Fluorous Effect in Proteins: *De Novo* Design and Characterization of a Four-α-Helix Bundle Protein Containing Hexafluoroleucine†

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ABSTRACT: Several studies have demonstrated that proteins incorporating fluorinated analogues of hydrophobic amino acids such as leucine and valine into their hydrophobic cores exhibit increased stability toward thermal denaturation and unfolding by guanidinium chloride. However, estimates for the increase in the thermodynamic stability of a protein ($\Delta\Delta G_{unfold}$) afforded by the substitution of a hydrophobic amino acid with its fluorinated analogue vary quite significantly. To address this, we have designed a peptide that adopts an antiparallel four-helix bundle structure in which the hydrophobic core is packed with leucine, and investigated the effects of substituting the central two layers of the core with L-5,5,5,5′,5′,5′-hexafluoroleucine (hFLeu). We find that $\Delta\Delta G_{unfold}$ is increased by 0.3 kcal/mol per hFLeu residue. This is in good agreement with the predicted increase in $\Delta\Delta G_{unfold}$ of 0.4 kcal/mol per residue arising from the increased hydrophobicity of the hFLeu side chain, which we determined experimentally from partitioning measurements on hFLeu and leucine. The increased stability of this fluorinated protein may therefore be ascribed to simple hydrophobic effects, rather than specific "fluorous" interactions between the hFLeu residues.

The incorporation of non-natural amino acid residues into proteins provides an opportunity to modify the physicochemical properties of a protein such as its thermal stability and free energy of folding (*1*−*4*). Because of this, there has recently been considerable interest in incorporating extensively fluorinated (fluorous) analogues of hydrophobic amino acids into proteins (*5*−*11*). On the basis of the properties of small fluorocarbon molecules, fluorous proteins should exhibit greater stability toward heat and chemical denaturants (*2, 12*). In addition, extensively fluorinated proteins are expected to exhibit self-segregating properties (the so-called fluorous effect), leading to the possibility of engineering novel protein−protein recognition elements mediated through fluorocarbon−fluorocarbon interactions. The self-segregating properties of small fluorous molecules have already been extensively exploited in organic synthesis in purifying products from complex reaction mixtures (*13, 14*).

Initial studies on the fluorous effect in proteins used peptides based on the "leucine zipper" region of the yeast transcription factor GCN4 that incorporated trifluoromethylleucine (tFLeu)[1] and/or trifluoromethylvaline (tFVal) at hydrophobic positions (*6*, *9*, *10*). As predicted, these peptides exhibited increased stability toward thermal and chemical denaturants. Importantly, the fluorous GCN-4 analogues retain their ability to bind DNA. However, the tFLeu and tFVal used in the synthesis of these peptides were racemic at the γ- and β-positions, respectively. Thus, the fluorous peptides comprised mixtures of many diastereomers so that only the aggregate properties of the mixture could be measured, and broad unfolding transitions were observed.

An interesting experiment designed to test whether fluorous partitioning could drive the specific self-association of two fluorous peptides has also been reported (*5*). A peptide designed to form a parallel coiled-coil dimer was synthesized that incorporated hexafluoroleucine (hFLeu) at the hydrophobic **a** and **d** positions together with a control peptide that retained leucine at these positions. The peptides did preferentially self-segregate as predicted; however, the fluorous peptide formed a tetramer, whereas the control peptide was dimeric (as intended). Thus, it is unclear whether segregation was driven by the fluorous nature, per se, of the hFLeu-containing peptide, or the incompatibility of hFLeu in a two-stranded coiled coil, which would be better ascribed to steric effects.

Although the experiments described above demonstrate the potential of fluorous amino acids to increase the stability of proteins and to mediate novel protein−protein interactions, quantitative data on the stabilizing effect of fluorous amino acids in proteins are lacking. Estimates of the stabilizing

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[1] Abbreviations: hFLeu, L-5,5,5,5′,5′,5′-hexafluoroleucine; tFLeu, L-5,5,5-trifluoroleucine; tFVal, L-4,4,4-trifluorovaline; ANS, 8-anilinophthalenesulfonic acid; CD, circular dichroism; TFA, trifluoroacetic acid; GuHCl, guanidinium chloride.

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effect of tFLeu, obtained from measurements on the chemically heterogeneous peptides described above, range from 0.4 (*9*) to ~0.07 kcal/mol (*6*). In contrast, incorporation of tFVal into NTL9, a small natural protein, led to an increase in stability of up to 1.4 kcal/mol (*15*).

Accurate data for a homogeneous system would both contribute to a better understanding of how fluorous amino acids modify the properties of proteins and serve to guide further design efforts in this field. Therefore, we have designed a peptide that adopts a structurally robust antiparallel four-helix bundle topology and measured the effect of incorporating a limited number of hFLeu residues into the hydrophobic core on stability. The additional stability imparted by the hFLeu residues agrees well with the increase in hydrophobicity associated with the fluorinated side chain, as determined by solvent partitioning measurements.

MATERIALS AND METHODS

Reagents. Rink Amide resin, Fmoc-protected and *t*-Boc-protected amino acids, *N*-hydroxybenzotriazole (HOBt), and 2-(1*H*-benzotriazol-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. 8-Anilinophthalenesulfonic acid (ANS) was purchased from Aldrich Chemical Co. Guanidinium chloride (>99% pure) was obtained from Gibco BRL. L-5,5,5,5′,5′,5′-Hexafluoroleucine was synthesized as described previously (*16*) 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 the mixture 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.

Peptide α_4-F_2 was 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.* (*17*), 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 from 0 to 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 of 3299.8 amu, detected mass of 3300.1 amu; expected mass for peptide α_4-F_2 of 3515.7 amu, detected mass of 3515.8 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 (CD). CD spectra of peptides were recorded with an Aviv 62DS spectropolarimeter at 25 °C. Mean residue ellipticities, [θ], were calculated using eq 1

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

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 containing 200 μM peptide (concentration of monomer) in 100 mM potassium phosphate buffer (pH 7.0) were prepared, one with 8.0 M GuHCl and one without. The solutions were mixed in various proportions to produce samples with different GuHCl concentrations, and after equilibration for several minutes, the ellipticity at 222 nm was measured.

Fluorescence Measurements. Fluorescence spectra of the ANS dye in the presence of peptides were measured using a Cary Eclipse fluorescence spectrophotometer with a cuvette with a path length of 5 mm. Peptides were titrated against a constant concentration of ANS (5 μM) in 100 mM potassium phosphate buffer (pH 7.0). The excitation wavelength was 370 nm.

Gel Filtration Chromatography. Peptides were subjected to chromatography using an FPLC system equipped with a Pharmacia "Peptide" Superose column equilibrated in 100 mM potassium phosphate buffer (pH 7.0). The flow rate was 0.25 mL/min, and the initial peptide loading concentration was 500 μM. Peptides were detected by their absorbance at 280 nm.

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-dimensional proton spectra were acquired on a Bruker 500 MHz spectrometer using standard solvent suppression pulse sequences.

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman XLA analytical ultracentrifuge equipped with scanning UV−visible optics (*18*). 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 a single species using the *Ultrascan* software package (B. Demeler, University of Texas Health Science Center, San Antonio, TX; www.ultrascan.uthscsa.edu). Partial specific volumes were calculated using the method of Cohn and Edsall (*19*); the partial specific volume of α_4-H was calculated to be 0.74 cm^3 g^{-1}, and the partial specific volume of α_4-F_2 was calculated to be 0.69 cm^3 g^{-1}.

Preparation of p-Nitrobenzoyl Amino Acid Derivatives. *N*-*p*-Nitrobenzoylglycine was purchased from Aldrich Chemical Co and converted to *N*-*p*-nitrobenzoylglycine carboxamide by being heated in concentrated ammonium hydroxide. Leucine and hFLeu were converted to their *N*-*p*-nitrobenzoyl derivatives by reaction with *N*-*p*-nitrobenzoyl chloride using standard procedures and subsequently converted to their *N*-*p*-nitrobenzoylcarboxamide derivatives by heating in concentrated ammonium hydroxide. The amino acid derivatives

were recrystallized from methanol and shown to be pure by ^{1}H NMR and reverse-phase HPLC (C_{18} column, equilibrated in 5% acetonitrile and developed with a gradient of 5 to 50% acetonitrile).

Curve Fitting. The denaturation profiles for the peptides were analyzed assuming a two-state equilibrium between the unfolded monomeric peptide and the folded, tetrameric bundle, with no significantly populated intermediates being present, as described previously (*20*). Igor Pro software (Wavemetrics, Inc.) was used to fit the denaturation curves.

RESULTS

Partitioning Studies with Amino Acids. To determine the likely energetic contribution of a single hFLeu side chain buried within the hydrophobic core of a protein to its stability, we first sought to quantify the effect of fluorination on the hydrophobicity of the leucine side chain. We therefore synthesized the *N*-*p*-nitrobenzoyl carboxamide derivatives of leucine, hFLeu and glycine, and examined their partitioning between water and either *n*-heptanol or its fluorous counterpart, *n*-1,1-H_2-perfluoroheptanol. *n*-Heptanol was chosen for these studies (rather than *n*-octanol which is more commonly used) as perfluorinated *n*-octanol is a solid at room temperature. The *p*-nitrobenzoyl group was chosen to provide a convenient chromophore for quantitation of the amino acid derivatives. Solutions of each amino acid derivative were allowed to partition between water and the organic solvent with vigorous shaking overnight at 25 °C. The two phases were separated, and the concentration of amino acid derivative in each phase was determined by reverse-phase HPLC.

From these data, two partitioning coefficients were calculated for leucine and hFLeu, $^{H}\Pi$, referring to partitioning between water and heptanol, and $^{F}\Pi$, referring to partitioning between water and *n*-1,1-H_2-perfluoroheptanol. $^{X}\Pi$ is calculated from eq 2

$$^{X}\Pi = \log(^{X}D_{\text{amino acid}}) - \log(^{X}D_{\text{glycine}}) \qquad (2)$$

where *D* is the distribution coefficient between water and the organic solvent, X, and the subscript refers to the amino acid derivative.

$^{H}\Pi_{Leu}$ is 1.58, a number that agrees well with previously published hydrophobicity scales for amino acid side chains (*21*, *22*). $^{F}\Pi_{Leu}$ is 1.56, a value that is not significantly different from $^{H}\Pi_{Leu}$. Both the glycine and leucine derivatives partition more heavily into perfluoroheptanol, consistent with it being a more hydrophobic solvent. In comparison, $^{H}\Pi_{hFLeu}$ is 1.87, indicating that the hFLeu side chain is significantly more hydrophobic than leucine. Perhaps surprisingly, $^{F}\Pi_{hFLeu}$ is also 1.87. This finding suggests that the six fluorines associated with the hFLeu side chain are insufficient to induce fluorous effects on their own; otherwise, we would have expected the hFLeu derivative to partition preferentially into the fluorous solvent over the hydrocarbon solvent. From these Π values, the free energies for partitioning of the leucine and hFLeu side chains into *n*-heptanol are as follows: $\Delta G_{Leu} = -2.1$ kcal/mol and $\Delta G_{hFLeu} = -2.5$ kcal/mol. The free energies for partitioning of these amino acid derivatives into *n*-1,1-H_2-perfluoroheptanol are essentially the same. These data suggested that hFLeu may stabilize a protein by up to an additional 0.4 kcal/mol per residue over leucine due to its increased hydrophobicity.

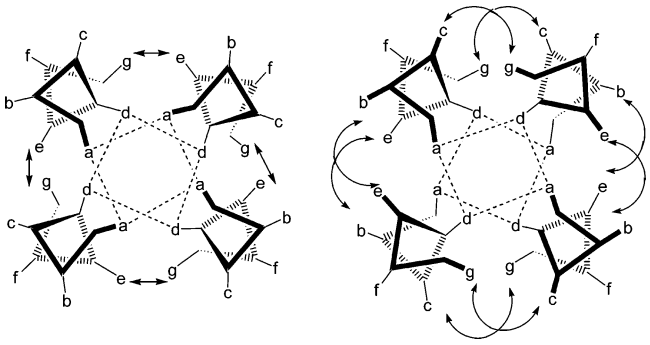

FIGURE 1: Helical wheel diagrams illustrating the differences in side chain interactions for parallel (left) and antiparallel (right) four-helix bundle proteins. The parallel arrangement results in only one type of interfacial interaction between **g** and **e** positions, whereas the antiparallel arrangement results in two different interhelical interactions between **b** and **e** positions and **c** and **g** positions.

Design of the Antiparallel Four-Helix Bundle Peptide. The choice of protein structure is an important consideration in the design of experiments for measuring the effects of incorporating fluorous amino acid residues on the stability of a protein. We chose to focus on the antiparallel four-helix bundle structure as such proteins are widely distributed in nature, and have been the subject of previous *de novo* design efforts (*23*−*25*). Although both parallel (C_4 symmetric) and antiparallel (D_2 symmetric) four-helix bundles have been designed, the antiparallel topology is far more conformationally stable because, as illustrated in Figure 1, this arrangement results in two different interhelix interfaces that are unique to this topology (*26*). These are designated the **b**−**e** and **c**−**g** interfaces in the standard heptad notation. In contrast, the parallel arrangement results in only one type of helix−helix interaction, between the **e** and **g** positions, that is very similar for tetrameric, trimeric, and dimeric bundles. As a result, subtle changes in sequence or buffer conditions can readily change the oligomerization state of parallel helical bundles (*25*, *27*), a phenomenon that has also been encountered in the design of parallel fluorous coiled coils (*5*).

A 27-residue peptide, α_4-H, intended to adopt an antiparallel four-helix bundle topology, was designed with the aid of the Insight II software package, and was based on a previously described antiparallel four-helix bundle, Coil-LL (*26*). α_4-H comprises three heptad repeats and incorporates leucines at each of the hydrophobic **a** and **d** positions, any of which can, in principle, be substituted with the fluorous analogue hFLeu. This arrangement results in a regularly packed hydrophobic core comprising six layers each packed with four leucine residues, two from **a** positions and two from **d** positions of the opposing helices. An antiparallel topology was enforced through complementary electrostatic interactions between residues at the **b** and **e** positions and **c** and **g** positions; residues intended to promote "capping" of the helix were incorporated at the N- and C-termini of the peptide (*28*). The N-terminus of the peptide is acetylated and the C-terminus amidated. The sequence of α_4-H and a helical wheel diagram illustrating the design are shown in Figure 2A.

Although the substitution of fluorine for hydrogen is often considered to be a sterically conservative one, the hFLeu residue, while maintaining a shape similar to that of leucine, is calculated to be ~37 Å^3 larger than leucine (*29*). Therefore,

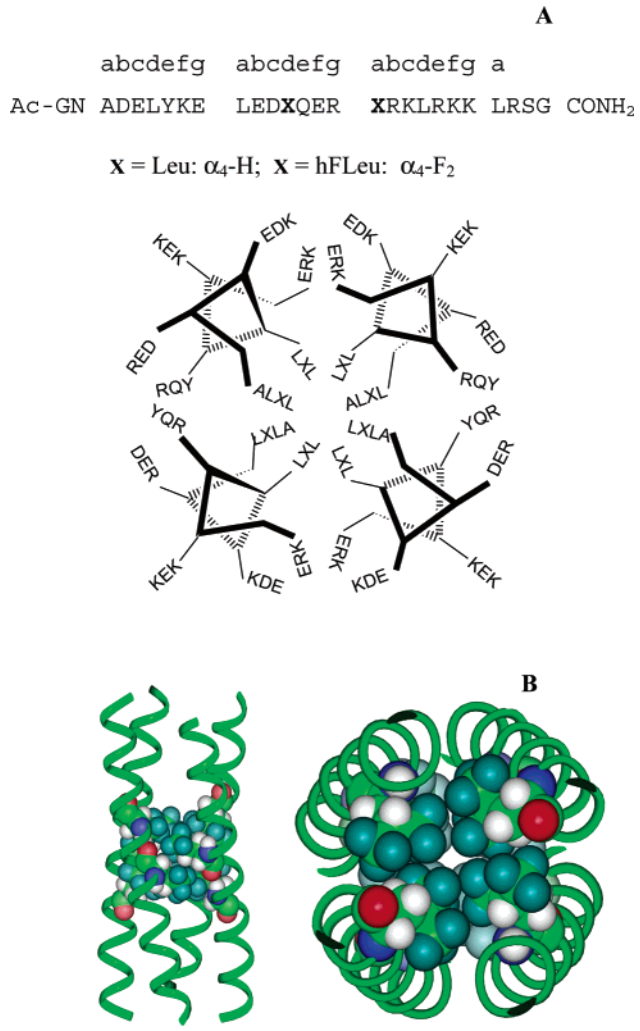

FIGURE 2: Design of helical peptides used in this study. (A) Sequence of α_4-H (X = Leu) and α_4-F_2 (X = hFLeu) peptides. The positions of the amino acid side chains in the antiparallel four-helix bundle are illustrated by the helical wheel diagram for the three heptad repeat units of the peptide. (B) Model showing the packing of hFLeu in the central two layers of α_4-F_2 (fluorine atoms are colored dark green).

to minimize the possibility of large-scale structural changes due to the increased volume of the hydrophobic core, we decided to repack only the central two layers of the hydrophobic core with hFLeu by incorporating this residue at positions 13 and 17; this peptide was called α_4-F_2. On the basis of the partitioning studies described above, these substitutions could potentially stabilize the protein by an additional 3.2 kcal/mol, and this difference should be readily measurable in unfolding experiments. Furthermore, these central positions are the most deeply buried, and substitutions made here are likely to be easier to interpret than changes at the ends of the peptide, which are more likely to be conformationally labile and partially exposed to solvent. A model, generated with Insight II, illustrating the packing of the hFLeu residues in α_4-F_2 is shown in Figure 2B.

The nonfluorous peptide, α_4-H, was synthesized without difficulty by standard automated methods using Fmoc-protected amino acids. However, this approach proved to be unsuccessful when we attempted to synthesize hFLeu-containing peptides. In general, the yields of the full-length peptide were very low, and MALDI-TOF analysis indicated the presence of failure sequences that may be attributed to very low yields in the coupling steps for the second and third amino acids following incorporation of hFLeu. The reason for this is unclear. Neither double coupling nor the inclusion of chaotropic solvents, which might break up secondary structure, led to any marked improvement in yield. Fortunately, α_4-F_2 could be synthesized manually in good yield using protocols employing *t*-Boc-protected amino acids (*17*).

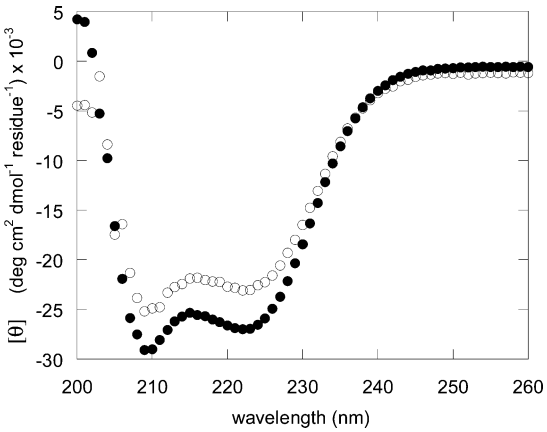

FIGURE 3: CD spectra of α_4-H (●) and α_4-F_2 (○) (peptide concentration of 30 μM) in 100 mM potassium phosphate buffer (pH 7.0).

Characterization of Peptides. At neutral pH, both α_4-H and α_4-F_2 peptides exhibited CD spectra characteristic of extensively α-helical secondary structure with minima at 208 and 222 nm (Figure 3). Interestingly, the mean residue ellipticity, [θ], of α_4-F_2 is somewhat lower than that of the nonfluorinated peptide, suggesting that the helical content may be slightly lower, perhaps because repacking two layers of the bundle with the larger hFLeu side chain introduces some distortion into the helical structure.

The oligomerization state of the peptides was examined by gel filtration. Both α_4-H (M_r = 3300) and α_4-F_2 (M_r = 3516) exhibited elution volumes very similar to that of cytochrome *c* (M_r = 12 500), indicating that they were adopting a predominantly tetrameric structure, as intended. Whereas cytochrome *c* eluted in a sharp symmetrical peak, characteristic of a monodisperse species, both peptides exhibited significant trailing of lower-molecular weight material (data not shown), which is consistent with the peptides existing in equilibrium between tetrameric and monomeric species.

Analytical Ultracentrifugation. Analytical sedimentation equilibrium centrifugation was used to investigate the oligomerization states of α_4-H and α_4-F_2. The initial peptide concentrations varied between 70 and 500 μM. At high peptide concentrations, both peptides sedimented as homogeneous species with apparent molecular masses of 13 300 and 13 000 Da for α_4-H and α_4-F_2, respectively, which are close to those expected for a tetrameric structure (expected molecular masses for α_4-H and α_4-F_2 tetramers of 13 200 and 14 064 Da, respectively). Fits of the curves assuming either a trimeric or pentameric structure for the peptides exhibited significant systematic deviation of the residuals from the data (Figure 4). At lower concentrations, the sedimentation curves are better described by a monomer−tetramer equilibrium.

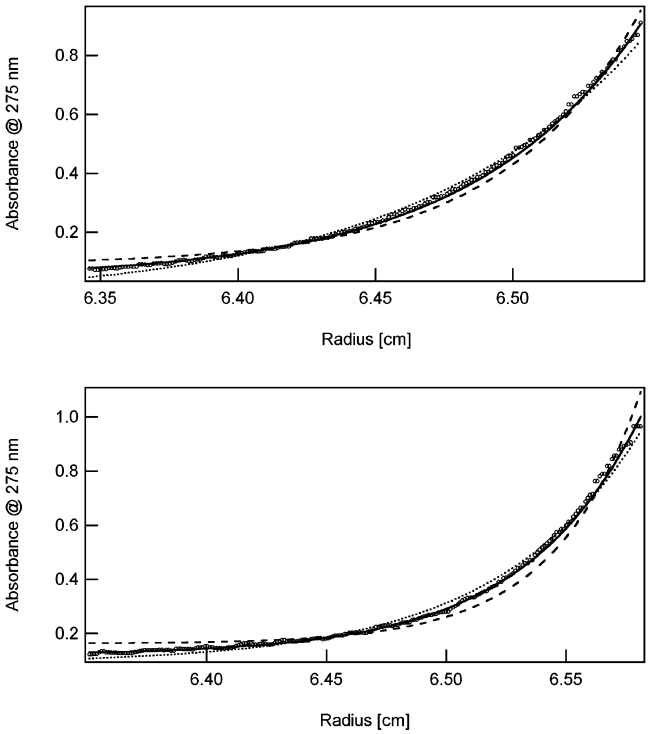

FIGURE 4: Representative sedimentation equilibrium traces obtained from analytical ultracentrifugation of α_4-H (top panel) and α_4-F_2 (bottom panel). The fit to a single sedimenting species is shown as a solid line, and for both peptides gives an apparent molecular weight close to that for a tetramer. Theoretical fits to a trimer (dotted line) and a pentamer (dashed line) are shown for comparison. The initial peptide loadings were 500 μM in 100 mM potassium phosphate buffer (pH 7.0), and the temperature was 298 K.

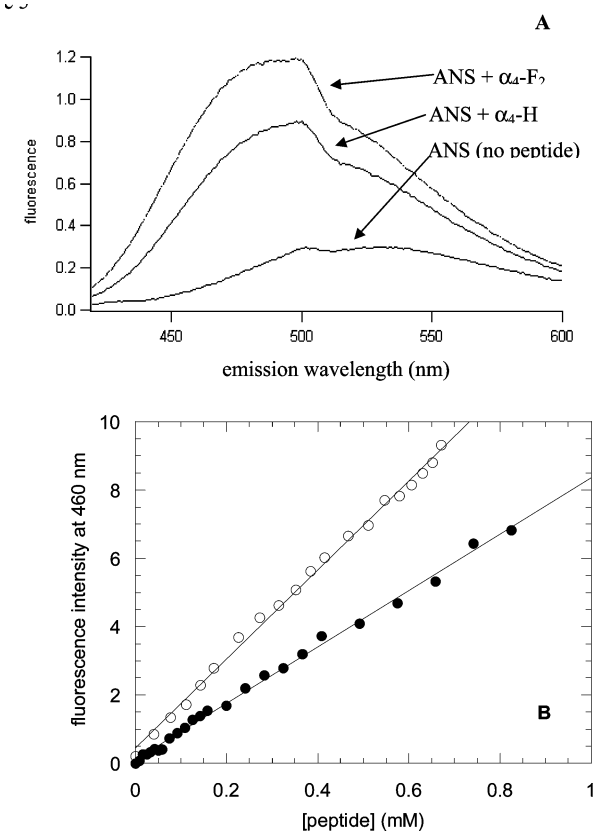

FIGURE 5: Hydrophobic dye binding by peptides. (A) Fluorescence emission spectra of α_4-H and α_4-F_2 in the presence of 5 μM ANS, with the emission spectrum of ANS alone shown for comparison. The buffer was 100 mM potassium phosphate (pH 7.0), and the excitation wavelength was 370 nm. (B) Titration of ANS with increasing concentrations of peptides: α_4-H (●) and α_4-F_2 (○).

Hydrophobic Dye Binding. To qualitatively assess how replacing two leucine residues in α_4-H with hFLeu in α_4-F_2 might affect the hydrophobic packing of the interior of the bundle, we investigated the binding of the hydrophobic dye 8-anilinophthalenesulfonic acid (ANS) to the peptides. ANS is commonly used as a probe for molten globule behavior in proteins because whereas well-folded proteins do not bind the dye strongly, those possessing molten hydrophobic cores bind ANS, typically with micromolar affinities, resulting in a large increase in fluorescence (*30*, *31*).

When α_4-H was titrated against ANS, only a small increase in fluorescence was observed, which increased linearly across the concentration range that was used (Figure 5). This result indicates weak, nonspecific binding of ANS, rather than intercalation of the dye into the hydrophobic core of the peptide. This suggests that the core is well-packed, rather than molten globule-like. In this respect, α_4-H differs from Coil-LL, the peptide upon which it is based. Coil-LL has the same all-leucine core but was found to be molten globule-like (*26*). A similar result was obtained when α_4-F_2 was titrated against ANS (Figure 5). Although the fluorescence intensity is slightly higher with this peptide, there is no evidence for specific binding of ANS. As a positive control, the fluorescence spectrum of ANS (2 μM) was also recorded in the presence of 30 μM α-lactalbumin in 0.1 M KCl at pH 2.0, where it is known to adopt a molten globule state (ref *32* and data not shown). Whereas ANS fluorescence is only increased 3−5-fold in the presence of the peptides, fluorescence was increased ~135-fold and the emission maximum was blue-shifted to 470 nm by α-lactalbumin. This further suggested the peptides were not molten globule-like.

NMR Spectra. The proton NMR spectra of α_4-H and α_4-F_2 were recorded in potassium phosphate buffer (pH 7.0) containing 10% D_2O. The spectra are shown in Figure 6. The spectrum of α_4-H is well-dispersed, and the peaks are sharp, indicative of a well-folded protein. The spectrum of α_4-F_2 exhibits a similarly disperse set of amide resonances; however, the peaks are significantly broader, indicating that these protons are exchanging more rapidly, which in contrast to the ANS binding studies might suggest a more dynamic structure. The aliphatic regions of the spectra of both proteins exhibit sharp peaks, and it is apparent that substitution of leucine with hFLeu causes significant changes in this region of the spectrum.

Denaturation Experiments. The GuHCl-induced unfolding of α_4-H and α_4-F_2 was followed using CD spectroscopy by monitoring the ellipticity of the peptides at 222 nm as a function of the increasing GuHCl concentration. The peptides exhibit well-defined unfolding transitions, as shown in Figure 7, that appear to be well fitted by assuming a two-state equilibrium between unstructured monomeric peptides and a folded tetrameric helical bundle. From fits to these curves, the free energy of unfolding (ΔG_{unfold}) is 20.2 ± 0.2 kcal/mol for α_4-H and 22.7 ± 0.2 kcal/mol for α_4-F_2. The proportionality constants (m) were 2.4 ± 0.1 and 2.6 ± 0.2 kcal mol^{-1} M^{-1} for α_4-H and α_4-F_2, respectively. From these data, the additional stabilization afforded to α_4-F_2 by the

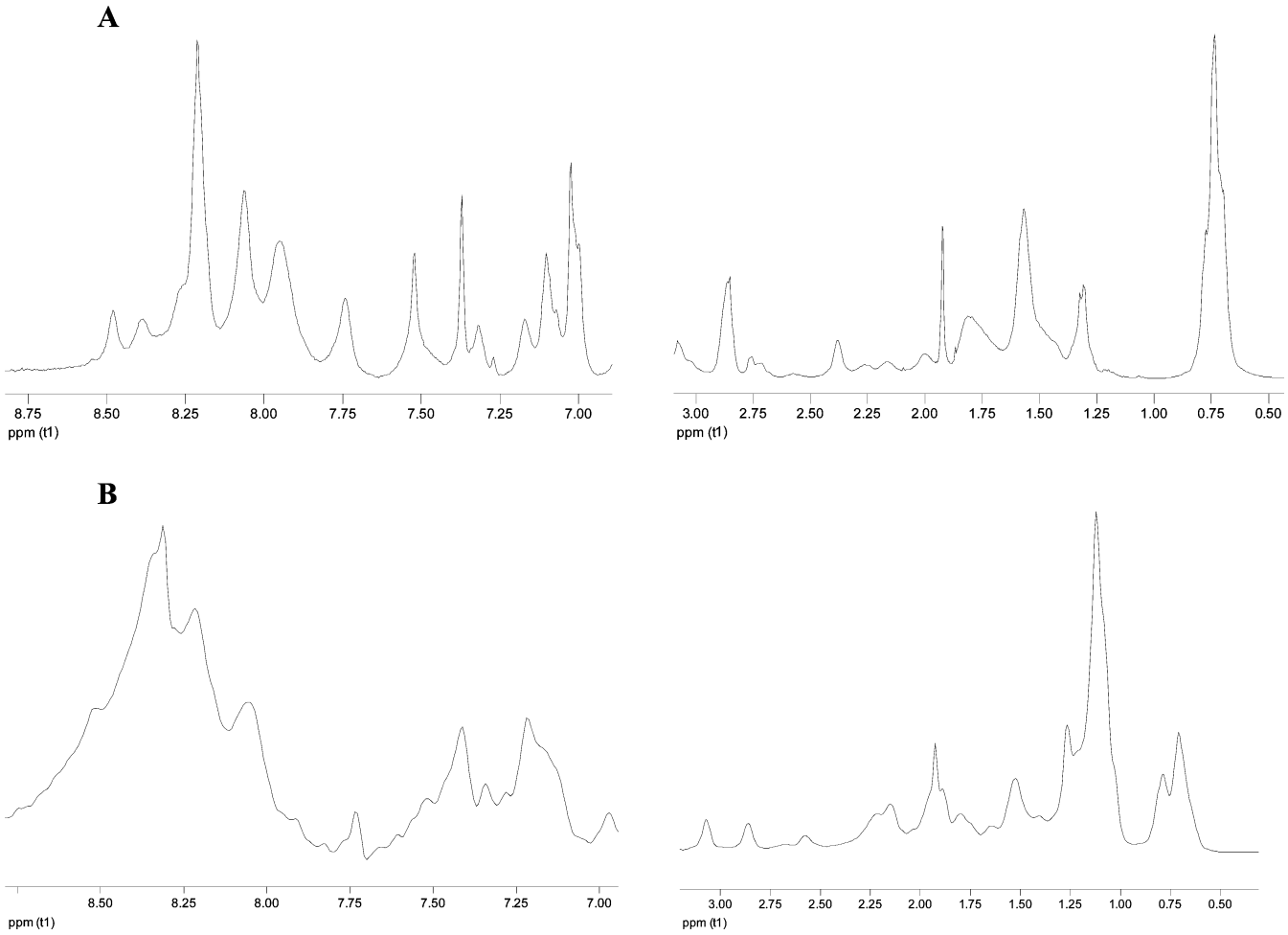

FIGURE 6: Proton NMR spectra of (A) α_4-H and (B) α_4-F_2, showing the amide and aliphatic regions of each peptide. Each region of the spectra has been scaled to normalize the peak intensities. The spectra were recorded in 10 mM potassium phosphate (pH 7.0) containing 10% D_2O.

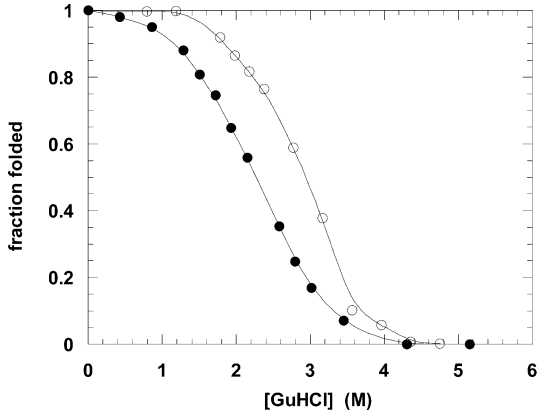

FIGURE 7: Unfolding of peptides in GuHCl. Plots of fraction folded vs GuHCl concentration for α_4-H (●) and α_4-F_2 (○). Unfolding was followed by CD spectroscopy by monitoring changes in ellipticity at 222 nm; the buffer was 100 mM potassium phosphate (pH 7.0), and the peptide concentration was 200 μM.

hFLeu residues is 2.5 ± 0.3 kcal/mol, or approximately 0.3 kcal/mol per hFLeu residue.

One caveat to the above interpretation of the data should be noted. It is possible that the unfolding transition may not be two-state, even though the data are well-fitted by a two-state model. If intermediate structures are significantly populated during unfolding, this will lead to inaccuracies in calculating ΔG_{unfold} using this assumption. This is more of a concern for the α_4-F_2 peptide in view of the dynamic behavior that is evident from its NMR spectrum. On balance, though, we think it less likely that intermediates would be significantly populated in the folding pathway of these simple helical peptides than in the folding of larger natural proteins where such behavior has been encountered.

The thermal denaturation of α_4-H and α_4-F_2 was also investigated, using CD spectroscopy to monitor the elipticity at 222 nm. However, in neither case did the peptides exhibit significant unfolding up to 95 °C, the highest temperature that can be accessed in the spectrometer (data not shown).

DISCUSSION

As we had anticipated, the antiparallel four-helix bundle provides a sufficiently robust structure that incorporating the larger hFLeu residue into the hydrophobic core does not appear to grossly alter the peptide's folding. Both α_4-F_2 and α_4-H exhibit CD spectra characteristic of extensively α-helical proteins. Importantly, the α_4-F_2 peptide retains its tetrameric structure as judged by gel filtration and analytical ultracentrifugation. This illustrates the advantage of antiparallel coiled coils for comparing the effects of sequence changes on protein stability; in contrast, subtle changes in hydrophobic packing have been shown to result in changes to the oligomerization state of parallel coiled-coil peptides (*27*). However, the substitution of hFLeu is not entirely

without structural consequences. The mean residue ellipticity of α_4-F_2 is smaller in magnitude than that of α_4-H, and the NMR spectra indicate that the amide protons are in more rapid exchange in α_4-F_2 than in α_4-H. These observations suggest that repacking the center of the bundle with hFLeu may cause some distortion of the α-helices.

The additional stability imparted to α_4-F_2 by the hFLeu residues ($\Delta\Delta G_{unfold} = 0.3$ kcal/mol per residue) is in good agreement with that predicted from the increase in the free energy of partitioning of the fluorinated side chain from *n*-heptanol into water ($\Delta\Delta G = 0.4$ kcal/mol). Therefore, it appears to be reasonable to ascribe the increased stability exhibited by α_4-F_2 to the more hydrophobic nature of hFLeu, rather than specific fluorous interactions between the hFLeu side chains in the hydrophobic core. If fluorous interactions were playing a significant role in α_4-F_2 folding, we might expect that the per-residue increase in $\Delta\Delta G_{unfold}$ would exceed that calculated from solvent partitioning measurements. One caveat to this interpretation is that there may be some energetic penalty incurred by incorporating hFLeu if, as seems likely, this results in some distortion of the protein structure.

It is perhaps not surprising that α_4-F_2 does not exhibit any additional fluorous stabilization, as each peptide monomer only contains two hFLeu residues for a total of four trifluoromethyl groups. On the basis of the solubility properties of simple fluorocarbon molecules, this is probably insufficient for inducing self-segregation. In their studies on self-segregating coiled coils, Kumar and colleagues used a peptide containing seven hFLeu residues (*5*). They calculated that the self-association of the fluorinated peptides was more favorable by ~2 kcal/mol than the formation of mixed hydrocarbon−fluorocarbon coiled coils, although the actual free energies of folding were not determined for these peptides.

Previous studies have produced estimates for the additional stability imparted by incorporating a trifluoromethyl group into a peptide that vary quite significantly. Fluorous analogues of the coiled-coil region of yeast transcription factor GCN4 have been synthesized, both biosynthetically (*9*) and by chemical synthesis (*10*), that incorporate tFLeu in place of leucine. For the biosynthetically produced peptide, a $\Delta\Delta G_{unfold}$ of 0.4 kcal/mol per buried trifluoromethyl group was estimated (assuming six buried CF_3 groups per monomer), based on thermal unfolding measurements. However, introduction of tFLeu at four positions of the leucine zipper region of GCN4 by chemical synthesis only resulted in an estimated increase in stability of 0.12−0.3 kcal/mol per buried trifluoromethyl group. In another case, a GCN4 analogue was synthesized that incorporated both tFLeu at four **a** positions and tFVal in place of valine at three **d** positions (*6*). These substitutions increased the apparent free energy of unfolding by ~1.1 kcal/mol, or an average of 0.08 kcal/mol per buried trifluoromethyl group. In all these cases, measurements were taken on peptides that comprised a mixture of diastereomers because tFLeu and tFVal were racemic at C-4 and C-3, respectively, so these values represent aggregate properties of the mixture. Even so, it is unclear why these values should vary so much, given the overall similarity in sequence and structure of these peptides.

We find that in our designed peptide, hFLeu stabilizes the folded structure by an average of ~0.3 kcal/mol per hFLeu, or ~0.15 kcal/mol per trifluoromethyl group. In comparison with the previous studies discussed above, this value appears to be quite reasonable, lying midway between the upper and lower estimates. It is also reasonable on physicochemical grounds given the difference in hydrophobicity between leucine and hFLeu.

The design of α_4-H is based on that of Coil-LL, a 27-residue peptide designed to fold into an antiparallel four-helix bundle (*26*). Coil-LL binds ANS, indicating it possesses a dynamic, molten globule-like hydrophobic core. Therefore, we were surprised to find that α_4-H and α_4-F_2 do not appear to be molten globule-like by this test, even though they share the same simple packing arrangement that uses only leucine. The well-packed nature of α_4-H is further supported by the NMR spectrum of this peptide, which exhibits good spectral dispersity and sharp peaks. α_4-F_2 on the other hand appears to be less well structured by NMR and probably has some molten character, even though it does not appear to bind ANS.

α_4-H incorporates 10 changes to the sequence of Coil-LL, mainly to the interfacial **b**, **c**, **e**, and **g** positions. Although it was not a goal of our studies, it is certainly noteworthy that these substitutions at interfacial residues also appear to have resulted in a better-packed hydrophobic interior. Most of the changes introduced into α_4-H were from neutral or hydrophobic residues to charged residues, made with the aim of reinforcing antiparallel topology in the design; in particular, a methionine at position 9 (**g** position) was changed to glutamate. Interestingly, Hill and DeGrado (*33*) have demonstrated that changing even a single glutamate residue at an exposed **b** position in the well-folded, *de novo*-designed four-helix bundle, α_2D, was sufficient to induce conformational heterogeneity in the folded protein.

By incorporating two hFLeu residues per chain, we have increased ΔG_{unfold}, from 20.2 kcal/mol for α_4-H to 22.7 kcal/mol in α_4-F_2, an increase in thermodynamic stability of more than 10%. This suggests that a fully fluorous version of α_4-H, incorporating six hFLeu residues per helix, might be more stable than α_4-H by more than one-third. It is an interesting question whether a similar increase in thermodynamic stability would be observed if hFLeu (or other fluorous amino acids) were to be substituted into the core of a natural protein.

Natural proteins generally exhibit structurally well-defined hydrophobic cores that incorporate a diverse collection of hydrophobic residues, rather than simple packing arrangement of our *de novo*-designed peptides. They are also usually somewhat less stable than these peptides; ΔG_{unfold} is typically in the range of 10−15 kcal/mol. Substitution of a single tFVal residue for valine in the small (56-residue), marginally stable protein NTL9 has recently been reported to increase ΔG_{unfold} between 0.8 and 1.4 kcal/mol, depending upon the position of substitution (*15*). This is a much greater increase in stability than would have been predicted simply on the basis of the likely increase in hydrophobicity afforded by the CF_3 group, suggesting additional favorable interactions may be formed by the CF_3 group. Nevertheless, this interesting observation, together with the results described herein and previous studies, suggests that the potential of fluorous amino acids for stabilizing natural proteins would be significant, especially for those that exhibit marginal stability under normal conditions of temperature and pH.

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SUPPORTING INFORMATION AVAILABLE

Comparison of fluorescence spectra of peptides and α-lactalbumin in the molten globule state and a description of the fitting procedure used to determine ΔG_{unfold} from GuHCl denaturation curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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