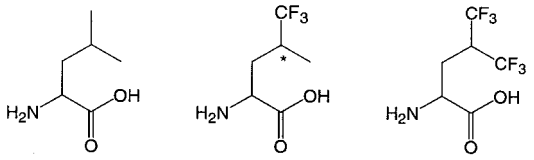
Fluorinated Coiled-Coil Proteins Prepared In Vivo Display Enhanced Thermal and Chemical Stability**

Yi Tang, Giovanna Ghirlanda, Wendy A. Petka, Tadashi Nakajima, William F. DeGrado, and David A. Tirrell*

Incorporation of noncanonical amino acids provides a route to proteins and peptides with unique structural and chemical features.[1] Functionality orthogonal to that of the naturally occurring amino acids, including alkenes,[2] alkynes,[3] aryl halides,[4] and electroactive side chains[5] has been incorporated efficiently into proteins prepared in bacterial cultures. We present here a general approach to the stabilization of leucine-zipper peptides and coiled-coil proteins by incorporation of the hyperhydrophobic leucine (**1**) isostere trifluoroleucine (Tfl, **2**). Trifluoroleucine was reported more than 30 years ago to support bacterial cell growth and to be incorporated into nascent proteins in the absence of leucine.[6]



1 **2** **3**

Leucine-zipper domains form coiled-coil structures comprising generic heptad repeats designated *abc**d**efg*, where the ***d*** positions are occupied predominantly by leucine residues.[7] Our work was motivated by the conjecture that replacement of leucine with Tfl at the ***d*** positions of the heptad repeat should stabilize the coiled-coil dimer with respect to thermal and chemical denaturation. The choice of trifluoroleucine was based on several factors, the most important of which is the observation that many fluorocarbons behave as though they are more hydrophobic than their hydrocarbon analogues.[8] Trifluoroleucine has been reported to be activated and charged by the *E. coli* leucyl tRNA-synthetase (LeuRS) and can be incorporated into recombinant proteins produced in vivo.[6] Finally, the trifluoromethyl group is chemically inert and nearly isosteric to the methyl group,[9] suggesting that proteins and peptides outfitted with Tfl might adopt folded structures similar to those of their "wild-type" analogues.

The leucine-zipper protein A1 (Figure 1a) was used as a test protein in assays for incorporation of Tfl and hexafluoroleucine (Hfl, **3**).[10] The design of this protein has been

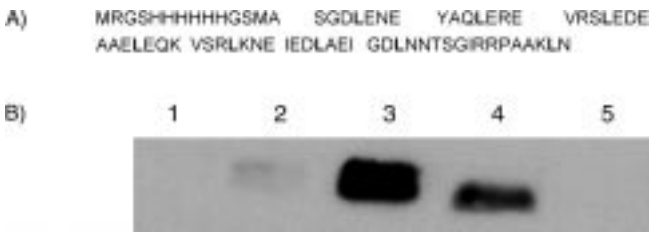


Figure 1. A) Amino acid sequence of A1. The leucine positions are bold. B) Western blot analysis of A1 expression. Cultures were supplemented with leucine or with one of the analogues Tfl or Hfl. Lane 1: uninduced sample; lane 2: induced sample without supplementation; lane 3: induced sample supplemented with leucine (150 μM); lane 4: induced sample supplemented with Tfl (220 μM); lane 5: induced sample supplemented with Hfl (170 μM).

discussed previously;[10] the primary sequence contains eight leucine residues, of which six are distributed at the ***d*** positions of the six heptad repeats. The capacity of each of these amino acids to support protein synthesis was determined by induction of gene expression in leucine-free culture media supplemented either with Tfl or with Hfl. Accumulation of the target protein was visualized by western blotting as shown in Figure 1b. As expected from previous results,[6, 11] Tfl (lane 4, Figure 1b) clearly supports protein synthesis, although the yield of protein is reduced in comparison to that obtained from leucine-supplemented medium (lane 3, Figure 1b). In contrast, Hfl (lane 5, Figure 1b) does not support measurable protein synthesis under the conditions examined in this work as shown by comparison with negative controls (lanes 1 and 2 Figure 1b). The increased electrophoretic mobility of the fluorinated protein (cf. lanes 3 and 4, Figure 1b) has been observed previously for other fluorinated proteins produced by similar methods.[11a]

To control the level of substitution of leucine by Tfl, we varied the concentration of leucine in the medium, while keeping the concentration of Tfl (as the D,L mixture) at 550 μM. The concentration of leucine ranged from 0 to 330 μM (as the L-amino acid). The extent of replacement was determined by amino acid analysis of the purified proteins. We also performed matrix-assisted laser desorption ionization (MALDI) mass spectrometry on the protein products; in each case, the most intense signal in the mass spectrum corresponds closely to the substitution level calculated from amino acid analysis.

The results are shown in Table 1. The extent of leucine replacement decreased from 92% in medium supplemented only with Tfl, to levels below the limit of detection in medium supplemented with 330 μM L-leucine. We were not able to reach 100% incorporation of Tfl, even in the absence of added leucine, possibly due to trace amounts of leucine liberated by cellular protein degradation. Protein yield

[*] Prof. D. A. Tirrell, Y. Tang
Division of Chemistry and Chemical Engineering
California Institute of Technology
Pasadena, CA 91125 (USA)
Fax: (+1) 626-793-8472
E-mail: tirrell@caltech.edu
Dr. G. Ghirlanda, Prof. W. F. DeGrado
Department of Biochemistry & Biophysics
University of Pennsylvania
Philadelphia, PA 19104 (USA)
Dr. W. A. Petka, Dr. T. Nakajima
Department of Polymer Science and Engineering
University of Massachusetts
Amherst, MA 01003 (USA)

[**] This work was supported by a grant from the U.S. Army Research Office. Y. Tang is supported by a Whitaker Graduate Research Fellowship. We thank Dr. Gary Hathaway for performing matrix-assisted laser desorption/ionization analyses.

 © WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2001 0044-8249/01/11308-1542 $ 17.50+.50/0

Table 1. Wild-type and fluorinated proteins produced in vivo.

Protein	[Leu] in culture [μM][a]	Yield [mg L^{-1}]	%Tfl by AAA[b]	MALDI mass[c] (Tfl sites)
A1-WT	330	40	0	8307.9 (0)
FA1-17	114	27	17	8361.2 (1)
FA1-29	83	25	29	n.d.[d]
FA1-79	23	21	79	8631.9 (6)
FA1-92	0	20	92	8739.8 (8)

[a] Concentration of L-leucine. The concentration of Tfl in culture was 550 μM (D,L mixture). [b] % Substitution determined from the diminution of the leucine mole fraction by amino acid analysis (AAA). [c] Mass of the most intense signal in the MALDI mass spectrum. Numbers in parentheses indicate corresponding numbers of sites occupied by Tfl. [d] n.d.: not determined.

decreased as the concentration of leucine in the culture medium was reduced. The yield of A1 obtained from leucine-enriched medium was 40 mg L^{-1}, twice that from medium supplemented only with 550 μM Tfl.

The secondary structures of the wild-type protein (A1-WT) and the variant containing 92% Tfl (FA1-92) were examined by circular dichroism (CD) spectroscopy (Figure 2). Both

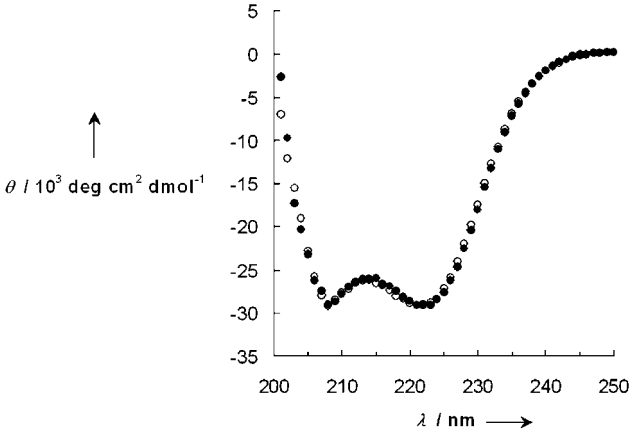


Figure 2. CD spectra of A1 (●) and FA1-92 (○) at 0 °C (10 μM protein concentration, PBS buffer, pH 7.4). θ: molar ellipticity. PBS = phosphate-buffered saline.

proteins are more than 90% helical as determined from the molar ellipticity at 222 nm, and the extent of overlap of the spectra suggests essentially identical secondary structures. Equilibrium sedimentation indicated that both peptides formed stable dimers with dissociation constants $\leq$10 μM. At high μM concentrations the peptides further associated to form higher-order species. The data were well described by a dimer-to-tetramer equilibrium, yielding dimer-to-tetramer equilibrium constants of 80.5 μM and 380.2 μM for A1-WT and FA1-92, respectively.

Previous examples of replacement of ***d***-position leucine residues in leucine-zipper peptides by other natural amino acids have all resulted in reduction of coiled-coil stability.[12] The reduction in stability observed in such experiments has been attributed to the fact that these substitutions are usually of the "large" to "small" type and cause losses in hydrophobic packing efficiency.[13] In marked contrast, thermal and chemical unfolding studies show that fluorinated variants of A1 are highly resistant to denaturation. The "melting temperature" (T_m) of FA1-92 is elevated by 13 °C compared to A1-WT (Figure 3), while the midpoint concentration (C_m) for urea denaturation for FA1-92 is increased to 7 M (vs. 2.8 M for A1-WT) (Figure 4). Surprisingly, proteins with low levels of fluorination (FA1-17 and FA1-29) exhibit pronounced increases in stability. In FA1-17, for example, the single substitution of a side chain methyl group by a trifluoromethyl group results in an increase in T_m by 6 °C and in C_m by about 2 M.

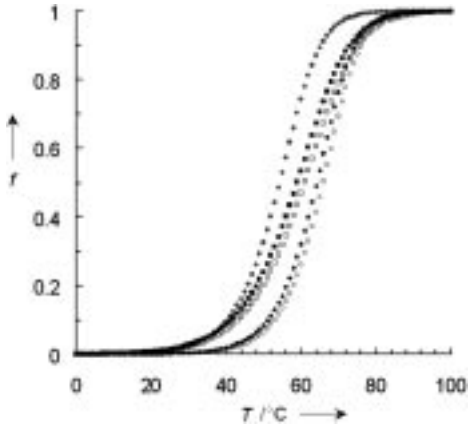


Figure 3. Thermal denaturation of partially fluorinated A1 proteins: A1-WT (●), FA1-17 (■), FA1-29 (□), FA1-79 (◆) and FA1-92 (○) (10 μM protein concentration, PBS buffer, pH 7.4). *f*: fraction unfolded.

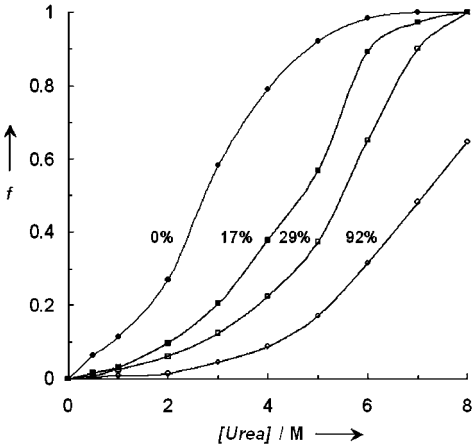


Figure 4. Urea titration of WT-A1 (●), FA1-17 (■), FA1-29 (□), and FA1-92 (○) at 0 °C. *f*: fraction unfolded.

Global thermodynamic fitting was used to obtain the thermodynamic quantities associated with the monomer-to-dimer transitions of A1-WT and FA1-92 (Table 2).[14] The intermediate samples (FA1-17 and FA1-29) were not analyzed in this way because the compositional heterogeneity of these samples precludes application of a two-state transition model. It must be emphasized, however, that FA1-92 is also heterogeneous, since Tfl is incorporated into bacterial proteins as an equimolar mixture of the (2*S*,4*S*) and (2*S*,4*R*) diastereomers.[11a] The free energy of folding for FA1-92 is 2.4 kcal mol^{-1} (of monomer) more negative than for A1-WT at

Table 2. Thermodynamic data for the folding of A1-WT and FA1-92.

Protein	T_m[a] (10 μM)	T_m[b] (1M)	$\Delta G^{\ominus}$ (37 °C)[c]	$\Delta\Delta G^{\ominus}$	ΔH_m[d] (1M)	ΔC_p[e] (1M)	$C_{urea,50\%}$[f] (0 °C)
A1-WT	54	103	−10.7	–	−70.9	−252	2.8
FA1-92	67	116	−13.1	−2.4	−77.0	−272	7

[a] Midpoint of the thermal denaturation curve at 10 μM protein concentration (PBS, pH 7.4). Units for T_m are °C. [b] Midpoint of the thermal denaturation curve extrapolated to 1M standard state using non-linear least squares fit. [c] The free energy of folding at 37 °C at 1M standard state. Units for $\Delta G^{\ominus}$ and $\Delta\Delta G^{\ominus}$ are kcal mol^{-1} of monomers. [d] The enthalpy of folding at the midpoint temperature extrapolated to 1M standard state; units are in kcal mol^{-1} of monomers. [e] Heat capacity change upon folding at 1M standard state; units are in cal mol^{-1} K^{-1}. Uncertainties in T_m (1M), $\Delta G^{\ominus}$, ΔH_m, and ΔC_p are ±1.5 °C, 1.2, 4.8 kcal mol^{-1}, and 120 cal mol^{-1} K^{-1}, respectively. [f] Midpoint urea denaturation concentration at 0 °C in M of urea.

37 °C, which corresponds to 0.4 kcal mol^{-1} of stabilization for each Tfl site localized at the dimer interface. Consideration of the large numbers of leucine residues packed in the hydrophobic cores of many proteins leads to the conclusion that the additive stabilizing effects of Tfl might in some cases be quite substantial.

The results reported here show that one can incorporate Tfl efficiently into proteins produced in vivo, control the level of incorporation, maintain secondary and higher-order protein structure, and elevate protein stability with respect to thermal and chemical denaturation. The implications of these observations are under investigation.

Experimental Section

Materials: Tfl was prepared in an overall yield of 22% in seven steps from *β*-trifluoromethylcrotonic acid, according to the procedure of Rennert and Anker with slight modifications.[6] Hfl was prepared by modification of the procedures reported by Zhang et al.[15] A leucine auxotrophic variant of *E. coli* strain mc1000 (F$^-$lacΔx74anaD139/(Ana Abioc-leu)Δ7679 gal U gal K rspL), designated LAM1000, was provided by Prof. M. J. Fournier of the University of Massachusetts, Amherst. Expression vector pQEA1, which contains the A1 coding sequence, was obtained from the U.S. Army Natick RD&E Center (Natick, MA).

Analogue incorporation assay: LAM1000 strains were transformed with pQEA1 and pREP4 to yield the expression strain LAM1000/pQEA1/pREP4. Tfl and Hfl were assayed for translational efficiency using a medium shift technique.[3] Amino acid concentrations after the medium shift were **1**: 150 μM; **2**: 220 μM; **3**: 170 μM. Proteins were detected by western blotting of the whole cell lysate with an antibody specific for the N-terminal His-Tag of A1.

Protein expression and purification: M9AA medium (1 L) supplemented with 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.4 wt% glucose, 1 mg L^{-1} thiamine and the antibiotics ampicillin (200 mg L^{-1}) and kanamycin (25 mg L^{-1}) was inoculated with 10 mL of fresh overnight culture (M9) of the expression strain. After the culture had grown to OD_{600} of 1.0, the medium shift procedure was applied. The cell pellet was then resuspended in 1 L M9 medium supplemented with Tfl (550 μM) and leucine in appropriate concentrations (Table 1). IPTG (1 mM) was added after 10 min to induce protein expression. Cells were collected after 3 h by centrifugation (5000 g, 15 min, 4 °C). The pellets were resuspended in Buffer A (20 mL; 8M urea, 0.1M NaH_2PO_4, 0.01M Tris, pH 8.0) and stored at −80 °C overnight. The cells were thawed rapidly at 37 °C, cell debris was sedimented (22500 g, 50 min, 4 °C), and the supernatant was applied to a Ni-NTA column (1 × 5 cm). The column was washed with 25 mL portions of Buffer A at pH 8.0, 6.5 and 5.9, sequentially. The target protein was eluted at pH 4.5.

Protein characterization: Ultracentrifugation and circular dichroism analyses were performed as described previously.[10, 14] Analysis of CD thermal melting data was performed according to a previously described procedure using a two-state model.[14] For A1-WT, the concentrations used for curve fitting were 10 and 100 μM, while concentrations of 2 and 100 μM were used to fit data for FA1-92. The thermodynamic quantities T_m, ΔH_m, ΔC_p and K_d were parameters of the fitting procedure and are reported in the 1M standard state (Table 2).

Received: January 2, 2001 [Z16355]

[1] a) S. M. Hecht, *Acc. Chem. Res.* **1992**, *25*, 545–552; b) V. W. Cornish, D. Mendel, P. G. Schultz, *Angew. Chem.* **1995**, *107*, 677–690; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 621–633; c) D. R. Liu, T. J. Maghery, M. Pastrnak, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10092–10097; d) R. Furter, *Protein Sci.* **1998**, *7*, 419–426; e) D. R. Liu, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4780–4785.

[2] J. C. M. van Hest, D. A. Tirrell, *FEBS Lett.* **1998**, *428*, 68–70.

[3] J. C. M. van Hest, K. L. Kiick, D. A. Tirrell, *J. Am. Chem. Soc.* **2000**, *122*, 1282–1288.

[4] N. Sharma, R. Furter, P. Kast, D. A. Tirrell, *FEBS Lett.* **2000**, *467*, 37–40.

[5] S. Kothakota, M. J. Fournier, D. A. Tirrell, T. L. Mason, *J. Am. Chem. Soc.* **1995**, *117*, 536–537.

[6] O. M. Rennert, H. S. Anker, *Biochemistry* **1963**, *2*, 471.

[7] For example, see: a) A. D. McLachlan, M. Stewart, *J. Mol. Biol.* **1975**, *98*, 293–304; b) S. Y. M. Lau, A. K. Taneja, R. S. Hodges, *J. Biol. Chem.* **1984**, *259*, 13253–13261; c) E. K. O'Shea, R. Rutkowski, P. S. Kim, *Science* **1989**, *243*, 538–542; d) E. K. O'Shea, J. D. Klemm, P. S. Kim, T. A. Alber, *Science* **1991**, *254*, 539–544; e) H. Wendt, A. Baici, H. R. Bosshard, *J. Am. Chem. Soc.* **1994**, *116*, 6073–6074; f) L. Gonzales, R. A. Brown, D. Richardson, T. Alber, *Nat. Struct. Biol.* **1996**, *3*, 1002–1100; g) K. T. Kenar, B. Garcia-Moreno, E. Freire, *Protein Sci.* **1995**, *4*, 1934–1938; h) S. F. Betz, P. A. Liebman, W. F. DeGrado, *Biochemistry* **1997**, *36*, 2450–2458; i) S. F. Betz, W. F. DeGrado, *Biochemistry* **1996**, *35*, 6955–6962, and references therein.

[8] a) C. A. Gough, D. A. Pearlman, P. Kollman, *J. Chem. Phys.* **1993**, *99*, 9103–9110; b) J. Hine, P. K. Mookejee, *J. Org. Chem.* **1975**, *40*, 292–297.

[9] V. P. Kukhar, V. A. Soloshonok, *Fluorine Containing Amino Acids—Synthesis and Properties*, Wiley, Chichester, **1995**.

[10] W. A. Petka, J. L. Harden, K. P. McGrath, D. Wirtz, D. A. Tirrell, *Science* **1998**, *281*, 389–392.

[11] a) S. Kothakota, Ph.D. Thesis, University of Massachusetts, Amherst, **1993**; b) W. A. Petka, Ph.D. Thesis, University of Massachusetts, Amherst, **1997**.

[12] a) J. Moitra, L. Szilak, D. Krylov, C. Vinson, *Biochemistry* **1997**, *36*, 12567–12573; b) R. S. Hodges, N. E. Zhou, C. M. Kay, P. D. Semchul, *Pept. Res.* **1990**, *3*, 125–137; c) B. Tripet, K. Wagschal, P. Lavigne, C. T. Mant, R. S. Hodges, *J. Mol. Biol.* **2000**, *300*, 377–402.

[13] a) W. Sandberg, T. Terwilliger, *Science* **1989**, *245*, 54–57; b) E. Baldwin, J. Xu, O. Hajiseyedjavadi, W. A. Baase, B. W. Matthews, *J. Mol. Biol.* **1996**, *259*, 542–559; c) H. Kono, M. Nishiyama, M. Tanokura, J. Doi, *Protein Eng.* **1998**, *11*, 47–52.

[14] J. P. Schneider, J. D. Lear, W. F. DeGrado, *J. Am. Chem. Soc.* **1997**, *119*, 5742–5743.

[15] C. Zhang, C. Ludin, M. K. Eberle, H. Stoeckli-Evans, R. Keese, *Helv. Chim. Acta* **1998**, *81*, 174–181.

 © WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2001 0044-8249/01/11308-1544 $ 17.50+.50/0