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Stabilization of β-Peptide Helices by Direct Attachment of Trifluoromethyl Groups to Peptide Backbones

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Received 00th January 2012, Accepted 00th January 2012
DOI: 10.1039/x0xx00000x

The 14-helix structure of oligo-β-peptides was significantly stabilized by direct attachment of CF₃ groups to their backbones. Our studies indicate this stabilization originates from the CF₃-promoted increase in the intramolecular hydrogen-bonding ability of their backbone amides, leading to a novel strategy to stabilize peptide folding.

Tailor-made amino acids with an appropriately designed side chain provide powerful tools for tuning structure and properties of peptides. Among them, amino acids bearing a perfluoroalkyl (Rf) group have attracted particular attention recently, because they offer a reliable method to control the tertiary and quaternary structure of peptides. When such amino acids are appropriately incorporated into a peptide so that they get crowded to form a fluorocarbon-rich domain, the peptide tends to self-aggregate due to the omniphibicity of fluorinated domains, allowing for the formation of higher order structure, such as the bundle of helices. In this design strategy, the Rf groups are usually located at ‘remote’ positions from peptide backbones (Fig. 1a, type vi), connected typically via a one or two carbon spacer, so that the Rf groups occupy the surface region of peptides. Such ‘remote’ fluorination is suitable to tune the interpeptide interactions along with retaining the original conformation of each peptide.

Meanwhile, the incorporation of fluorne atoms (Fig. 1a, types i and iv) or Rf groups (types ii, iii, and v) in ‘direct proximity’ to the peptide backbones is expected to expand the scope and utility of fluorination in peptide engineering. Owing to their strong electron-withdrawing nature, Rf groups generally increase the acidity and hydrogen-bond donating ability of adjacent protic functionalities, including amide NHs (Fig. 1b). Therefore, such modification can bring a significant influence on the stability of intramolecular hydrogen bonds between amide NHs and carbonyls in a peptide backbone, which would lead to a novel methodology to control the secondary structure of peptides.

Despite the simplicity and promise, the aforementioned strategy has not been well examined so far, most likely due to the difficulty of designing suitable amino acid components. In order to maximize the inductive effect of fluorne atoms, they should be introduced at positions near to the amino group, such as α- and β-carbons. However, as far as using a α-amino acid skeleton, such fluorination can not ensure the chemical/stereochemical stability of the amino acids or has difficulty in peptide elongation (Fig. 1a, types i–iii). To date, only a few types of peptides and peptidomimetics bearing fluorne atoms or Rf groups in ‘direct proximity’ to their backbones have been reported, and the effect of Rf-group incorporation on the hydrogen-bonding properties of adjacent amide NHs has not been studied. As an alternative class of amino acid components, we focused on fluorinated β-amino acids. Without

Fig. 1  (a) Types of fluorinated α- and β-amino acids. In parentheses: limitations as building blocks of peptides (types i–iii) and expected positions of fluorinated units in peptides (types iv–vi). (b) Effect of a perfluoroalkyl (Rf) group directly attached to a peptide backbone on the acidity of the adjacent amide NH.
ambiguity, β-peptides are one of the most important mimics of natural peptides, as demonstrated by Seebach et al. and Gellman et al. In terms of chemical/stereochemical stability and peptide elongation capability, the fluorination of α-amino acids is more feasible than that of ω-amino acids. Indeed, Soloshonok et al. predicted that fluorination/perfluoroalkylation of β-peptide backbones can modulate the structure and properties of the resultant peptides. Here we report that trifluoromethyl (CF₃) groups, directly attached to β-peptide backbones, can significantly enhance the stability of the folded conformation of peptides (Fig. 1a, type v).

Because of this aim of this work is to fairly evaluate the effects of the CF₃-activated intramolecular hydrogen bonds on the conformational stability of individual peptides, the formation of higher order structure, potentially induced by the omniphic properties of CF₃ groups particularly in aqueous media, should be excluded. Thus, the present β-peptides were designed suitable for dissolution in nonaqueous media. As a fluorinated β-amino acid component, we employed a fluorinated homoalanine (Fig. 2a, F), of which stereocontrolled synthesis and peptide elongation have been well established. For comparison, the homologated 1-alanine (A) was used as a non-fluorinated analogue of F. For accurate evaluation of the conformational stability of peptides, a simple and well-explored structural motif is favorable. Therefore, we selected the 14-helix structure formed by β-peptides mainly composed of a cyclohexane-based α-amino acid (C). Owing to the conformationally restricted structure, C is one of the most promising β-amino acids to construct β-peptides that adopt the 14-helix structure.

In the present work, three kinds of new β-peptides were designed and synthesized based on the hexameric C sequence (Boc-C-C-C-C-C-OBn) by replacing two residues with either of F or A (Fig. 2b). As the modification sites, the 2nd and 5th residues from the N-terminus were chosen because (1) for simplicity and generality, modification at special sites such as the peptide termini (the 1st and 6th residues) is not suitable, and (2) to ensure the 14-helix formation, the center of the peptides (the 3rd and 4th residues) may well be composed of C rather than conformationally more flexible F or A. Also regarding the hydrogen-bond donating ability of amide NHs, the 2nd and 5th residues seem suitable as the modification sites. The 2nd amide NH is known to participate in the intramolecular hydrogen-bond network of 14-helix. Meanwhile, the 5th amide NH is also expected to stabilize the 14-helix structure via the intramolecular hydrogen bond with the carbonyl at C-terminus, considering the recent reports on oligo-β-peptides and our own ¹H NMR studies (Fig. S10–S17, ESI†). Hereafter, the hexa-β-peptides Boc-C-C-C-C-C-OBn, Boc-C-F-C-C-C-C-OBn, Boc-C-F-C-C-C-C-OBn, and Boc-C-A-C-C-C-C-OBn will be denoted as pep-CC, pep-FF, pep-FA, and pep-AA, respectively (Fig. 2b).

Among these hexa-β-peptides, pep-FF furnished single crystals suitable for X-ray crystallography upon slow evaporation of its solution in CH₃OH/CHCl₃ (1:1, v/v). The X-ray structure showed that the conformation of pep-FF in the solid state is quite similar to that of pep-CC (Fig. 3 and Fig. S1, ESI†). pep-FF adopts the left-handed 14-helix structure by forming all of the four possible 14-membered ring hydrogen bonds between the backbone amides (NH···N=C=O, i = 1–4), generating two helical turns (Fig. 3a). The two CF₃ groups incorporated in the single peptide align along the same face of the helix and are not in contact with each other (Fig. 3b). The similarity between the crystal structure of pep-FF and that of pep-CC also exists in terms of the packing of the molecules. In both cases, each peptide associates with the neighboring peptides at its N- and C-termini via one or two intramolecular hydrogen bonds to form a one-dimensional columnar array (Fig. S1, ESI†).

In solution, the 14-helix structure of the new β-peptides was unambiguously confirmed by 2D NMR spectroscopy. On the NOESY spectra measured at 25 °C in CD₃OH/CDCl₃ (2:1, v/v), all of the β-peptides showed three long-range NOEs C.H ↔ C.H (i = 1–3) characteristic of the 14-helix (Tables S1–S3 and Fig. S2–S4, ESI†). For all of the β-peptides, population of the 14-helical conformation is considered to be high at the present conditions, because NOEs for both termini (i = 1 and 3) were clearly observed. As we envisioned, these β-peptides are not likely to form higher order structure in organic media that were used in the measurements of the present work (methanol, butanol, chloroform, etc.). Indeed, their circular dichroism (CD) and ¹H NMR profiles showed essentially no concentration dependency (Fig. S5 and S6, ESI†). Their apparent molecular masses, determined by sedimentation equilibrium in an analytical ultracentrifuge, also proved that they exist as monomeric peptides in solution (Fig. S7, ESI†).

CD spectroscopy allowed for the qualitative and quantitative comparison of the secondary structure of the β-peptides in solution. In butanol, all of them showed a broad negative peak maximum around 216 nm, which is characteristic of the left-handed 14-helix (Fig. 4). Quite importantly, variable-temperature CD spectroscopy revealed that the thermal stability of the 14-helix notably enhances as the number of fluorinated residue increases. Upon heating from –10 to 100 °C, the CD intensity at 216 nm of pep-AA decreased by 60%, while that of pep-FF and pep-FA decreased only by 22 and 33%, respectively (Fig. S8, ESI†). These results clearly indicate that the thermal stability of the 14-helix can be significantly improved by introducing CF₃ side chains. Worth to note is that the 14-helix of pep-FF is more heat tolerant than that of pep-CC, even though pep-CC is generally recognized as an ideal hexa-β-peptide for the formation of stable 14-helix (Fig. S8, ESI†).
To clarify the effects of the CF<sub>3</sub> side chains on the kinetic events of β-peptides, H/D exchange of the amide NHs in these peptides (NH<sub>i</sub>, i = 1–6; Fig. 2b) was examined by using <sup>1</sup>H NMR spectroscopy (Fig. 5 and Fig. S10–S13, ESI†). To fairly compare the H/D exchange kinetics between the peptides, we focused on NH<sub>1</sub> and NH<sub>5</sub>, because (1) the amide NHs at the termini (NH<sub>1</sub> and NH<sub>5</sub>) are anticipated to suffer terminal effects and (2) the amide NHs in the modification sites (NH<sub>2</sub> and NH<sub>3</sub>) vary their acidity depending on the neighboring side-chain groups (CH<sub>3</sub> or CF<sub>3</sub>), so that their H/D exchange kinetics does not simply reflect the conformational stability of peptides (Fig. S14–S16, ESI†). For example, in CD<sub>3</sub>OD/CDCl<sub>3</sub> (2:1, v/v) at 25 °C, the H/D exchange of NH<sub>2</sub> and NH<sub>3</sub> were suppressed more as the number of fluorinated residue increased (pep-FF < pep-FA < pep-AA). In the case of pep-AA, the H/D exchange of NH<sub>1</sub> and NH<sub>5</sub> completed within 4 h (Fig. 5a, right). Contrary to this, changes in the corresponding <sup>1</sup>H NMR signals of pep-FA were more sluggish, where residual signals (8 and 25% for NH<sub>1</sub> and NH<sub>5</sub>, respectively, in integration with respect to the initial state) were observed even after 16 h (Fig. 5a, center). Furthermore, these resonances of pep-FF remained almost unchanged within this time scale (Fig. 5a, left). As depicted in Fig. 5b, the rate constants of the H/D exchange (NH<sub>1</sub> and NH<sub>5</sub>) for pep-FF (0.0064 and 0.0068 h<sup>−1</sup>) and pep-FA (0.13 and 0.077 h<sup>−1</sup>) were 85–90 and 4–8 times smaller than those of pep-AA (0.55 and 0.61 h<sup>−1</sup>). The H/D exchange rates were hardly changed when they were measured in the coexistence of these β-peptides (Fig. S18, ESI†), which excludes the possibility that the observed difference in H/D exchange rates is due to some impurities that catalyze H/D exchange.

From the results of the variable-temperature CD spectroscopy and the H/D exchange kinetics, we can clearly conclude that the introduction of CF<sub>3</sub> side chains significantly enhances the stability of 14-helix structure of the β-peptides. Taking account of the fact that the β-peptides can hardly aggregate to form higher order structure under the above experimental conditions (Fig. S5–S7, ESI†), one of the most plausible reasons for the increased conformational stability is that the CF<sub>3</sub> groups, directly attached to the peptide backbones, enhance the hydrogen-bond donating ability of the neighboring amide NHs, which strengthens the intramolecular hydrogen bond(s) between the peptide backbone amides. Considering that there are non-negligible differences in the stabilities between pep-FF and pep-FA, the two activated amide NHs in pep-FF (NH<sub>2</sub> and NH<sub>3</sub>) seem to cooperatively retard the peptide unfolding that is mainly initiated by the fraying at the N- and C-termini. Although the electron-withdrawing nature of CF<sub>3</sub> groups is anticipated to reduce the hydrogen-bond accepting ability of the neighboring carbonyls (CF<sub>3</sub>–C=O and CF<sub>3</sub>–C=NH), this effect seems to be negligible, judging from relatively small changes in <sup>13</sup>C NMR chemical shifts of such carbonyls caused by the incorporation of the CF<sub>3</sub> group (+0.7 and −2.2 ppm, respectively; Fig. S19, ESI†). Most likely, the effect of the CF<sub>3</sub> group is attenuated by the three-bond spacers that connect the CF<sub>3</sub> group and the carbonyls.

In summary, we found that the 14-helix structure of hexa-β-peptides can be significantly stabilized by incorporating a fluorinated β-amino acid (F) into their peptide sequences. Systematic studies suggest that the explicit stabilization of the 14-helix, observed in both thermodynamic and kinetic aspects, was not induced by the self-aggregation of the peptides due to the omniphobicity of their fluorinated domains, but by the CF<sub>3</sub>-promoted increase in the hydrogen-bond donating ability of the amide NHs in their backbones. Since the intramolecular hydrogen-bonding interaction is one of the most crucial factors of peptide and protein folding, the direct attachment of fluoralkyl groups to peptide backbones would offer a general strategy to control the secondary structure of various types of peptides and proteins. Considering the recent remarkable progress on the study of αβγ-hybrid peptides, the hybridization of the fluorinated β-amino acid F and its derivatives with α- and γ-amino acid might further expand the utility of this methodology.

A part of this research was financially supported by the Grant-in-Aid for Young Scientists (B) 21750137 (MEXT, Japan).

Notes and references

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Fig. 4 Circular dichroism (CD) spectra (butanol, [peptide] = 20 μM) of pep-FF (left), pep-FA (center), and pep-AA (right) with changing temperature from −10 to 100 °C with a step of 10 °C.

Fig. 5 H/D exchange (CD<sub>3</sub>OD/CDCl<sub>3</sub>, 2:1, v/v; 25 °C; [peptide] = 3.0 mM) of the amide NHs of pep-FF, pep-FA, and pep-AA. (a) Changes in <sup>1</sup>H NMR spectra. The authentic spectra before H/D exchange ('Original') were obtained in CD<sub>3</sub>OH/CDCl<sub>3</sub> (2:1, v/v). (b) Plots of ln(|ω(t)|) versus time for NH<sub>1</sub> (circles) and NH<sub>2</sub> (squares). ω(t) denotes the integral of an amide NH resonance at time t.


24. For the choice of solvents in NMR and CD measurements, see Section 1 (page S2) of ESI.


† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c000000x/
Direct attachment of trifluoromethyl groups to peptide backbones was found to be a novel strategy for peptide folding stabilization, where the intramolecular hydrogen-bonding ability of backbone amides is enhanced.