CHAPTER 2

Homoisoleucine: A Translationally Active Leucine Surrogate of Expanded Hydrophobic Surface Area

This chapter first appeared as a communication in *ChemBioChem*: James A. Van Deventer, John D. Fisk, and David A. Tirrell. *ChemBioChem* **2011,** 12, 700-702.

Abstract

Homoisoleucine (Hil) serves as an effective surrogate for leucine with respect to protein translation in bacterial cells. Replacement of Leu by Hil stabilizes coiled-coil peptides, as shown by the elevation of the thermal denaturation temperature. The increase in denaturation temperature is larger than that observed previously for replacement of Leu by trifluoroleucine.

Introduction

Noncanonical amino acids (ncAAs) provide useful tools for the investigation and control of protein behavior (1-4). Several laboratories have used ncAAs to explore the role of hydrophobic forces in stabilizing proteins, including prion proteins, T4 lysozyme, chloramphenicol acetyltransferase, green fluorescent protein, and coiled-coil and helix-bundle proteins (5-12). Here we examine the consequences of introducing the leucine surrogate (2*S*,4*S*)-2-amino-4-methylhexanoic acid (homoisoleucine, Hil, **2**; scheme 2.1) (5) into the coiled-coil peptide A1 (figure 2.1) (13). A1 contains six heptad repeats, designated (*abcdefg*), that mediate self-association of the peptide in aqueous solutions. Previous studies have shown that replacement of leucine by (2*S*,4*R*)-trifluoroleucine (Tfl, **3**) or hexafluoroleucine (Hfl, **4**) at the *d* positions of the heptad repeats leads to substantial stabilization of the coiled-coil structure of A1 (9, 10, 12), presumably through enhanced hydrophobic interactions between fluorinated peptide strands. Similar results have been obtained in other fluorinated coiled-coil and helix-bundle systems (14-16).

Whitesides and coworkers have pointed out that hydrocarbons and fluorocarbons exhibit equivalent “intrinsic” hydrophobicities when changes in molecular surface area are

taken into account (17). Marsh and coworkers have argued that the "efficient packing" of the bulkier fluorinated amino acids in helix bundle cores can be more important than fluorination per se (18). Because the molecular surface areas of Hil and Tfl are nearly identical (and larger than that of Leu by 14–19 $Å^2$) (19), we imagined that replacement of Leu by Hil might stabilize coiled-coil peptides such as A1.

Results and Discussion

We first focused our attention on the translational activity of Hil in bacterial cells. Schultz and coworkers reported incorporation of Hil into proteins via chemical misacylation of suppressor tRNA and *in vitro* translation (5), but we are unaware of previous studies of incorporation of Hil into cellular proteins. *E. coli* strain LAM1000 (a previously reported leucine auxotroph) was cotransformed with expression plasmid pA1EL and repressor plasmid pREP4 (10). pA1EL codes for both the protein A1 and a constitutively expressed copy of the *E. coli* leucyl-tRNA synthetase (LeuRS) gene. Protein expression was induced in minimal medium depleted of Leu and supplemented with Hil (see materials and methods for details). Electrophoretic analysis of whole-cell lysates indicated high-level protein expression in media containing as little as 0.25 mM L-Hil, a concentration comparable to the concentrations of canonical amino acids in minimal media.

ATP-PP_i exchange assays confirmed that Hil is activated by the *E. coli* LeuRS, albeit at a rate substantially lower than that characteristic of the natural substrate (table 2.1). The reduced rate of activation of Hil is consistent with our observation that Hil supports high-level protein expression only when LeuRS is overexpressed in the bacterial host.

A1 samples containing Leu and Hil were purified from 25 mL cultures in yields of 15.9 ± 2.5 mg L^{-1} and 10.2 ± 1.1 mg L^{-1}, respectively. Liter-scale expression and purification improved the yields of A1 sequence variants containing Hil two- to threefold. Liquid chromatography/mass spectrometry (LC/MS) indicated replacement of at least 97% of Leu by Hil (see "Determination of amino acid replacement levels" in materials and methods).

Figure 2.2A shows circular dichroism spectra of 10 μM solutions of the Leu- (Leu-A1) and Hil- (Hil-A1) forms of A1. Strong minima at 208 and 222 nm confirm that both proteins assume α-helical structures; K2D2 (20) analysis indicates helical contents of 60%–69%, consistent with the fact that the putative heptad repeats constitute 57% of the peptide sequence. A1 is expected to exist primarily as dimers under the conditions employed here (21).

Replacement of Leu by Hil increases the denaturation temperature of A1, as expected. Figure 2.2B shows the molar ellipticities at 222 nm of solutions of Leu-A1 and Hil-A1 as functions of temperature. Fitting the CD data to a model of a two-state transition between folded dimer and unfolded monomer states (22) yielded melting temperatures of 58.7 ± 0.2 °C and 75.8 ± 0.1 °C for Leu-A1 and Hil-A1, respectively.

Table 2.2 compares the extent to which the thermal denaturation temperature of A1 is elevated by replacement of Leu by bulkier hydrocarbon and fluorocarbon surrogates. Replacement of Leu by Hil raises T_m by 17 °C, as compared to 10 °C for replacement of Leu by (2*S*,4*R*)-Tfl and 22 °C for replacement by Hfl. Expansion of hydrophobic side chain volume at the *d*-position of the heptad repeat constitutes an effective strategy for

stabilization of coiled-coil peptides, irrespective of the hydrocarbon or fluorocarbon character of the side chain.

We do not mean to suggest similar molecular origins for the hydrophobic properties of hydrocarbons and fluorocarbons. Although Hil and Tfl behave similarly with respect to elevation of the melting temperature of A1, other experiments suggest important differences in the behavior of water adjacent to hydrocarbon and fluorocarbon side chains. Recent studies via ultrafast spectroscopy indicate a marked slowing of water motions upon replacement of Leu by Tfl at solvent-exposed sites (23). In contrast, replacement of Leu by Hil is accompanied by increased rates of solvent reorganization. Much remains to be done to elucidate the origins of hydrophobic effects in proteins and other molecular systems.

In conclusion, we find that Hil serves as an effective surrogate for Leu with respect to protein translation in bacterial cells, and that replacement of Leu by Hil leads to substantial stabilization of recombinant coiled-coil peptides. The results reported here also highlight the value of amino acid replacement at multiple sites in peptides and proteins; replacement of a single Leu residue by Hil in T4 lysozyme has been reported to cause an increase of just 1.9 °C in the melting temperature of the protein (5). In contrast, replacement of six Leu residues in the putative coiled-coil domain of A1 raises T_m by 17 °C.

Materials and Methods

Materials. All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. Dry solvents were obtained from commercial suppliers and used as received. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Gold

Biotechnologies; Ni-NTA resin and spin columns were purchased from Qiagen. Sequencing grade modified trypsin was purchased from Promega; C18 Zip Tips, and Amicon Ultra-4 and Amicon Ultra-15 concentration devices were purchased from Millipore. A BCA assay kit was obtained from Pierce Protein Research Products. The *E. coli* expression strain LAM1000 outfitted with the plasmids pA1EL and pREP4 has been described previously (10).

Protein expression. For 25 mL and 200 mL scale preparations of Hil-A1 and small-scale preparation of Leu-A1, a single colony of LAM1000 transformed with pA1EL and pREP4 was used to inoculate an overnight culture of M9 minimal medium (M9 salts containing glucose (0.4% w/v), thiamine HCl (35 mg L^{-1}), $MgSO_4$ (1 mM), $CaCl_2$ (0.1 mM), and 20 amino acids (40 mg L^{-1})) supplemented with ampicillin (200 mg L^{-1}), and kanamycin (35 mg L^{-1}). Overnight cultures were diluted into fresh M9 medium containing all 20 amino acids and allowed to grow with agitation at 37 °C until reaching an OD_{600} of approximately 0.9–1.0. Cells were pelleted at 6000 × *g* for 7 minutes at 4 °C, washed 3 times in ice-cold sodium chloride solution (0.9% w/v) and resuspended in fresh M9 minimal medium lacking leucine. Aliquots of the resuspended cultures were supplemented with Hil (0.5 mM) or Leu (0.3 mM), shaken at 37 °C for 15 minutes, and induced by addition of IPTG (1 mM final concentration). After 3 hours, cells were harvested by centrifugation at 6000 × *g* for 7 minutes. In the case of small-scale production, cells were frozen at −80 °C either before or after addition of Qiagen buffer B (8 M urea, pH 8.0, buffered with NaH_2PO_4 (100 mM) and Tris·Cl (10 mM)). For the case of large-scale production, cells were frozen at −80 °C before addition of Qiagen buffer B.

For 200 mL scale production of Leu-A1, a single colony of LAM1000 transformed with pA1EL and pREP4 was used to inoculate an overnight culture of 2×YT medium supplemented with ampicillin (200 mg L^{-1}) and kanamycin (35 mg L^{-1}). The overnight culture was diluted 1:100 into fresh 2×YT supplemented with ampicillin and kanamycin and allowed to grow at 37 °C with shaking until reaching an OD_{600} of approximately 0.9–1.1. The culture was induced by addition of IPTG to a final concentration of 1 mM. After 3 hours, cells were harvested by centrifugation at 6000 × *g* for 7 minutes and frozen at −80 °C.

Protein purification. At small scales, the cell pellets were thawed, Qiagen buffer B was added as necessary, and the resuspended pellets were incubated at room temperature with gentle agitation for at least 1 hour. The pellets were then subjected to 20–40 minutes of sonication in an immersion sonicator. In some cases, the pellets were frozen again at −80 °C and the above procedure was repeated. The sonicated pellets were then centrifuged for 20–30 minutes at 10000 × *g*, and the supernatants were saved. The clarified lysates were then subjected to purification using Qiagen Ni-NTA spin columns according to the manufacturer's protocols (2000 Ni-NTA Spin Handbook) or using Ni-NTA agarose according to the manufacturer's protocols with slight modifications. When Ni-NTA agarose was used, Qiagen buffer C was supplemented with 50 mM imidazole, and Qiagen buffer E was supplemented with 250 mM imidazole.

At large scales, the cell pellets were resuspended in Qiagen buffer B and subjected to sonication using a microtip on a Misonix Sonicator 3000. The total sonication process time was 10 minutes, with 5 second sonication pulses and 5 second wait periods. The

pellets were then frozen again at −80 °C and subjected to a second round of sonication. The lysates were clarified by centrifugation at approximately 75000 × *g* for 10 minutes at 25 °C. The clarified lysate was then subjected to purification using Ni-NTA agarose with buffers as described above.

Peptide mass spectrometry. Small (1–3 μL) samples of Leu-A1 and Hil-A1 eluents from the purifications were diluted 50- to 100-fold into sodium bicarbonate (50 mM, pH 7.8) and digested overnight by treatment with sequencing grade modified trypsin (5 μL) at 37 °C. Portions of these samples were desalted using C18 Zip Tips according to the manufacturer's instructions with one slight modification. Prior to column equilibration in 0.1% trifluoroacetic acid, columns were wetted with a 50/50 mixture of 0.1% trifluoroacetic acid and acetonitrile. The cleaned samples were then analyzed using matrix-assisted laser desorption ionization (MALDI) mass spectrometry on an Applied Biosystems Voyager DE Pro instrument at the mass spectrometry facility of the Caltech Division of Chemistry and Chemical Engineering. All peptide masses were found to lie within the mass ranges of manufacturer-specified instrument tolerances. See figure 2.3 for typical results.

For quantitative analysis, the remainders of the trypsinized samples (not desalted) were submitted to the Protein and Peptide Mass Analytical Laboratory (PPMAL) of the Beckman Institute at Caltech for liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis. The samples were separated on a 6 cm long, 100 μm diameter C18 column using an Eksigent NanoLC-2D and then immediately injected into an Applied Biosystems QStar XL tandem mass spectrometer. Time-resolved data were analyzed using Analyst QS software provided by Applied Biosystems. Amino acid replacement levels

were determined by using information contained within extracted ion currents (XIC) of trypsin-digested protein samples. Peaks corresponding to peptides globally substituted with Hil were identified along with peaks corresponding to peptides substituted at only a fraction of the Leu positions. Assuming that substitution of Hil in place of Leu is a random event, the ratio of the peak areas yields the quantitative replacement level in a given sample. A full description of the method is provided below. Where possible, fragmentation of abundant peptide ions was used to confirm the sequences of substituted peptides.

Determination of amino acid replacement levels. Liquid chromatography tandem mass spectrometry (LC/MS/MS) was used to quantitate the Hil replacement levels in purified A1 protein samples. Figure 2.4 depicts results from a typical LC/MS/MS experiment. Figure 2.4A shows the total ion currents (TIC) from the trypsinized Hil-A1, and figure 2.4B–E show the mass spectra and mass-filtered extracted ion currents (XIC) from the doubly substituted peptide SXEDEAAEXEQK (X = Hil) and the mixture of singly substituted peptides SLEDEAAEXEQK and SXEDEAAELEQK. The unsubstituted peptide SLEDEAAELEQK was not detected in this experiment. The areas of the XICs permit an estimation of the substitution rate. Assuming that replacement of leucine is a random event, the distribution of peak areas should follow the binomial distribution,

$$A\left[(1-p)^2 + 2p(1-p) + p^2\right], \quad (2.1)$$

where A is a multiplication factor equal to the sum of the areas of the unsubstituted, singly substituted, and doubly substituted peaks. The quantity p is the probability of substitution at

a given site, and the three terms in the polynomial represent non-, singly-, and doubly-substituted peptides. The polynomial also has the property

$$(1-p)^2 + 2p(1-p) + p^2 = 1. \quad (2.2)$$

The ratios of the areas of two substituted peaks are more relevant in this particular case because the total area *A* cannot be determined from the available experimental data. The experimentally accessible peak area ratio of singly to doubly substituted peaks is

$$\frac{2p(1-p)}{p^2}, \quad (2.3)$$

which can be defined as *X*. Rearranging equation (2.2) yields the identity

$$\frac{2p-2p^2}{p^2} = \frac{2p(1-p)}{p^2} = X. \quad (2.4)$$

Solving for *p* yields the two roots

$$p = 0, \quad (2.5)$$

or

$$p = \frac{2}{X+2}. \quad (2.6)$$

Substituting the peak area ratio for *X* in the nonzero root yields the substitution rate.

The above analysis was used to determine the substitution of Hil in place of Leu in the peptides SLEDEAAELEQK and GSHHHHHHGSMASGDLENEVAQLER. A simpler calculation was also made to determine the incorporation of Hil based on the peptide AEIGDLNNTSGIR, which contains one leucine residue (The value *p* can be determined from the ratio of the XIC area of the substituted peptide to the sum of the substituted and unsubstituted peptide areas). When the XIC contained multiple peaks (often the case when searching for peptides of lower abundance), the areas of the clearly distinguishable peaks (generally having areas of 10 or more) were summed in order to ensure a conservative estimate of amino acid incorporation levels. The identities of the peptides in the SLEDEAAELEQK series were confirmed by tandem mass spectrometry. Two samples of Hil-A1 produced independently were analyzed to obtain the substitution levels summarized in table 2.3.

Protein characterization. To determine protein yields, portions of Leu-A1 and Hil-A1 produced at small scale and purified using Ni-NTA agarose resin were buffer exchanged into acetate buffer (100 mM NaCl, 10 mM sodium acetate, pH 4.0) using Amicon Ultra-4 concentration devices with a 3000 Da molecular weight cutoff. The concentrations of the exchanged samples were determined with a BCA assay kit.

Circular dichroism samples were prepared by dialyzing protein samples in Qiagen buffer E (containing 250 mM imidazole) against phosphate-buffered saline, pH 7.4. Concentrations were again determined using the BCA assay, but prior to performing the assay, samples were heated to 42 °C for approximately 20 minutes in order to ensure that

samples were fully dissolved. Samples were adjusted to 10 μM by concentration with Amicon Ultra-15 concentration devices (molecular weight cutoff = 3000 Da) as necessary and dilution in fresh PBS. Circular dichroism spectroscopy was performed on an Aviv 62DS spectropolarimeter in a 1 mm path length cell. All samples were heated to 42 °C and cooled on ice prior to measurement. Each experiment consisted of a wavelength scan performed at 1 °C followed by a temperature scan from 0 °C to 94.5 °C in 1.5 °C intervals with signal monitoring at 222 nm. During the temperature scans, the sample was allowed to equilibrate for one minute prior to performing readings at each temperature step. All data was referenced to background scans of PBS buffer acquired under identical conditions. Wavelength scans were analyzed using K2D2 (20), and temperature scans were analyzed using a Matlab implementation of a model for coiled-coil unfolding described previously (22).

ATP-PP_i exchange assays. *E. coli* LeuRS was expressed and purified as previously described and its concentration was determined from its absorbance at 280 nm under native conditions (24). Assays were run at room temperature in buffer containing HEPES (30 mM, pH 6.8), $MgCl_2$ (10 mM), dithiothreitol (1 mM), ATP (2 mM), and [^{32}P]-PP_i (2 mM, 3 μCi in 200 μL rxn volume). Activation of leucine was performed in solutions containing LeuRS (75 nM) and varying Leu concentrations (1.6 to 50 μM), while activation of homoisoleucine was measured using a higher concentration of LeuRS (300 nM) and varying Hil concentrations (16 to 500 μM). Aliquots (30 μL) were taken every 2 (Leu) or 6 (Hil) minutes and quenched in a suspension of activated charcoal (3% w/v) containing $HClO_4$ (7% w/v) and inorganic pyrophosphate (200 mM). The

charcoal was washed twice in a solution of $HClO_4$ (0.5% w/v) and inorganic pyrophosphate (10 mM), and added to 20 mL scintillation vials. Scintillation fluid (5 mL) was added to each vial and samples were counted using a Beckman Coulter liquid scintillation counter. Data were fitted with nonlinear regression using the program Igor.

Synthesis of homoisoleucine (2-amino-4-methylhexanoic acid, CAS # 3570-21-6). Silica chromatography was performed using 230-400 mesh silica gel 60 (EMD). TLC was run on Baker-flex silica gel IB-F plates, R_fs are reported under the same solvent conditions as columns unless otherwise noted. TLC was examined under UV light for fluorescent compounds or alternatively stained with $KMnO_4$, ceric ammonium molybdenate or *p*-anisaldehyde. NMR spectra were recorded on Varian spectrometers (300 MHz for ^1H) and processed with NUTS NMR software. NMR spectra were referenced to internal standards; proton spectra were referenced to tetramethylsilane and carbon spectra were referenced to solvent peaks. FAB mass spectrometry was performed at the Caltech Division of Chemistry and Chemical Engineering Mass Spectrometry Facility.

The following procedure was adapted from O'Donnell and Eckrich (25): aminoacetonitrile benzophenone imine (1.21 g, 5.5 mmol), benzyltriethylammonium chloride (0.1 g, 0.4 mmol), 50% aq NaOH (0.75 mL, 14 mmol) and toluene (1 mL) were combined in a 10 mL round bottom flask which contained a magnetic stir bar. The flask was chilled in an ice-water bath and stirred vigorously (~1200 rpm). (*S*)-2-methyl bromobutane (1.15 g, 0.94 mL, 7.6 mmol, 1.4 eq) was added portion-wise via syringe over 1 hour to the stirred solution. The reaction mixture was stirred for an additional 2 hours at 0 °C and then allowed to come to room temperature. Stirring was continued at room

temperature for 96 hours. The reaction mixture was transferred to a separatory funnel and diluted with H_2O (20 mL) and CH_2Cl_2 (40 mL). The aqueous layer was washed 3 times with CH_2Cl_2 (10 mL × 3) and the combined organic layers were washed 3 times with H_2O (10 mL × 3) and once with saturated NaCl (10 mL). The organic layers were dried over Na_2SO_4 and filtered, and the solvent was removed to yield a yellow oil (1.80 g). Purification by silica chromatography (eluting with 6% ethyl acetate in hexanes; R_f 0.18 in 10% ethyl acetate/hexanes) gave a yellow oil (1.40g, 88%); a mixture of diastereomers. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.73 (dd, 3H J = 4.5, 6.5 Hz), 0.82 (dd, 3H J = 7.5, 14.0 Hz), 1.00-1.37(m, 2H), 1.41-1.81(m, 2H), 1.82-2.14 (m, 1H), 4.27 (dd, 0.5H, J = 6.3, 8.0 Hz), 4.31 (dd, 0.5H, J = 6.3, 8.1 Hz), 7.15-7.26 (m, 2H), 7.27-7.37 (m, 2H), 7.37-7.58 (m, 4H), 7.58-7.82 (m, 2H) ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.724, 10.891, 18.416, 18.699, 28.865, 28.923, 30.680, 31.086, 41.266, 41.347, 51.037, 51.606, 119.747, 119.899, 127.108, 127.238, 128.039, 128.789, 129.205, 130.933, 135.007, 135.051, 138.243, 138.294, 172.259, 172.582. FAB MS calculated for $C_{20}H_{22}N_2$ (M+) 290.1783, observed 290.1787.

The following procedure was adapted from Dorizon et al. (26): HCl (1 M, 14 mL) was added dropwise to a solution of alkylated aminoacetonitrile benzophenone imine (290 mg, 1.00 mmol) in diethyl ether (7 mL). The mixture was stirred vigorously at room temperature for 24 hours. The aqueous phase was extracted twice with diethyl ether and evaporated to give 1-amino-1-cyano-3-methylpentane hydrochloride (180 mg, ~100%). This material was dissolved in 6 M HCl (3 mL) and heated to reflux for 72 h. The solution was evaporated under reduced pressure to give an off-white solid (168 mg, 93%). ^{1}H NMR (300 MHz, CD_3OD) δ 0.929 (t, 3 H J = 7.5 Hz), 0.936 (t, 3 H J = 7.5 Hz), 0.979 (d, 3 H J = 3.6), 0.999 (d, 3 H J = 3.4), 1.148-1.548 (m, 2 H), 1.554-2.010 (m, 3 H), 3.979 (m, 1H);

^{13}C NMR (75 MHz, CD_3OD) δ 10.036, 10.265, 17.665, 18.004, 28.846, 29.254, 30.649, 30.659, 37.601, 37.656, 51.117, 51.133, 171.117, 171.244; FAB MS calculated for $C_7H_{16}NO_2$ (M+H) 146.1176, observed 146.1205.

Acknowledgements

We thank Shelly Tzlil for assistance in implementing the model for thermal unfolding of coiled-coil peptides in Matlab. This research was supported by NIH grant GM62523. J. A. V. was supported in part by an N. D. S. E. G. Fellowship; J. D. F. was supported in part by an NIH Postdoctoral Fellowship.

References

1. Liu CC & Schultz PG (2010) Adding new chemistries to the genetic code. *Annu. Rev. Biochem.* **79,** 413-444.
2. Dougherty DA (2009) In Vivo Studies of Receptors and Ion Channels with Unnatural Amino Acids. *Protein Eng.,* Nucleic Acids and Molecular Biology, eds Kohrer C & RajBhandary UL (Springer-Verlag, Berlin), Vol 22, pp 231-254.
3. Budisa N (2006) *Engineering the Genetic Code: Expanding the Amino Acid Repertoire for the Design of Novel Proteins* (Wiley-VCH, Weinheim, Germany).
4. Connor RE & Tirrell DA (2007) Non-canonical amino acids in protein polymer design. *Polymer Reviews* **47,** 9-28.
5. Mendel D*, et al.* (1992) Probing protein stability with unnatural amino acids. *Science* **256,** 1798-1802.

6. Wolschner C*, et al.* (2009) Design of anti- and pro-aggregation variants to assess the effects of methionine oxidation in human prion protein. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 7756-7761.

7. Montclare JK & Tirrell DA (2006) Evolving proteins of novel composition. *Angew. Chem. Int. Ed.* **45,** 4518-4521.

8. Yoo TH, Link AJ, & Tirrell DA (2007) Evolution of a fluorinated green fluorescent protein. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 13887-13890.

9. Tang Y*, et al.* (2001) Fluorinated coiled-coil proteins prepared in vivo display enhanced thermal and chemical stability. *Angew. Chem. Int. Ed.* **40,** 1494-1496.

10. Tang Y & Tirrell DA (2001) Biosynthesis of a highly stable coiled-coil protein containing hexafluoroleucine in an engineered bacterial host. *J. Am. Chem. Soc.* **123,** 11089-11090.

11. Son S, Tanrikulu IC, & Tirrell DA (2006) Stabilization of bzip peptides through incorporation of fluorinated aliphatic residues. *ChemBioChem* **7,** 1251-1257.

12. Montclare JK, Son S, Clark GA, Kumar K, & Tirrell DA (2009) Biosynthesis and stability of coiled-coil peptides containing (2S,4R)-5,5,5-trifluoroleucine and (2S,4S)-5,5,5-trifluoroleucine. *ChemBioChem* **10,** 84-86.

13. Petka WA, Harden JL, McGrath KP, Wirtz D, & Tirrell DA (1998) Reversible hydrogels from self-assembling artificial proteins. *Science* **281,** 389-392.

14. Tang Y*, et al.* (2001) Stabilization of coiled-coil peptide domains by introduction of trifluoroleucine. *Biochemistry* **40,** 2790-2796.

15. Bilgicer B, Fichera A, & Kumar K (2001) A coiled coil with a fluorous core. *J. Am. Chem. Soc.* **123,** 4393-4399.

16. Lee KH, Lee HY, Slutsky MM, Anderson JT, & Marsh ENG (2004) Fluorous effect in proteins: De novo design and characterization of a four-alpha-helix bundle protein containing hexafluoroleucine. *Biochemistry* **43,** 16277-16284.

17. Gao JM, Qiao S, & Whitesides GM (1995) Increasing binding constants of ligands to carbonic-anhydrase by using greasy tails. *J. Med. Chem.* **38,** 2292-2301.

18. Buer BC, de la Salud-Bea R, Hashimi HMA, & Marsh ENG (2009) Engineering protein stability and specificity using fluorous amino acids: the importance of packing effects. *Biochemistry* **48,** 10810-10817.

19. Molecular surface areas of Leu, Hil, (*S*,*S*)-Tfl, and (*S*,*R*)-Tfl were determined using Spartan '08 (Wavefunction, Inc. Irvine, CA). Structures of amino acids were minimized using Hartree-Fock calculations with a 6-31G* basis in vacuum, followed by determination of the molecular surface areas.

20. Perez-Iratxeta C & Andrade-Navarro MA (2008) K2D2: estimation of protein secondary structure from circular dichroism spectra. *BMC Struct. Biol.* **8**.

21. Tang et al. confirmed the predominance of dimers in dilute solutions of A1 containing Leu or fluorinated analogs of Leu at neutral pH; the oligomerization state at low (10 μM) concentrations was found to be insensitive to fluorination (9, 10).

22. Schneider JP, Lear JD, & DeGrado WF (1997) A designed buried salt bridge in a heterodimeric coiled coil. *J. Am. Chem. Soc.* **119,** 5742-5743.

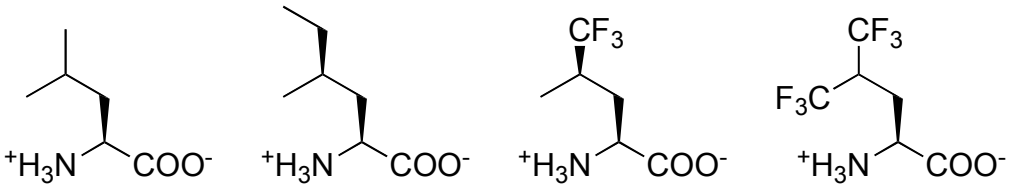
23. Kwon OH*, et al.* (2010) Hydration dynamics at fluorinated protein surfaces. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 17101-17106.

24. Tang Y & Tirrell DA (2002) Attenuation of the editing activity of the Escherichia coli Leucyl-tRNA synthetase allows incorporation of novel amino acids into proteins in vivo. *Biochemistry* **41,** 10635-10645.

25. Odonnell MJ & Eckrich TM (1978) Synthesis of amino acid derivatives by catalytic phase transfer alkylations. *Tetrahedron Lett.* 4625-4628.

26. Dorizon P*, et al.* (1999) Stereoselective synthesis of highly functionalized cyclopropanes. Application to the asymmetric synthesis of (1S,2S)-2,3-methanoamino acids. *J. Org. Chem.* **64,** 4712-4724.

27. Boniecki MT, Vu MT, Betha AK, & Martinis SA (2008) CP1-dependent partitioning of pretransfer and posttransfer editing in leucyl-tRNA synthetase. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 19223-19228.



1 2 3 4

Scheme 2.1. Amino acids used in study. **1**, leucine (Leu). **2**, (2*S*,4*S*)-2-amino-4-methylhexanoic acid (homoisoleucine, Hil). **3**, (2*S*,4*R*)-trifluoroleucine (Tfl). **4**, hexafluoroleucine (Hfl).

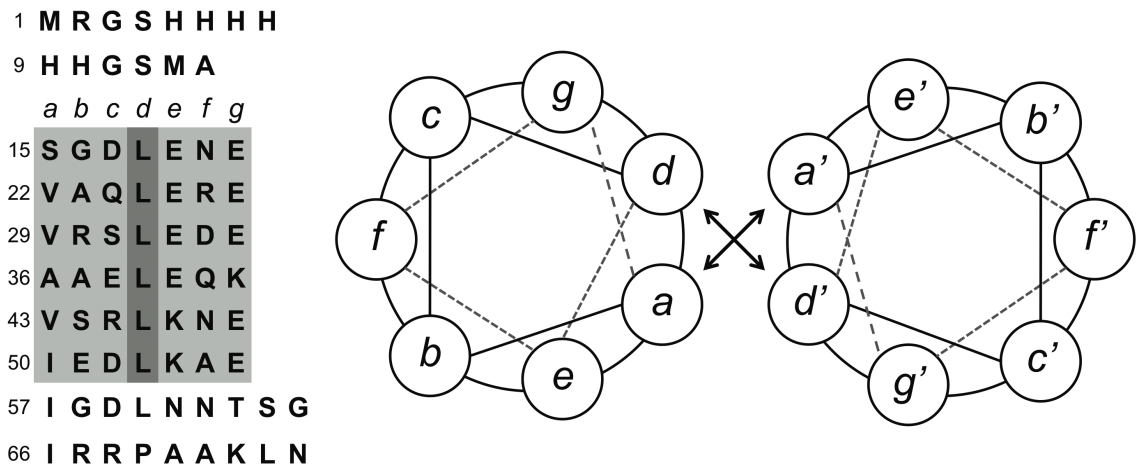


Figure 2.1. A1 peptide sequence and helical wheel representation of A1 homodimers. The amino acids that comprise the putative heptad repeats are highlighted in gray, with additional emphasis on the leucine residues at the *d* positions.

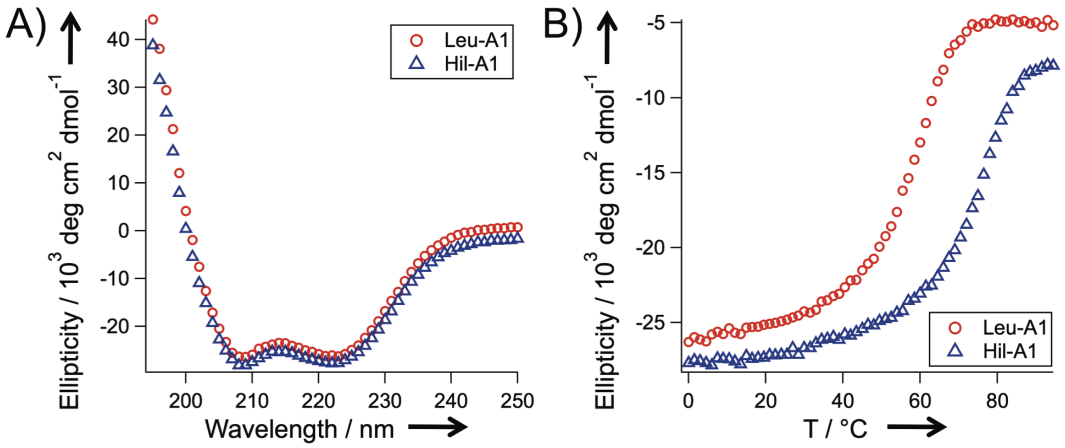


Figure 2.2. Circular dichroism spectra of Leu-A1 and Hil-A1. A) Wavelength scans performed at 1 °C. B) Ellipticity at 222 nm as a function of temperature. All experiments were performed with 10 μM peptide in PBS, pH 7.4.

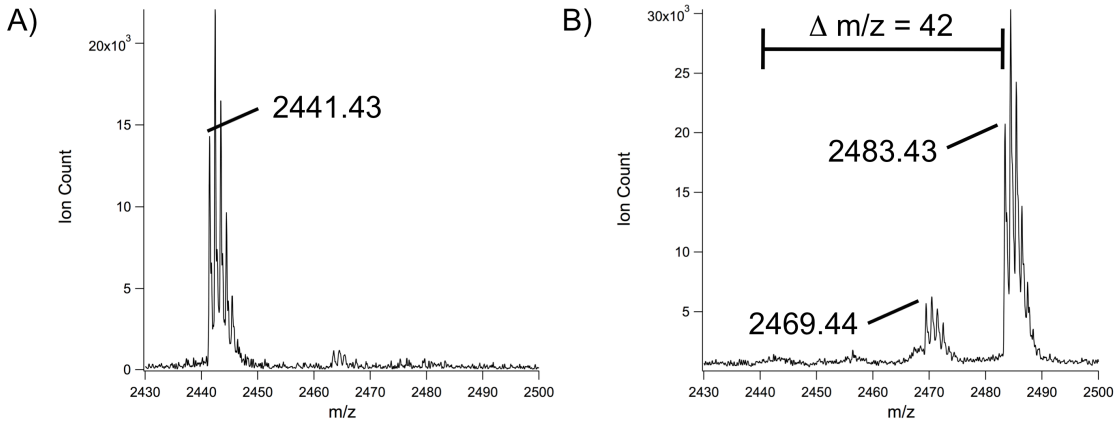


Figure 2.3. MALDI spectra of tryptic fragments of A) Leu-A1 and B) Hil-A1. The portions of the spectra shown encompass the m/z region in which the peptide LKNEIEDLKAEIGDLNNTSGIR appears with or without Hil substitution. In the Leu-A1 sample, the major peak appears at 2441.43 Da (calculated mass: 2442.28 Da). In the Hil-A1 sample, the largest peak appears at 2483.43 Da (calculated mass: 2484.32 Da), 42 mass units away from the unsubstituted peak, corresponding to complete replacement of Leu by Hil. A smaller peak at 2469.44 Da (calculated mass: 2470.31 Da) indicates the presence of some peptides that contain only two out of three Hil substitutions.

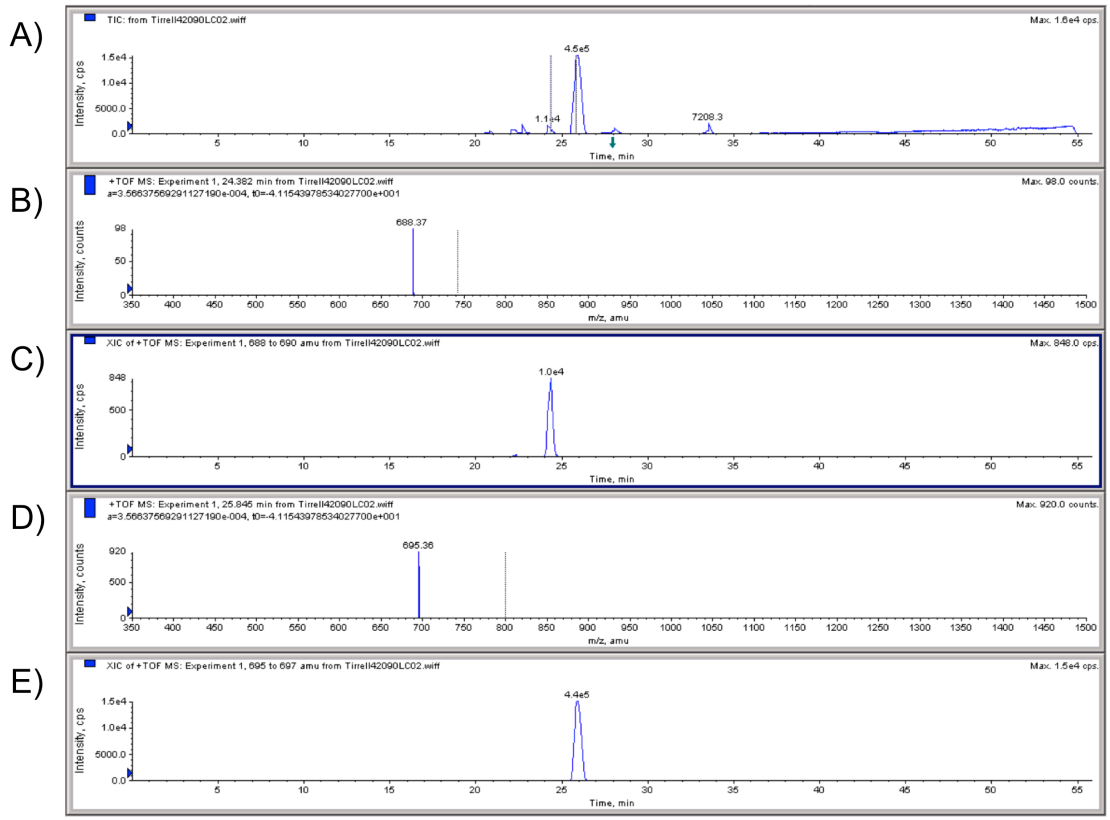


Figure 2.4. LC/MS results obtained on trypsinized sample of Hil-A1 (Sample A in table 2.3). A) Total ion current (TIC) of sample. B) Mass spectrum at time = 24.382 min, revealing a doubly charged ion having the mass corresponding to the masses of the singly substituted peptides SXEDEAAELEQK and SLEDEAAEXEQK (X = Hil). Calculated doubly charged ion 688.33 Da, observed 688.37 Da. C) Extracted ion current (XIC) of masses ranging from 688 to 690 amu. The mass filtering reveals a single, well-defined peak of integrated area 1.0×10^4 arbitrary units. D) Mass spectrum at time = 25.845 min, revealing a doubly charged peak having a mass corresponding to the mass of the doubly substituted peptide SXEDEAAEXEQK. Calculated doubly charged ion 695.34 Da, observed 695.36 Da. E) XIC of masses ranging from 695 to 697 amu. Again, a single, well-defined peak is observed and has an integrated area of 4.4×10^5 arbitrary units. The sequences of these peptides were confirmed with LC/MS/MS fragmentation.

Table 2.1. Kinetic parameters for activation of Leu and Hil by LeuRS

Substrate[a]	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [rel]
Leu[b]	15.1 ± 2.2	3.7 ± 1.9	1
Hil	0.4 ± 0.1	77 ± 65	1/690

[a] Leu was used as the L-isomer; Hil as a mixture of the D- and L- isomers. The concentrations of Hil reported here are those of the L-isomer. Kinetic parameters are reported as averages determined from three independent experiments with errors reported as averages of the 95% confidence intervals. [b] Parameters determined for activation of Leu are consistent with previous reports; the value of k_{cat} measured in this work is within the range of reported values, while the value of K_m reported here is lower than literature values by a factor of 2–10 (10, 12, 24, 27).

Table 2.2. Stabilization of A1 by replacement of Leu with noncanonical amino acids

Amino acid at *d* position	Leu	(2*S*,4*R*)-Tfl	Hil	Hfl[b]
ΔT_m[a]	0	10 (ref 12)	17	22 (ref 10)

[a] Increase in melting temperature (as compared to Leu-A1) determined from CD spectroscopy of 10 μM solutions of peptide in PBS, pH 7.4. [b] 74% replacement of Leu.

Table 2.3. Incorporation levels of Hil in A1 samples determined from multiple series of substituted peptides

Peptide series	Sample A	Sample B
SLEDEAAELEQK	98.9%	97.1%
GSHHHHHHGSMASGDLENEVAQLER	99.5%	99.0%
AEIGDLNNTSGIR	98.5%	ND[a]
[a] Not determined due to insufficient signal of unsubstituted ion.		