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Modulating Protein Structure with Fluorous Amino Acids: Increased Stability and Native-like Structure Conferred on a 4-Helix Bundle Protein by Hexafluoroleucine

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Abstract: There has recently been much interest in exploiting the unusual properties associated with fluorocarbons to modulate the physicochemical properties of proteins. Here we present a detailed investigation into the effect on structure and stability of systematically repacking the hydrophobic core of a model protein with the extensively fluorinated (fluorous) amino acid L-5,5,5,5′,5′,5′-hexafluoroleucine (hFLeu). The starting point was a 27-residue peptide, α_4-H, that adopts an antiparallel 4-α-helix bundle structure, and in which the hydrophobic core comprises six layers of leucine residues introduced at the "a" and "d" positions of the canonical heptad repeat. A series of peptides were synthesized in which the central two (α_4-F_2), four (α_4-F_4), or all six layers (α_4-F_6) of the core were substituted hFLeu. The free energy of unfolding increases by 0.3 (kcal/mol)/hFLeu on repacking the central two layers and by an additional 0.12 (kcal/mol)/hFLeu on repacking additional layers, so that α_4-F_6 is $\sim$ 25% more stable than the nonfluorinated protein α_4-H. One-dimensional proton, two-dimensional $^1H-^{15}N$ HSQC, and ^{19}F NMR spectroscopies were used to examine the effect of fluorination on the conformational dynamics of the peptide. Unexpectedly, increasing the degree of fluorination also appears to result in peptides that possess a more structured backbone and less fluid hydrophobic core. The latter only occurs in α_4-F_4 and α_4-F_6, suggesting that crowding of the hFLeu residues may restrict the amplitude and/or time scales for rotation of the side chains.

Introduction

Fluorocarbons possess unusual and useful physicochemical properties. They are extremely hydrophobic and chemically inert; properties that have been successfully exploited to develop inert materials such as Teflon, fire retardants, and anesthetics. Fluorocarbons also exhibit unusual phase segregation behavior. Extensively fluorinated molecules preferentially partition into fluorocarbon solvents, rather than hydrocarbon solvents—a phenomenon referred to as the "fluorous effect". Recently, the fluorous effect has found increasing use in organic synthesis to facilitate the purification of compounds tagged with long perfluorocarbon "tails".[1−4]

Fluorocarbons are quintessentially man-made compounds because, with one or two interesting exceptions,[5,6] fluorine is essentially absent from biological molecules. This raises the question of whether similarly novel and useful properties can be engineered into proteins that incorporate extensively fluorinated analogues of hydrophobic amino acids into their structures.[7,8] Therefore, recently there has been considerable interest in the properties of peptides incorporating fluorinated analogues of hydrophobic amino acids, in particular leucine and valine, within their hydrophobic cores.

Several studies have focused on synthesizing fluorous analogues of peptides designed to adopt dimeric α-helical coiled-coil structures typified by the "leucine zipper" region of the yeast transcription factor GCN4 dimerization domain. Incorporation of fluorinated analogues of leucine and valine have been shown to significantly increase the stability of coiled-coil peptides toward unfolding by heat and chemical denaturants.[9−15] The specific self-association of two α-helical peptides incorporating hexafluoroleucine (hFLeu) at the hydrophobic interface has also been accomplished.[9] Most recently, the fluorous effect

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has also been used to control the self-association of membrane-spanning α-helical peptides.[16]

Our laboratory has focused on a different structural motif, the antiparallel 4-α-helix bundle, in our investigations into the effect of incorporating fluorous amino acids on the physical properties of proteins.[17] The 4-α-helix bundle fold is a structurally robust motif that is widely distributed in Nature, being found in proteins such as the RNA-binding protein Rop, transcription factors, and cytokines and in the cores of many dinuclear metalloenzymes.[18−21] On the basis of de novo design principles established by DeGrado and co-workers, we have designed peptides that adopt an antiparallel 4-helix bundle fold in which the hydrophobic core is exclusively packed with leucine residues. This allows us to investigate the effect on protein structure and stability of systematically replacing leucine with the fluorous analogue hFleu.

Here we report the synthesis and characterization of series of peptides, designated α_4-F_n, (where $n = 2, 4, 6$) in which 2, 4, or 6 layers of the hydrophobic core have been repacked with hFLeu, as shown in Figure 1. This introduces progressively more trifluoromethyl groups into the hydrophobic core of the protein: thus α_4-F_2 contains 16 trifluoromethyl groups packed in the central 2 layers of the 4-helix bundle; α_4-F_4 contains 32 trifluoromethyl groups packed in the central 4 layers; and finally in α_4-F_6 a total of 48 trifluoromethyl groups pack all 6 layers. We find that increasing the number of hFLeu residues increases ΔG_{unfold} for the peptides in an almost linear fashion. And, unexpectedly, that increasing the number of hFLeu residues appears to result in better-structured peptides as judged by 1-D ^{1}H, ^{19}F NMR spectra and 2-D ^{1}H−^{15}N HSQC NMR spectra.

Materials and Methods

Materials. Rink Amide resin, Fmoc-protected and *t*-Boc-protected amino acids, *N*-hydroxybenzotriazole (HOBt), and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from NovaBiochem. Peptide synthesis grade *N*-methylpyrrolidinone (NMP), *N*,*N*-dimethylformamide (DMF), and trifluoroacetic acid (TFA) and ACS grade *N*,*N*-diisopropylethylamine (DIEA) and piperidine were purchased from Fisher. GuHCl, 99%+ purity, was obtained from Gibco BRL. L-5,5,5,5′,5′,5′-Hexafluoroleucine was synthesized as described previously [22] and converted to Fmoc- or *t*-Boc-protected derivatives by standard procedures.

Peptide Synthesis. Peptide α_4-H was synthesized using Fmoc-protected amino acids by standard protocols on an ABI 433A automated synthesizer. Peptides were cleaved from the resin by stirring for 2 h at room temperature with 10 mL of a mixture of 90% TFA, 3% ethanedithiol, 5% thioanisole, and 2% anisole. The resin beads were filtered off and rinsed with an additional 4 mL of TFA. TFA was evaporated from the filtrate under a stream of nitrogen, and 50 mL of cold ethyl ether was added to precipitate the peptide. The crude peptide was collected by filtration on a fritted funnel, dissolved in 10% aqueous acetic acid, and lyophilized.

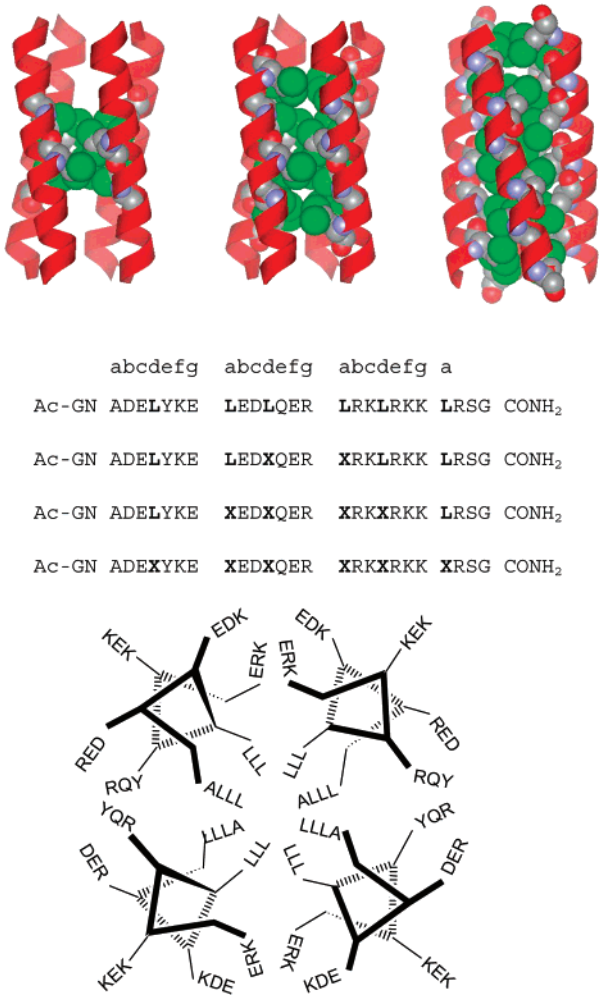

Figure 1. (Top) Models showing the packing of hFLeu in the hydrophobic cores of α_4-F_2, α_4-F_4, and α_4-F_6 peptides. The trifluoromethyl groups are represented by green spheres). (Middle) Sequences of α_4-H, α_4-F_2, α_4-F_4, and α_4-F_6 peptides (X = hFLeu). (Bottom) Helical wheel diagram illustrating the side-chain interactions between helices for the α_4-H sequence.

Peptides α_4-F_2, α_4-F_4, and α_4-F_6 were synthesized using *t*-Boc-protected amino acids for Merrifield manual solid-phase synthesis on MBHA resin; couplings were performed using the in situ neutralization/HBTU protocol described by Schnolzer et al.,[23] typically on a 0.25 mM scale. The peptide was cleaved from the resin using "high" HF conditions.

Peptide Purification. Peptides were redissolved at ~10 mg/mL in 10% aqueous acetic acid and purified by reverse-phase HPLC on a Waters semipreparative C_{18} column equilibrated in 0.1% TFA and eluted with a linear gradient of 0−90% acetonitrile containing 0.1% TFA. The peptides were determined to be pure by analytical HPLC and MALDI-TOF mass spectrometry: expected mass for peptide α_4-H = 3299.8 amu, detected mass = 3300.1 amu; expected mass for peptide α_4-F_2 = 3515.7 amu, detected mass = 3515.8 amu; α_4-F_4 = 3732.7 amu, detected mass = 3738.0 amu; α_4-F_6 = 3948.7 amu, detected mass = 3950.0 amu. The concentration of the peptides was determined by their absorbance at 275 nm due to the single tyrosine residue, using an extinction coefficient of 1420 cm^{-1} M^{-1}.

Circular Dichroism. Circular dichroism (CD) spectra of peptides were recorded with an Aviv 62DS spectropolarimeter at 25 °C. Mean residue ellipticities, [θ], were calculated using

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$$[\theta] = \theta_{obsd}/10lcn \quad (1)$$

where θ_{obsd} 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. To examine the unfolding of the peptide by GuHCl, two stock solutions were prepared containing 200 μM peptide (concentration of monomer) in 100 mM potassium phosphate buffer, pH 7.0, one with 8.0 M GuHCl and one without. The solutions were mixed in various proportions to obtain samples at different concentrations of GuHCl, and after equilibration for several minutes the ellipticity at 222 nm was measured.

NMR Spectroscopy. Peptides were dissolved in 10 mM potassium phosphate buffer, pH 7.0 containing 10% D_2O at concentrations ranging between 0.2 and 1 mM. One-dimension proton and ^{19}F spectra were acquired on a Bruker 500 MHz spectrometer using standard solvent suppression pulse sequences where appropriate. Natural abundance $^{1}H-^{15}N$ HSCQ spectra were acquired using standard pulse sequences on a Bruker 600 MHz spectrometer equipped with a cryogenic probe.

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman XLA analytical ultracentrifugation equipped with scanning UV−visible optics.[24] Initial peptide concentrations ranged from 500 to 70 μM in 100 mM phosphate buffer, pH 7.0. The temperature was 298 K. The samples were centrifuged at 35 000, 38 000, 41 000, 44 000, and 47 000 rpm and were judged to have obtained equilibrium when successive radial scans were indistinguishable. The data were fitted to either monomer−*n*-mer equilibria 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:[25] the partial specific volume of α_4-H was calculated as 0.74 cm^3g^{-1}; the partial specific volumes of α_4-F_2, α_4-F_4, and α_4-F_6 were calculated as 0.71, 0.69, and 0.66 $cm^3 g^{-1}$, respectively.

Curve Fitting. The denaturation profiles for the peptides were analyzed assuming a two-state equilibrium between unfolded monomeric peptide and folded, tetrameric bundle, with no significantly populated intermediates being present, as described previously.[17,26] Igor Pro software (Wavemetrics, Inc.) was used to fit the denaturation curves.

Results

Choice of Model Structure. The choice of protein structure is an important consideration in the design of experiments to measure the effects of incorporating fluorous amino acid residues on the stability of a protein. We chose to focus on the antiparallel 4-helix bundle structure as such proteins are widely distributed in Nature and have been the subject of previous de novo design efforts.[21,27,28] The packing of 4 leucine side chains in each layer of the bundle results in a relatively large hydrophobic core that may better accommodate the increase in volume that occurs when leucine is substituted by hFleu. As we and others have discussed previously,[17,29] antiparallel bundles are more conformationally stable toward changes in the hydrophobic core than are parallel bundles, in which subtle changes in sequence or buffer conditions can readily change the oligomerization state between dimer, trimer, and tetramer.[28,30] This phenomenon has also been encountered in the design of parallel fluorous coiled-coils.[9]

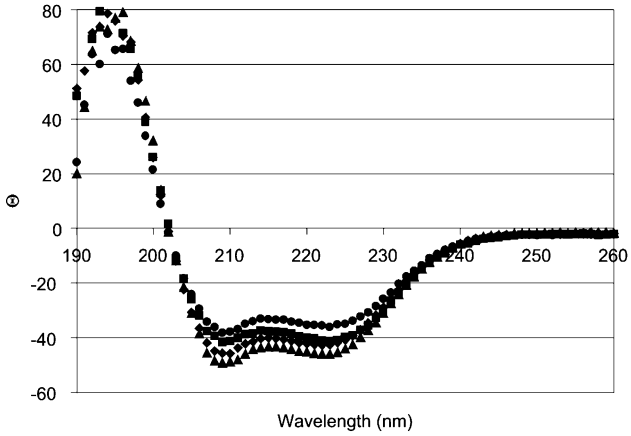

Figure 2. CD spectra of α_4-H (◆) α_4-F_2 (▲), α_4-F_4 (●), and α_4-F_6 (■) (peptide concentration, 30 μM) in 100 mM potassium phosphate buffer, pH 7.0.

We have previously described the design of a 27-residue peptide, α_4-H, that adopts an antiparallel 4-helix bundle topology.[17] α_4-H comprises 3 heptad repeats and incorporates leucines at each of the hydrophobic "a" and "d" positions. Initially, we investigated the effect on protein stability of substituting the central 2 layers, positions 13 and 17, of α_4-H with hFLeu. The resulting peptide, called α_4-F_2, was stabilized against unfolding by GuHCl by approximately 2.5 kcal/mol, a value that could be explained by the increased hydrophobicity of the hFLeu side chain. To better understand the effects of fluorination on the structure and stability of proteins, we decided to investigate the effect of progressively increasing the number of hFLeu residues in the peptide. This was accomplished by the design of two further peptides in the series, α_4-F_4 in which positions 10, 13, 17, and 20 are substituted with hFLeu and α_4-F_6 in which positions 6, 10, 13, 17, 20, and 24 are substituted with hFLeu. These peptides were synthesized manually using standard protocols and *t*-Boc-protected amino acids as described in Materials and Methods.

Comparison and Characterization of Peptides. The CD spectra obtained at neutral pH for all four peptides, α_4-H, α_4-F_2, α_4-F_4, and α_4-F_6, are shown in Figure 2. All the peptides exhibited spectra characteristic of extensively α-helical secondary structure with minima at 208 and 222 nm. Although there is some variation in the intensity of the spectra between the different peptides (all the spectra were recorded at 30 μM concentration, using the absorption of the single tyrosine residue to determine concentration), we feel it is unlikely that these differences are indicative of significant differences in the secondary structure of the peptides.

Analytical Ultracentrifugation. Analytical sedimentation equilibrium centrifugation was used to investigate the oligomerization states of the peptides. The initial peptide concentrations varied between 70 and 500 μM. At high peptide concentrations the peptides sedimented as homogeneous species with apparent molecular weights that are close to those expected for a tetrameric structure. These data are summarized in Table 1. Fits of the curves assuming either a trimeric or pentameric

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Table 1. Determination of Oligomerization State of Peptides by Equilibrium Analytical Ultracentrifugation

peptide	calcd M_r for tetramer	exptl M_r
α_4-H	13 200	11 900 ± 1000
α_4-F_2	14 060	13 400 ± 1000
α_4-F_4	14 926	16 300 ± 1000
α_4-F_6	15 788	17 200 ± 1000

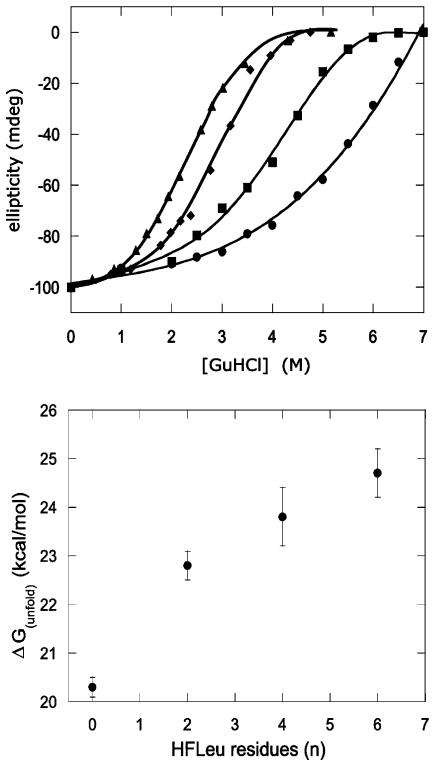

Figure 3. Unfolding of peptides in GuHCl. (Top) Plots of ellipticity versus GuHCl concentration for α_4-H (◆), α_4-F_2 (▲), α_4-F_4 (●), and α_4-F_6 (■). Unfolding was followed using CD spectroscopy to monitor changes in ellipiticity at 222 nm, and the buffer was 100 mM potassium phosphate, pH 7.0; the temperature was 25 °C. To follow unfolding, it was necessary to use different concentrations of each peptide (between 50 and 200 μM); therefore the ellipticity values have been arbitrarily scaled to be between −100 and 0 mdeg to facilitate comparison. (Bottom) Variation of $\Delta G°_{unfold}$ as a function of the number of hFLeu residues.

structure for the peptides exhibited significant systematic deviation of the residuals from the data. At lower concentrations the sedimentation curves were better described by a monomer−tetramer equilibrium; however, attempts to extract equilibrium constants from the fits yielded unreliable results.

Denaturation Experiments. The GuHCl-induced unfolding of the peptides was followed using CD spectroscopy by monitoring the ellipticity of the peptides at 222 nm as a function of increasing GuHCl concentration. Because the unfolding of the peptides is concentration-dependent, concentrations of peptide between 50 and 200 μM were used. The most reliable data were obtained for α_4-H and α_4-F_2 at 200 μM concentration (as described previously[17]), whereas for the more stable α_4-F_4 better data were obtained at 100 μM. These peptides exhibit well-defined unfolding transitions, as shown in Figure 3, that appear to be well-fitted by assuming a two-state equilibrium between unstructured monomeric peptides and folded tetrameric helical bundle. From fits to these curves the free energy of unfolding, $\Delta G°_{unfold}$, and the proportionality constants, m, were determined; these data are presented in Table 2. However, the

Table 2. Summary of Thermodynamic Parameters Determined from GuHCl-Induced Unfolding of Peptides

peptide	$\Delta G°_{unfold}$ (kcal/mol)	m ((kcal/mol)/M_{GuHCl})
α_4-H	20.3 ± 0.2	2.4 ± 0.1
α_4-F_2	22.8 ± 0.3	2.6 ± 0.2
α_4-F_4	23.8 ± 0.6	1.6 ± 0.1
α_4-F_6	24.7 ± 0.5	1.4 ± 0.1

upper baseline for the unfolding transition of α_4-F_6 could not be observed even at 50 μM peptide, the lowest concentration at which changes in ellipticity could be reliably followed. Therefore, the value of $\Delta G°_{unfold} = 24.7 \pm 0.5$ kcal/mol must be regarded as an estimate. As shown in Figure 3(bottom), there is an initial increase in the stability of the 4-helix bundle of about 2.5 kcal/mol upon incorporating the first two hFLeu residues corresponding to an additional stabilization approximately 0.3 kcal/mol per hFLeu residue. The addition of further hFLeu residues results in a smaller and almost linear further increase in $\Delta G°_{unfold}$ of 0.12 kcal/mol per hFLeu residue.

NMR Spectroscopy. The CD and ultracentrifuge data described above established that the substitution of hFLeu for Leu in the α_4 peptides does not result in gross structural changes to the peptides in that they remain tetrameric and extensively helical. To obtain more detailed information on the effects of fluorination on the structure of the peptide, we turned to NMR spectroscopy. Initially, 1-D proton NMR spectra of α_4-H, α_4-F_2, α_4-F_4, and α_4-F_6 were recorded in 10% D_2O at pH 7.0. These spectra are shown in Figure 4. All the spectra are reasonably well dispersed and typical of folded small proteins. There are noticeable differences in the amide region where α_4-F_2 seems to exhibit somewhat sharper peaks with narrower chemical shift dispersion than α_4-H, whereas both α_4-F_4 and α_4-F_6 exhibit a wider range of amide chemical shifts. In the aliphatic region the leucine methyl groups are clearly identified as a broad resonance at 0.5 ppm in the spectrum of α_4-H that, as expected, is reduced in relative intensity in α_4-F_2 and α_4-F_4, and absent in α_4-F_6. With the possible exception of α_4-F_2 the chemical shifts of the individual methyl groups of the leucine residues in the peptide are not resolved. This is not surprising given the homogeneous nature of the hydrophobic core that comprises only leucine residues.

To better compare the spectral changes in the amide region caused by repacking the hydrophobic core with hFLeu, we recorded natural abundance 2-D $^1H-^{15}N$ HSQC spectra of α_4-H and α_4-F_6. The spectra were recorded at 600 MHz at room temperature in 10% D_2O, at pH 7.0, with peptide concentration ~3 mM and are shown in Figure 5. Both spectra are characteristic of well-structured small proteins. Many of the peaks in the two spectra either overlap one another, as might be expected given the similarity between both the sequences and the overall structures of the two peptides. However there are a number of differences between the two spectra. In particular, greater chemical shift dispersion is observed in the α_4-F_6 resonances compared to α_4-H, with many of the intense resonances in α_4-H being resolved into individual peaks in α_4-F_6. This is particularly evident in the region between 111 and 116 ppm in the ^{15}N dimension. Furthermore, although two glycine residues are present in these peptides, at the N- and C-termini, only one is observed in α_4-H (in characteristic glycine 111−112 ppm region in ^{15}N dimension), both are observed in α_4-F_6. This suggests that end-fraying effects may be reduced in α_4-F_6.

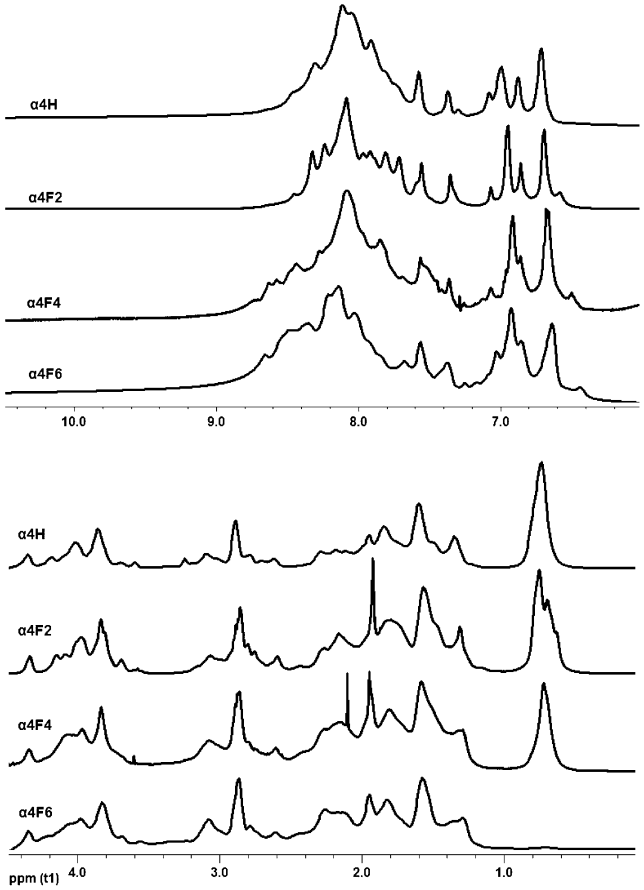

Figure 4. Proton NMR spectra of the amide and aromatic region (top panel) and aliphatic region (bottom panel) of the α_4 peptides. Spectra from top to bottom are of α_4-H, α_4-F_2, α_4-F_4, and α_4-F_6 and were recorded at 25 °C, pH 7.0 in 10% D_2O.

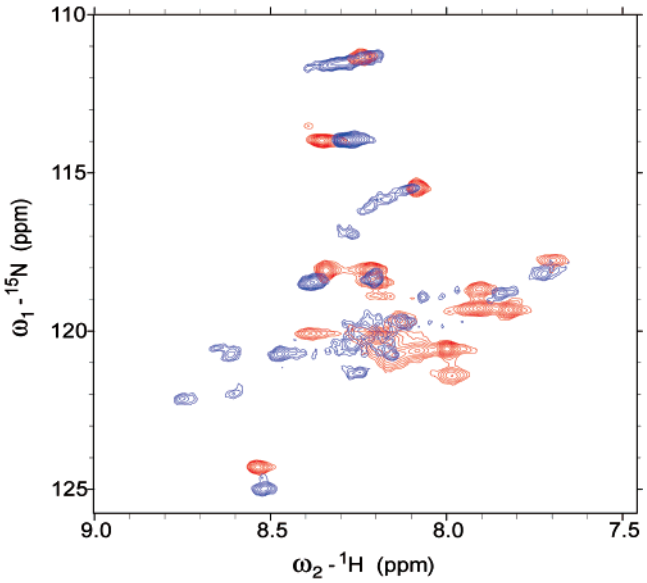

Figure 5. Overlaid 2D ^{1}H−^{15}N HSQC NMR spectra comparing the amide regions of α_4-H (red) and α_4-F_6 (blue). The spectra were recorded using unlabeled (natural abundance ^{15}N) peptides at 25 °C, pH 7.0 in 10% D_2O using a cryogenic probe.

Overall, 18 amide peaks are clearly resolved in the α_4-H spectrum, whereas for α_4-F_6 ~24 amide resonances are visible, which is in better agreement with expected 26 backbone amide resonances. We note that some of these resonances are expected to be absent if their protons are exchanging rapidly with the solvent. The large number of resonances and greater chemical shift dispersion tends to suggest that the α_4-F_6 peptide is more structured than α_4-H. There are also 5−6 peaks that exhibit clear differences in chemical shifts, indicating that these sites experience different chemical environments. These are evident in the α_4-F_6 spectrum between 8.8 and 8.5 ppm, and in the α_4-H spectrum between 7.8 and 8.0 ppm in the ^{1}H dimension. These differences could either arise from differences in the conformation of the peptides or be a direct effect on the chemical shift of some residues caused by the substitution of hydrogen by fluorine.

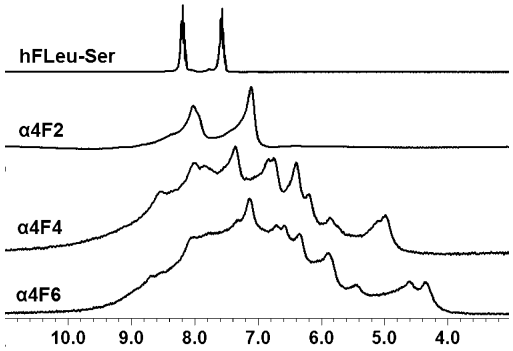

Figure 6. ^{19}F spectra of (from top to bottom) hFLeu-Ser dipeptide, α_4-F_2, α_4-F_4, and α_4-F_6 recorded at 25 °C and neutral pH. All the spectra are referenced to TFA.

Last, we have used ^{19}F NMR spectroscopy to examine the effect of fluorination on the hydrophobic core of the peptides. ^{19}F nucleus exhibits a wide range of chemical shifts that are sensitive to changes in environment, a property that has been exploited in various studies on protein structure.[31−35] One-dimensional ^{19}F spectra were recorded for α_4-F_2, α_4-F_4, and α_4-F_6 at pH 7.0 at 25 °C and referenced to trifluoroacetate as an internal standard. To aid in interpreting these spectra, the ^{19}F spectrum of a simple dipeptide, hFLeu-Ser, was also recorded under the same conditions. The spectra are shown in Figure 6.

The spectrum of hFLeu-Ser shows two resonances for the diastereotopic trifluoromethyl groups at 7.8 and 8.2 ppm, which are split into multiplets due to coupling between the fluorines and the γ-hydrogen. The ^{19}F spectrum of α_4-F_2 is quite simple, showing only two peaks that presumably correspond to the two diastereotopic CF_3 groups. The peaks are broadened, as expected for fluorine incorporated in a macromolecule, and somewhat asymmetric—this may be due to the two hFleu residues being in slightly different environments with distinct rotational averaging properties. The centers of the peaks are shifted to slightly higher field (7.1 and 8.0 ppm relative to TFA) than in the dipeptide, consistent with the fluorines now being in a more hydrophobic environment.

The incorporation of additional hFLeu residues dramatically changes the spectra of α_4-F_4 and α_4-F_6. The spectra become much more complex, even allowing for the fact that there are more fluorinated residues in the peptides. Most interestingly,

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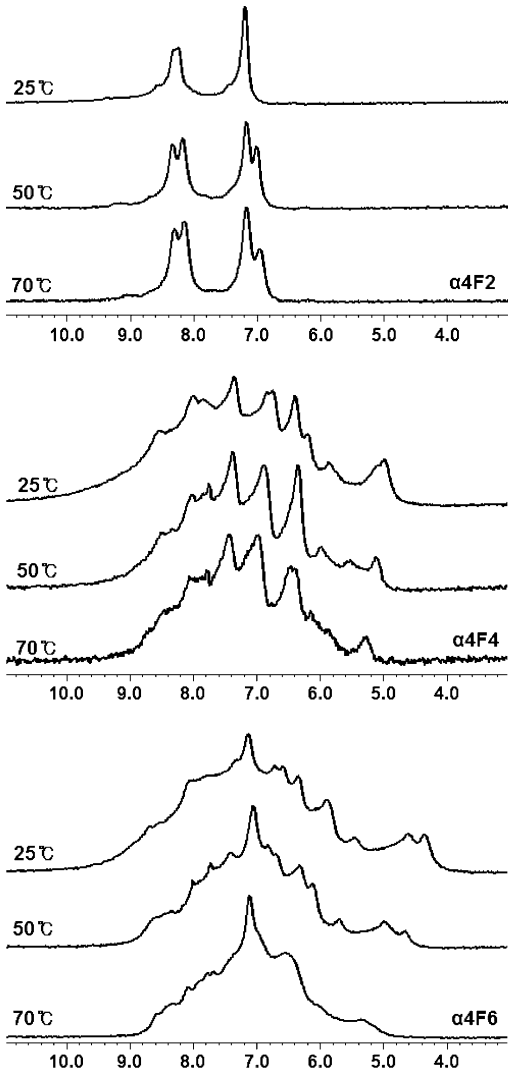

Figure 7. ^{19}F spectra of α_4-F_2, α_4-F_4, and α_4-F_6 recorded at 25, 50, and 70 °C. (Top) α_4-F_2; (middle) α_4-F_4; (bottom) α_4-F_6. All the spectra are referenced to TFA.

the range of chemical shifts increases to cover the region from 4 ppm to ~9 ppm in the case of α_4-F_6. These changes suggested that the additional hFLeu residues lead to a situation in which side chains adopt multiple well-defined conformations that interchange slowly at the NMR time scale.

To gain more insight into the unexpectedly complex ^{19}F spectra of α_4-F_4 and α_4-F_6 the spectra of all three peptides were recorded at 50 and 70 °C (Figure 7). At higher temperatures the spectra of all the peptides become sharper, as would be expected due to decreased solvent viscosity, and the spectral envelope is narrowed. For α_4-F_2 the two sets of hFLeu resonances become much sharper and are clearly resolved at 50 °C. The spectra of both α_4-F_4 and α_4-F_6 become somewhat sharper and some of the peaks begin to coalesce; this is more noticeable for the spectrum of α_4-F_4. The dispersion becomes smaller, suggesting faster exchange (relative to NMR time scales) among the "frozen out" side chain conformations. None of the peptides undergoes thermal unfolding, as judged by CD spectroscopy, below 95 °C (data not shown). Therefore these spectral changes are most consistent with the hydrophobic core becoming more dynamic at higher temperatures.

Discussion

Several studies have examined the effects of introducing extensively fluorinated amino acid residues into peptides and largely confirmed the prediction that fluorination would increase the stability of the peptides against unfolding and that fluorous interaction could mediate specific protein−protein interactions.[9−15,17] This study is the first in which the effects of systematically increasing the degree of fluorination on the stability and structure of a folded peptide have been investigated.

First, we have demonstrated that it is possible to significantly increase the volume of the hydrophobic core of the 4-helix bundle without grossly perturbing the structure. Thus, the introduction of 6 hFLeu residues in α_4-F_6 increases the volume by about 30%; however, the peptide retains its helical tetrameric structure. As we have discussed previously,[17] the antiparallel 4-helix bundle is more tolerant of amino acid substitutions in the hydrophobic core than parallel coiled-coil peptides, in which subtle changes in hydrophobic packing have been shown to change the oligomerization state of the peptide.[30]

Previous studies have produced estimates for the additional stability imparted by incorporating a trifluoromethyl group into a peptide that vary quite significantly. Much of the variation may be explained by differences in experimental design and probably depend on the context in which the fluorinated residue is introduced. By systematically increasing the number of hFLeu residues in the 4-helix bundle, we have been able to obtain a better appreciation for how the degree of fluorination affects the stability of the bundle.

As expected, increasing the number of fluorous residues increases $\Delta G^\circ_{\text{unfold}}$ in an approximately linear fashion, although the initial increase in stability imparted by two hFLeu residues in α_4-F_2 ($\Delta\Delta G^\circ_{\text{unfold}} = 0.3$ (kcal/mol)/residue) is significantly larger than the additional stability imparted by introducing more hFLeu residues in α_4-F_4 and α_4-F_6 ($\Delta\Delta G_{\text{unfold}} = 0.12$ (kcal/mol)/residue). We previously measured the free energy of partitioning of the HFLeu side chain from *n*-heptanol into water as $\Delta\Delta G = 0.4$ kcal/mol.[17] The increased stability imparted by fluorination appears to be readily ascribed to the more hydrophobic nature of hFLeu, rather than specific "fluorous" interactions between the hFLeu side chains in the hydrophobic core. Indeed, one might expect that fluorous interactions would provide additional stability to the more extensively fluorinated peptides, resulting in an increase, rather than a decrease, in the per-residue stability for α_4-F_4 and α_4-F_6.

Whereas the stabilizing effect of increasing the number of hFLeu residues was predicted, the NMR evidence obtained regarding the structuring of the backbone and the observed "freezing out" of structured side chain states within the hydrophobic core was not anticipated. Two lines of evidence point to the hydrophobic core of α_4-F_6 being less conformationally dynamic than α_4-H. First, HSQC spectra show that α_4-F_6 has more well-resolved amide resonances than α_4-H, Second, ^{19}F spectra of α_4-F_6 and α_4-F_4 exhibit a complex and spectrally disperse envelop of resonances that become somewhat sharper and less disperse at higher temperatures. These changes in the spectra are consistent with the core beginning to melt at higher temperature to give a more dynamic molten globule-like interior. Interestingly, the incorporation of 2 hFLeu residues does not

seem to be sufficient to introduce this structural change, as α_4-F_2 exhibits a fairly simple ^{19}F spectrum even at room temperature.

We previously examined the binding of ANS to α_4-H and α_4-F_2, which is a widely applied test for the molten globule state.[17] We saw no evidence for intercalation of ANS into either peptide and concluded that neither peptide was molten globule-like. However, ANS binding is not a definitive test for molten globules, and indeed proteins may exhibit a range of dynamic behavior from completely ordered "natural" fold through to the highly mobile and poorly specified molten globule state. It seems reasonable that the α_4 series of peptides may exhibit a range of internal dynamic behavior while maintaining well-formed secondary structure. And although the α_4-F_4 and α_4-F_6 peptides seem to exhibit better packed, less dynamic interiors, this does not necessarily imply that the hFLeu residues pack in a unique arrangement as would be expected in a natural protein.

The unexpected ordering imposed by the inclusion of 4 or 6 hFLeu residues may explain why the α_4-F_4 and α_4-F_6 peptides show smaller per-residue increases in stability than α_4-F_2. The hFLeu side chains in α_4-F_2 retain their mobility in the folded structure, thereby maximizing the free energy of transfer from water into the hydrophobic core. In contrast, the burying of hFLeu side chains in the hydrophobic cores of α_4-F_4 and α_4-F_6 is presumably accompanied by a loss of entropy associated with reduced side-chain mobility so that free energy of folding is relatively less favorable.

It is unclear why the incorporation of multiple hFLeu residues should induce ordering of the hydrophobic core. One explanation is that there is simply less room for the larger hFLeu side chains to rotate within the hydrophobic core. Thus, packing 2 layers of the core with hFLeu does not restrict rotation sufficiently, but packing further layers does impede the ability of the central layers to rotate, leading to a less dynamic structure and the slowing down of internal motions sensed by the ^{19}F NMR spectra. A further possibility is that dipole−dipole interactions arising from the relatively large permanent dipole moments of the trifluoromethyl groups may become an important factor in the interactions of the hFLeu residues when they are pre-organized in the hydrophobic core of the protein. This so-called "polar" hydrophobicity of fluorocarbons[36] has been invoked to understand the interactions of fluorinated carbohydrate analogues with enzymes, but has not to our knowledge been considered in the context of fluorous proteins. A fuller explanation will most likely have to await the solution of an X-ray structure for α_4-F_6 and more detailed thermodynamic studies on these peptides.

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Supporting Information Available: Analytical ultracentrifugation of α_4 peptides, figures showing representative sedimentation equilibrium traces obtained from analytical ultracentrifugation of several peptides, and a table listing molecular weights of several peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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