Evaluation of the Molecular Interactions of Fluorinated Amino Acids with Native Polypeptides

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Investigation of the properties of fluorinated amino acids in a native polypeptide environment increases our ability to use the manifold functionalities and varied structural features of this class of non-natural amino acids for protein engineering.[1] Fluorine as a functional group is not found within the natural amino acid pool.[2] Due to their unique electronic properties, fluorinated amino acids have dramatic effects on protein stability, protein–protein as well as ligand–receptor interactions, and the physical properties of protein-based materials.[3] Furthermore, incorporation of fluorine into peptides and proteins provides an opportunity to study their conformational properties and metabolic processes by ^{19}F NMR.[4] α-Helical coiled-coil peptides could serve as a model system of such a natural protein environment for studying the impact of fluorine substitution on distinct hydrophobic as well as charge interactions. Fluorinated amino acids can be incorporated into different positions of a heptad repeat motif, and their influence on coiled-coil peptide formation assessed.It has been shown that the substitution of leucine in the hydrophobic surface of an α-helical coiled-coil peptide by trifluoroleucine[5] or hexafluoroleucine[6] results in enhanced thermal as well as structural stability of the resulting peptide or protein assembly. These results suggest that the increase in stability is based on stronger interactions between fluoroalkyl groups than between their hydrocarbon analogues. Furthermore, it could be shown that fluoroalkyl substituents in peptides tend to cluster and to avoid interaction with hydrocarbon site chains;[7] an effect that is well known from fluorine-based materials.

The steric bulk of a trifluoromethyl group is still a controversial issue. Tirrell et al. consider substitution of hydrogen by fluorine in an amino acid to be isosteric.[3a] Therefore, they have used trifluoro or hexafluoro leucine as substitutes for leucine in a coiled-coil peptide, as, according to their theory, these substitutes should have space-filling properties similar to leucine. On the other hand, according to Nagai et al., the volume of a trifluoromethyl group should be close to that of an isopropyl group.[8] Considering this controversy, fluorinated ethyl glycines may serve as good starting points for screening the space-filling properties of fluoroalkyl substituents within the hydrophobic core of an α-helical coiled-coil peptide.

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The ability of fluoroalkylated groups to participate in hydrogen bonding has been the subject of another controversial discussion for many years. The C–H–F–C interaction is supposed to be one of the weakest hydrogen bonds,[9] and its importance for the folding of fluoro-modified peptides as well as the action of peptide-based drugs has not been studied systematically yet. Thus, the interaction pattern of fluorinated amino acids with proteinogenic amino acids remains to be investigated.

The development of two simple screens based on the coiled-coil motif provides a systematic gauge of the influence of fluorinated substituents on the stability and folding of α-helical structure elements in proteins. Moreover, the results of these studies will contribute to elucidating complex questions about the space-filling properties of fluoroalkylated amino acid side chains as well as the influences of their unique electronic properties on peptide and protein folding.

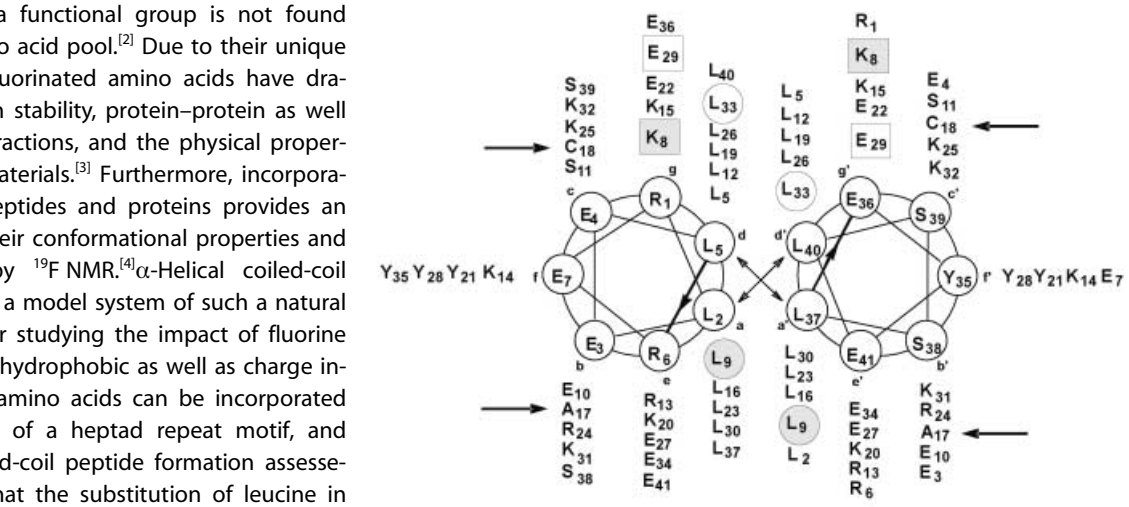

Figure 1. *Helical-wheel representation of the α-helical coiled-coil peptides and amino acid sequences.[15] The substitution positions g,g′-K8 and a,a′-L9 (shaded in gray) and their interaction partners g′,g-E29 and d′,d-L33 (unshaded) are highlighted with squares (charged interface) and circles (hydrophobic interface). The ligation sites of the electrophilic and nucleophilic fragments are marked with arrows.*

One screen of the interactions between a broad range of fluorinated amino acids and native amino acids measures the stability of such modified α-helical coiled-coil dimers (Figure 1).

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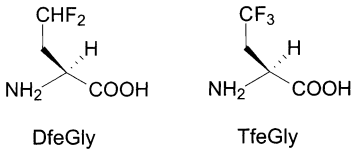
The single helices of the coiled-coil peptide consist of 4–3 heptad repeats. The hydrophobic surface formed by residues at positions a and d provides the thermodynamic driving force for the formation of the coiled coil, whereas the complement between charged amino acid residues in positions e and g provides for the specificity of folding. This system has been shown to respond very sensitively to even single mutations within either of the recognition domains.[10]

A second screen uses the property of peptides with hydrophobic heptad repeats to self-replicate.[11] The efficiency of template-assisted peptide-bond formation strongly depends on the degree of complement of interacting amino acid side chains of both recognition domains.

We designed a control peptide that consists of five full heptad repeats (Figure 1) that fold into an antiparallel coiled coil. We also designed six variants of the control sequence. Four contain the fluorinated amino acids (*S*)-trifluoroethyl glycine (TfeGly) and (*S*)-difluoroethyl glycine (DfeGly)[12] either at



position 9 within the hydrophobic core or position 8 within the charged surface; two others contain either one hydrophobic amino acid (Ala) at position 8 of the charged surface or one charged amino acid (Glu) at position 9 of the hydrophobic core. The last two variants were designed as points of comparison with the fluorinated peptides. Melting curves for all seven peptides (control plus six variants) were measured by temperature-dependent CD spectroscopy in guanidine hydrochloride (Table 1). The use of urea as denaturizing reagent did not lead to a sufficient destabilization of the coiled-coil peptides even at high concentrations (8 M).

Table 1. *Melting temperatures of the α-helical coiled-coil control sequence and its variants.*

Peptide	*Tm*[a] [°C]
control sequence	73.9
K8DfeGly	68.3
L9DfeGly	54.4
K8TfeGly	68.9
L9TfeGly	59.0
K8A	71.3
L9E	62.5

[a] T_m is defined as the temperature at which 50% of the peptide is unfolded.

All of the substitutions are placed within the part of the peptide that serves as electrophilic fragment in thioester-mediated chemical ligation with the nucleophilic peptide to produce the full-length sequence. Thus, the rate of the self-replication will give evidence of the sensitivity of the chosen system in responding to subtle changes in structure and properties of fluorinated side chains.

To prove that the control sequence has self-replicating properties, E_O and Nu were incubated at pH 7.0 with and without added full-length peptide. Addition of increasing amounts of template results in acceleration of product formation; this indicates an autocatalytic system[13] (results not shown).

The rates of product formation for all of the variant peptides differ from the original sequence; however, substitutions within the hydrophobic domain have a stronger impact than substitutions within the charged surface (Figure 2). Incorporation of a charged amino acid (Glu) instead of Leu in the hydrophobic core leads to a dramatically slower reaction rate; this is consistent with the L9Glu variant-unique character of not forming a dimer at room temperature in 5 M GnHCl (Figure 3).

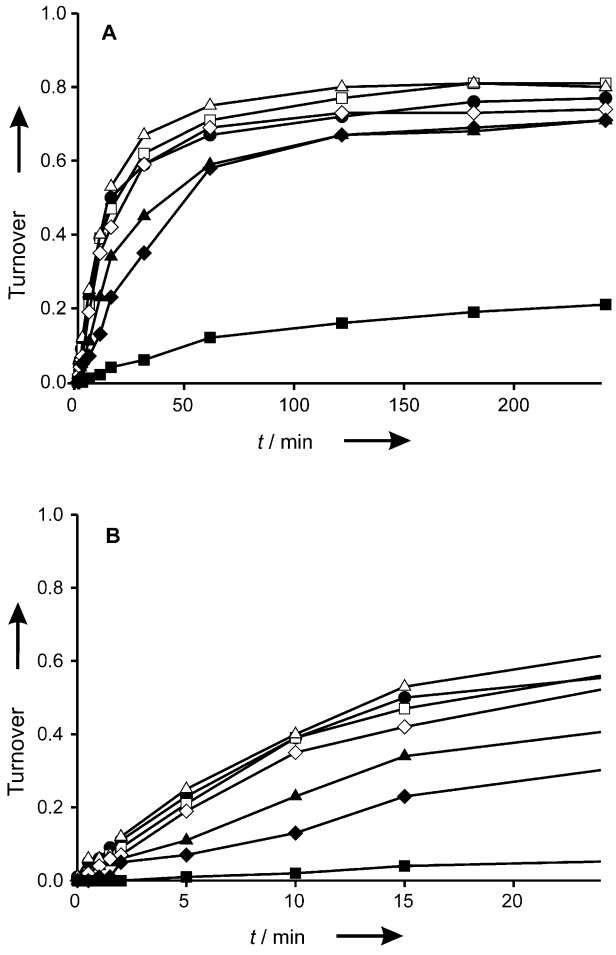


Figure 2. *A) Complete recorded and B) initial replicase turnover rates of electrophilic peptide fragments with the control sequence (circles) and substitutions of DfeGly (triangles) and TfeGly (diamonds) in positions K8 (open symbols) and L9 (closed symbols), Ala in position K8 (open squares) and Glu in position L9 (closed squares). Reactions were performed in 200 mM phosphate buffer at pH 7.0 with 100 μM peptide concentrations of both electrophilic and nucleophilic fragments. Tris(2-carboxyethyl)phosphine hydrochloride was added to a final concentration of 250 μM to avoid dimerization of the nucleophilic fragment and the reaction products through disulfide bond formation. Reactions were started by addition of the electrophilic fragment. At definite times aliquots were taken.*

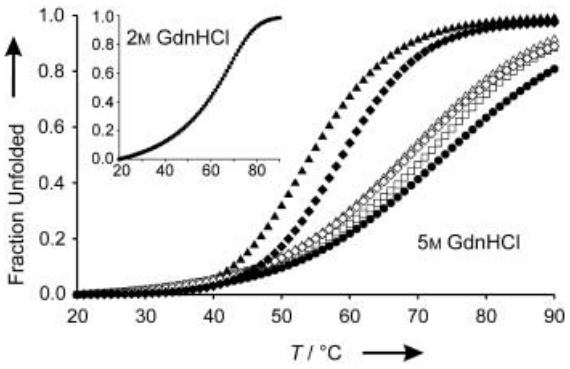


Figure 3. *Thermal unfolding profiles of: control sequence (circles), DfeGly (triangles), and TfeGly (diamonds) in positions K8 (open symbols) and L9 (closed symbols), Ala in position K8 (open squares) and Glu in position L9 (closed squares). Melting curves were recorded by observing the CD signal at 222 nm with peptide concentrations of 20 μM at pH 7.4 in 5 M guanidinium chloride in a 0.1 cm cuvette with a slope of 3 °C min^{-1}. The peptide with Glu in position 9 (hydrophobic core) was already fully unfolded at 20 °C in 5 M GdnHCl (data not shown), but a temperature-dependent denaturation of this peptide could be recorded in 2 M GdnHCl (inset diagram). The thermal melting profiles were fitted by using sigmoidal 5 parameter curve fit.*

Clearly, the Glu variant is an extreme example of the influence of polarity within the hydrophobic core on coiled-coil stability. Incorporation of either fluorinated amino acid into the hydrophobic surface causes a strong retardation of the replication rate relative to the control sequence, with the trifluoroethyl substitution showing the stronger influence. In contrast, the melting temperature of the DfeGly variant is more suppressed. The electron-withdrawing effect of the two fluorine substituents of the Dfe group causes a high acidity of the proton attached.[9] Thus, the acidic proton seems to disturb the interactions within the hydrophobic core and can be detected.

Substitution of Lys in position 8 within the charged surface by fluorinated amino acids or Ala, leads to subtle effects. Ala substitution, as a reference for a small hydrophobic substitution is relatively unperturbing. DfeGly substitution increases the rate slightly, while TfeGly substitution decreases it. Dimer stabilities, expressed by melting points, of all three K8 variants are decreased relative to the control peptide; this is most likely due to the lack of the positively charged Lys ε-amino group. This effect is slightly stronger for both of the fluoroethyl glycines than for K8A.

The thermal unfolding experiments allow straight conclusions about the differences in secondary structure stability between the variants and thus give direct information about the interactions of the fluorinated amino acids with the proteinogenic ones. In contrast, a higher turnover rate in the replicase screen indicates a variant to be a better catalyst in this system. Catalytic activity strongly depends on accessibility of the catalytically active template and, thus, on the dissociation rate of the dimeric coiled-coil product. The DfeGly shows weaker hydrophobic interactions in position 9 of the coiled coil than TfeGly, characterized by a lower melting temperature. This may result in the higher turnover rate of the L9DfeGly compared with the L9TfeGly variant due to a faster dissociation of the dimer. The reaction rate of the background reaction, which is the template-independent chemical ligation, was shown to be uninfluenced by the type of substitution in position 9 of the coiled-coil peptide (Figure 4). However, both fluorinated ethyl

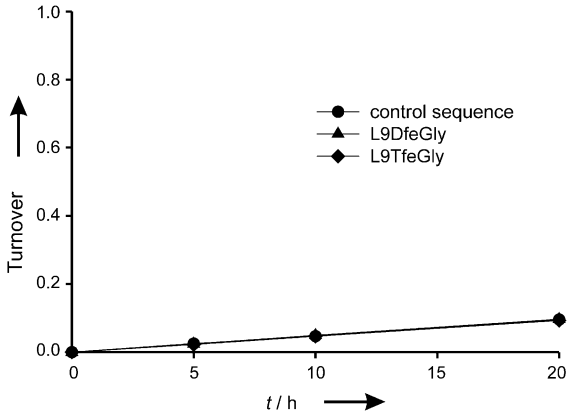


Figure 4. *Replicase turnover rates of the control sequence and two selected variants in the absence of autocatalysis. Reactions were performed in 6 M guanidinium hydrochloride. All remaining reaction conditions conform to the protocol described in Figure 2.*

glycine derivatives show weaker interactions in the hydrophobic core of the coiled-coil peptide than the native leucine peptide. Considering the hypothesis that the steric bulk of trifluoroethyl glycine is similar to leucine and the postulated high lipophilicity of fluoroalkyl groups,[14] the opposite result should have been expected. However, our results conform with studies on self-sorting parallel coiled-coil peptides with hydrophobic cores composed entirely of leucine or hexafluoroleucine.[7] Disulfide-exchange assays resulted in less than 2% heterodimeric assemblies containing interactions of native and fluorosubstituted amino acids; this indicates that fluoroalkyl–fluoroalkyl interactions are much stronger than mixed hydrocarbon–fluorocarbon cores. We conclude that either the space filling of fluoroalkyl groups is much overestimated and/or the high electronegativity of fluorine disturbs a throughout formation of a hydrophobic core. Both facts would prevent the formation of an intact helical coiled coil. In order to clarify these issues, the screening system introduced here will be used for the investigation of fluoroalkyl-substituted amino acids by systematically varying side chain size and fluorine content.

Fluorinated amino acids have been incorporated into a heptad repeat motif at different positions, and their influence on coiled-coil peptide formation as been studied. The screens introduced here are sufficiently sensitive to detect the difference of even one single fluorine atom in the side chain of an amino acid. On this basis, a general protocol is being developed to systematically study the steric, electronic, and hydrophobic effects of a variety of fluorinated amino acids that differ in the content of fluorine as well as the position of the fluorination on peptide modification.

Acknowledgements

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Keywords: amino acids · coiled-coil peptides · fluorine · helical structures · protein modifications · self replication

[1] a) J. C. M. van Hest, D. A. Tirrell, *Chem. Commun.* **2001**, 1897 – 1904; b) T. W. Muir, B. H. Kent, *Curr. Opin. Biotechnol.* **1997**, *4*, 420 – 427.
[2] There is one exception: 4-fluorothreonine as secondary metabolite of the bacterium *Streptomyces cattleya*.
[3] a) Y. Tang, G. Ghirlanda, N. Vaidehi, J. Kua, D. T. Mainz, W. A. Goddard III, W. F. DeGrado, D. A. Tirrell, *Biochemistry* **2001**, *40*, 2790 – 2796; b) B. Bilgicer, A. Fichera, K. Kumar, *J. Am. Chem. Soc.* **2001**, *123*, 4393 – 4399; c) "Biomedical Frontiers of Fluorine Chemistry", B. Koksch, N. Sewald, H.-D. Jakubke, K. Burger, *ACS Symposium Series* **1996**, *639*, 42 – 58; f) B. Koksch, N. Sewald, H.-J. Hofmann, K. Burger, H.-D. Jakubke, *J. Peptide Sci.* **1997**, *3*, 157 – 167.
[4] A. S. Ulrich in *Encyclopedia of Spectroscopy and Spectrometry*, (Eds.: J. Lindon, G. Tranter, J. Holmes), Academic Press, **2000**, pp. 813 – 825.
[5] Y. Tang, G. Ghirlanda, W. A. Petka, T. Nakajima, W. F. DeGrado, D. A. Tirrell, *Angew. Chem.* **2001**, *113*, 1542 – 1544; *Angew. Chem. Int. Ed.* **2001**, *40*, 1494 – 1496.
[6] Y. Tang, D. A. Tirrell, *J. Am. Chem. Soc.* **2001**, *123*, 11 089 – 11 090.
[7] B. Bilgicer, K. Kumar, *Tetrahedron* **2002**, *58*, 4105 – 4112.
[8] T. Nagai, G. Nishioka, M. Koyama, A. Ando, T. Miki, I. Kumadaki, *J. Fluorine Chem.* **1992**, *57*, 229 – 237.
[9] a) J. A. Erickson, J. I. McLoughlin, *J. Org. Chem.* **1995**, *60*, 1626 – 1631; b) W. Caminati, S. Melandri, P. Moreschini, P. G. Favero, *Angew. Chem.* **1999**, *111*, 3105 – 3107; *Angew. Chem. Int. Ed.* **1999**, *38*, 2924 – 2925; c) S. C. F. Kui, N. Zhu, M. C. W. Chan, *Angew. Chem.* **2003**, *115*, 1666 – 1670; *Angew. Chem. Int. Ed.* **2003**, *14*, 1628 – 1632.
[10] A. Saghatelian, Y. Yokobayashi, K. Soltani, M. K. Ghadiri, *Nature* **2001**, *409*, 797 – 801.
[11] K. Severin, D. H. Lee, A. J. Kennan, M. R. Ghadiri, *Nature* **1997**, *389*, 706 – 709.
[12] DfeGly was synthesized according to: D. Winkler, K. Burger, *Synthesis* **1999**, *12*, 1419 – 1422. TfeGly was synthesized according to T. Tsushima, K. Kawada, S. Ishihara, N. Uchida, O. Shiratori, J. Higaki, M. Hirata, *Tetrahedron* **1988**, *17*, 5375 – 5387.
[13] G. von Kiedrowski, *Bioorg. Chem. Front.* **1993**, *3*, 113 – 146.
[14] T. Fujita. *Prog. Phys. Org. Chem.* **1983**, *14*, 75 – 113.
[15] Peptides were synthesized on an ACT90 synthesizer by using standard Fmoc protocol. The nucleophilic peptide was synthesized on a Wang resin support, then cleaved and deprotected with trifluoroacetic acid in the presence of triisopropylsilane/water (85:10:5, *v/v/v*). The electrophilic peptides were synthesized on 4-sulfamylbutyryl resin and cleaved according to: R. Ingenito, E. Bianchi, D. Fattori, A. Pessi, *J. Am. Chem. Soc.* **1999**, *121*, 11 369 – 11 374 in order to obtain the C-terminal benzylthioesters. Deprotection was performed with trifluoroacetic acid/triisopropylsilane/water (85:10:5, *v/v/v*). All peptides were purified by using C4 reversed-phase HPLC (column: Vydac; 22 × 250 mm × 10 μm). Analytical C_4 reversed-phase HPLC (column: Vydac; 4 × 250 mm × 10 μm) and MALDI-TOF mass spectrometry were used to prove the purity and identity of each peptide product.

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