sedimenting species were involved in a monomer−tetramer equilibrium. The association constants are plotted as a function of X_{TFE} in Figure 4. The data show a clear trend in which log K_a decreases as a function of increasing TFE concentration. Log K_a is significantly larger for α_4F_6 at low concentrations of TFE, consistent with the peptide forming a more stable 4-helix bundle in water. However, as the TFE concentration increases, log K_a for both peptides converge, such that the transition from predominantly tetrameric to predominantly monomeric species occurs for both peptides at $X_{TFE} = \sim 0.15$. There is no evidence that TFE preferentially dissociates α_4F_6, as would be predicted if "fluorous" partitioning of the hFLeu side chain into TFE was occurring.

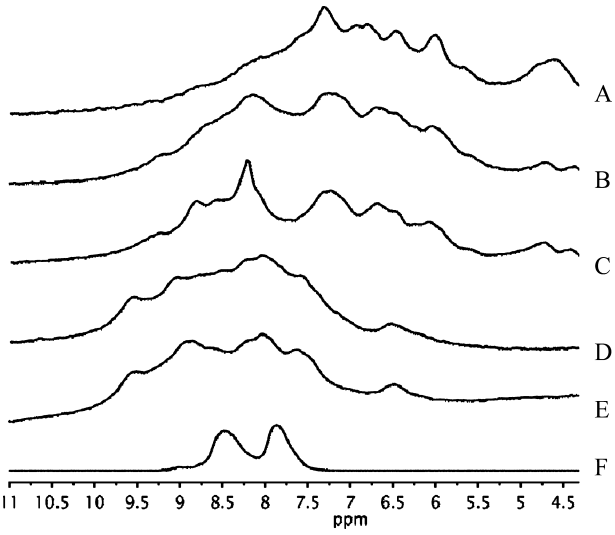

FIGURE 5: ^{19}F NMR spectra of α_4F_6 in the presence of increasing concentrations of α_4H. The spectra are (A) 2 mM α_4F_6, (B) 2 mM α_4F_6 and 0.67 mM α_4H, (C) 2 mM α_4F_6 and 2 mM α_4H, (D) 2 mM α_4F_6 and 6 mM α_4H, (E) 1 mM α_4F_6 and 6 mM α_4H, and (F) 0.5 mM α_4F_6, in 8 M urea, pH 2.0, where the peptide is fully denatured. All of the spectra [except (F)] were recorded at 25 °C and neutral pH and are referenced to trifluoroacetate.

Interestingly, the break in the plot of TFE concentration versus relative helicity of the peptides, shown in Figure 3, bottom right, occurs at the same concentration of TFE as the monomer−tetramer transition observed by ultracentrifugation. This suggests that the initial steep increase in Θ_{222} observed by CD results from the transition of the peptides from a 4-helix bundle structure, in which the α-helices are distorted by superhelical coiling, to monomeric α-helices which are free to adopt an undistorted structure.

Evaluation of Self-Segregating Properties of α_4H and α_4F_6. The self-segregating properties of small fluorinated molecules (*2, 33*) lead to the interesting prediction that it should be possible to design specific protein–protein interactions based on fluorous interactions between fluorinated amino acid residues. If this is the case, we would predict that α_4F_6 and α_4H should not form mixed heterotetramers because hydrocarbon−fluorocarbon interactions of Leu and hFLeu residues comprising the core of an α_4F_6−α_4H heterotetramer should be energetically unfavorable compared to the corresponding hydrocarbon−hydrocarbon interactions between Leu residues in the hydrophobic core of the α_4H tetramer. We have used ^{19}F NMR spectroscopy to probe for interactions between the α_4F_6 and α_4H peptides because the ^{19}F chemical shift is extremely sensitive to changes in the chemical environment (*34, 35*) that would occur if the peptides are interacting with each other.

The 1-D ^{19}F NMR spectrum of the folded α_4F_6 is shown in Figure 5 (spectrum A); for comparison, the spectrum of the peptide unfolded in 8 M GuHCl at pH 2 is also shown (spectrum F). As we have discussed previously, the folded spectrum exhibits a high degree of dispersity and appears more complex than can be accounted for simply by the signals from 12 CF_3^- groups in which the three fluorine atoms are chemically equivalent, suggesting that the fluorine atoms are in an anisotropic environment (*21*). As shown in Figure 5, titration of the α_4F_6 peptide with increasing concentrations of α_4H peptide results in a progressive change to the ^{19}F NMR spectrum of α_4F_6. As the concentration of α_4H increases, the spectrum of α_4F_6 becomes less disperse, and the envelope of signals shifts downfield. This suggests that the fluorine atoms are experiencing a less hydrophobic environment, consistent with the α_4F_6 peptide forming helical bundles with the less hydrophobic α_4H peptide. Clearly, if the peptides exhibited true self-segregating behavior, the addition of α_4H should have no effect on the spectrum of α_4F_6.

The CD spectra of the peptides did not change appreciably upon mixing the peptides (data not shown), indicating that the peptides remain highly helical. As a further test we analyzed the peptide mixtures by ultracentrifugation to confirm that mixing the peptides had not changed the oligomerization state, as might occur if, for example, a particularly stable heteropeptide dimer could form. The average apparent molecular weights were determined for three mixtures of α_4H and α_4F_6 at molar ratios of 3:1 ($M_{r\,app}$ 12700 ± 1000), 1:1 ($M_{r\,app}$ 15700 ± 1000), and 1:3 ($M_{r\,app}$ 15600 ± 1000), with the total peptide concentration constant at 200 μM. Within the limits of experimental error, the average molecular weight of each mixture is consistent with an ensemble of tetrameric peptides. We note that the accuracy of the technique does not allow us to determine the stoichiometry of the heterotetrameric complexes formed [e.g., to distinguish between an $(\alpha_4H)_2(\alpha_4F_6)_2$ tetramer and an $(\alpha_4H)(\alpha_4F_6)_3$ tetramer] because the differences in molecular weight would be too small to be detected.

From these observations taken together we conclude that the peptides do *not* exhibit the self-segregating behavior predicted by the fluorous effect.

DISCUSSION

Our experiments have demonstrated that incorporation of extensively fluorinated side chains, such as hFLeu, into a protein can significantly stabilize the protein against proteolysis and unfolding by "conventional" organic solvents. These properties can be attributed to the greater thermodynamic stability of α_4F_6 compared to α_4H, which, as we have discussed previously (*18, 19*), can be explained by the increase in hydrophobicity of the hFLeu side chains that comprise the hydrophobic core of the protein. Notably, however, our experiments have failed to find any evidence for the "fluorous" behavior predicted for α_4F_6, i.e., unfolding of the protein in fluorocarbon solvents, or self-segregation of the α_4F_6 protein from its hydrocarbon homologue.

We found that α_4H becomes increasingly more unfolded by methanol, ethanol, and 2-propanol, respectively. This trend is qualitatively described by the increasingly hydrophobic nature (as measured by log *P*) of these alcohols. This is consistent with the idea that the more hydrophobic solvents are better able to solvate the hydrophobic leucine residues that pack the protein core. That these alcohols do not effectively unfold α_4F_6 is not surprising, given that the fluorocarbon core of this protein is intrinsically more hydrophobic.

The slight increase in helicity seen for α_4H at intermediate concentrations of ethanol and for α_4F_6 at the highest concentrations of 2-propanol, and for both peptides in TFE, is a phenomenon that has also been observed for other proteins in high concentrations of alcohols (*36*). The

structuring effect is generally thought to be due to interactions between the alcohols and the protein that displace solvating water molecules (*30, 32*). This, in turn, decreases the local dielectric constant resulting in backbone hydrogen bonds of the α-helix becoming stronger and stabilizing the helix.

Contrary to our hypothesis, TFE did not unfold the α_4F_6 peptide in a manner analogous to the unfolding of α_4H by ethanol and 2-propanol. One might question whether TFE has a sufficiently high fluorine content that it may be legitimately considered a "fluorous" solvent, especially as it is also quite polar and has a dielectric constant similar to ethanol. Nevertheless, on the basis of the behavior of α_4H in ethanol and 2-propanol we might have expected that TFE would at least partially unfold α_4F_6. Instead, the peptide remains highly helical, although it is dissociated into monomers. Similar results were also obtained with the more highly fluorinated alcohol hexafluoro-2-propanol (HFIP) (L. M. Gottler, unpublished data).

Although it is unclear why TFE and HFIP do not unfold α_4F_6, we note that the helix-inducing properties of fluorinated alcohols such as TFE and HFIP are well-known and that the interactions of these solvents with proteins in aqueous media are complicated by the fact that they form nanoscale aggregates in water (*30, 31*). Indeed, although the unfolding of proteins in water−organic solvent mixtures is often simply ascribed to hydrophobic effects, the situation is actually more complex (*36*). A variety of physical parameters, such as log *P*, polarity index, dielectric constant, and E_T (*30*), have been examined for their ability to describe the effectiveness of solvents to unfold or inactivate proteins, but such measures do not appear to have a general predictive value (*27, 37*). Other empirically derived measures of solvent-induced unfolding such as denaturation capacity (*38*) have met with more success, but the behavior of fluorinated solvents remains poorly understood.

As discussed in the introduction, Kumar and co-workers observed self-segregating behavior with peptides designed to form a parallel coiled-coil dimer (*12*); one peptide, **H**, contained six Leu residues at the "a" and "d" positions, whereas the other peptide, **F**, contained six hFLeu residues. On the basis of their result, it is surprising that we did not observe self-segregation of α_4H and α_4F_6 because these peptides are of a similar size and contain the same number of hFLeu residues. Even substoichiometric concentrations of α_4H peptide perturbed the ^{19}F NMR spectrum of α_4F_6, arguing that there is little, if any, preference for the peptides to segregate; indeed, mixed peptide bundles may even be favored.

Interpretation of the experiment with **F** and **H** peptides was complicated by the fact that the **F** peptide was found, by analytical ultracentrifugation, to form a tetrameric coiled-coil structure rather than the intended dimeric structure. It was suggested that steric effects, imposed by the larger hFLeu side chain, prevented the **F** peptide from forming dimers (*10*). Thus it could be argued that steric effects, rather than its fluorous nature of the peptide *per se*, may have contributed to the self-segregating behavior. In the present case, one could also argue that steric effects might result in favorable packing of leucine and hFLeu residues within the hydrophobic core of the protein, which would offset any tendency for the peptides to self-segregate due to fluorous interactions. We also note that another difference between the **HH/FF** system and the α_4H/α_4F_6 system is that the former incorporated an asparagine residue at one of the "a" positions to enforce a parallel orientation of the helices. This may or may not contribute to the self-segregating behavior of the **H** and **F** peptides.

Clearly, the contrast between the self-segregating behavior of the **FF/HH** peptides and that of the α_4H/α_4F_6 peptides implies that there is still much to learn about modulating protein–protein interactions through fluorinated amino acids. On the other hand, the resistance exhibited by α_4F_6 to solvent denaturation and proteolytic degradation are certainly useful properties that may find biotechnological applications; for example, both our group and Kumar's group have recently shown that hFLeu can be used to modulate the properties of antimicrobial peptides (*39, 40*).

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