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SHORT COMMUNICATION

Compatibility of the conformationally rigid CF_3-Bpg side chain with the hydrophobic coiled-coil interface

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Abstract 3-(Trifluoromethyl)bicyclopent-[1.1.1]-1-yl glycine (CF_3-Bpg) has previously been established as a useful ^{19}F NMR label to analyse the structures of oligomeric membrane-active peptides or transmembrane segments. To systematically examine the effect of side chain volume, conformational rigidity, and hydrophobicity of CF_3-Bpg in polypeptide environments the amino acid was incorporated into an established coiled-coil based screening system. A single substitution of either valine (position a16) or leucine (position d19) within the hydrophobic core of the heteromeric coiled coil has practically no effect on its structure. Despite its comparatively high hydrophobicity, however, the stiff and bulky side chain of CF_3-Bpg is not so well accommodated by the hydrophobic core as it leads to a more pronounced destabilization than observed for other, more polar fluorinated amino acids which carry more flexible side chains. CF_3-Bpg is therefore a useful ^{19}F NMR label, though not for monitoring the stability of such helix–helix interactions.

Keywords Fluorinated amino acids · CF_3-Bicyclopentyl glycine · α-Helical coiled coil · Solid state ^{19}F NMR · ^{19}F labelling

Introduction

Fluorinated amino acids are valuable building blocks for the de novo design of peptides and proteins with advantageous properties such as increased metabolic and thermodynamic stability (Yoder and Kumar 2002; Jäckel and Koksch 2005; Marsh et al. 2009). Furthermore, the ^{19}F isotope represents a powerful NMR label for biomolecular structure analysis, because the ^{19}F nucleus is highly sensitive, natural background signals are absent, and it has a broad chemical shift range (Cobb and Murphy 2009; Sternberg et al. 2009). ^{19}F labelled amino acids are particularly valuable for studying the structures and dynamic interactions of membrane-active peptides in the natural environment of a lipid bilayer using solid state ^{19}F NMR (Ulrich 2005; Afonin et al. 2008; Wadhwani et al. 2008; Grage et al. 2010; Kubyshkin et al. 2010). The new amino acid 3-(trifluoromethyl)bicyclopent-[1.1.1]-1-yl glycine (CF_3-Bpg; Fig. 1a) was recently designed as an ideal label for this purpose (Mikhailiuk et al. 2006, 2010). By attaching the CF_3-reporter group to the α-carbon via a rigid bicyclopentane moiety, this stiff connection ensures that the anisotropic ^{19}F NMR parameters of the CF_3-group reveal directly the orientation of the corresponding peptide backbone segment. CF_3-Bpg was incorporated into the antimicrobial peptide PGLa as a substitute for isoleucine or alanine without perturbing its α-helical structure

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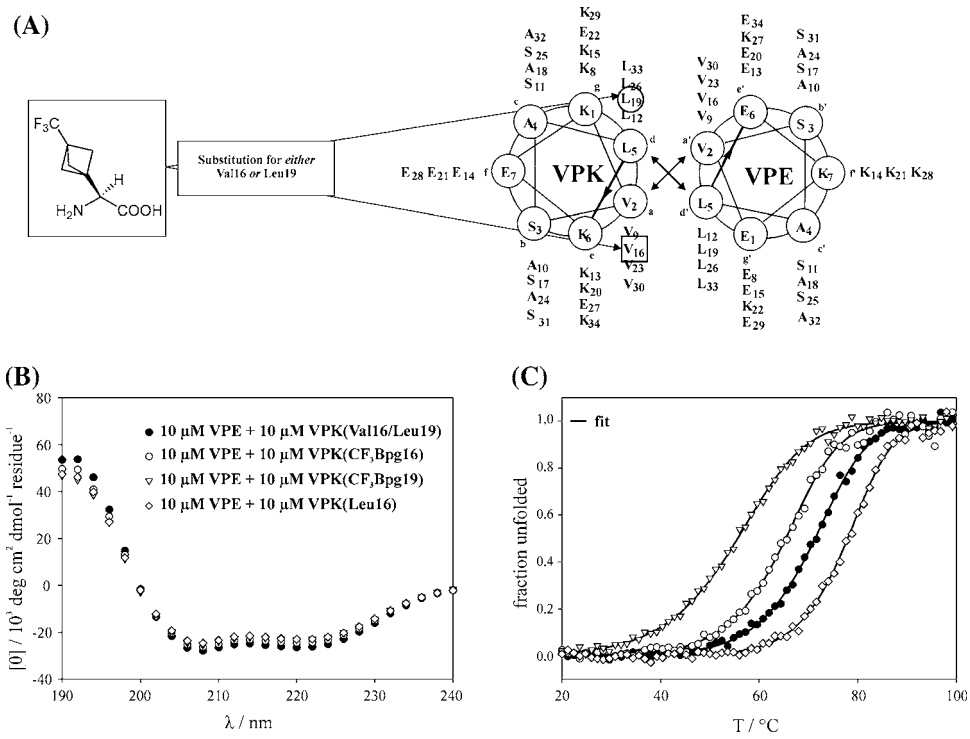

Fig. 1 Helical wheel representation of the coiled-coil based model system and structure of CF_3-Bpg (**a**), CD spectra of unmodified VPK, the CF_3-Bpg substituted variants, and one variant carrying Leu at a16 mixed with equimolar amounts of VPE at an overall peptide concentration of 20 µM at 20°C (**b**), and thermal unfolding profiles of all the variants measured using the CD signal at 222 nm (**c**). All measurements were carried out at at pH 7.4 (100 mM phosphate buffer)

(Mikhailiuk et al. 2006; Afonin et al. 2007; Strandberg et al. 2009). Furthermore, it was used as a substitute for leucine in SAP, a synthetic cell penetrating peptide, with no alteration of its polyproline II conformation (Mikhailiuk et al. 2008). We were now interested in examining the effect of this new building block on helix–helix interactions, as to whether it might affect oligomerization of membrane-active peptides or of transmembrane segments. Provided that the helix–helix interface does not get significantly disturbed by the stiff CF_3-Bpg label, this new amino acid will open up unique possibilities for highly sensitive NMR distance measurements over long-range (>10 Å). Given that coiled coils represent the most intimate helix–helix assemblies to be critically tested, we chose a model system (Fig. 1a) that has already been used to study the interactions of various other fluorinated amino acids with native residues (Jäckel et al. 2004, 2006). We have shown that within this parallel dimeric coiled coil the effects of fluorination are unequal at the hydrophobic positions a and d (Salwiczek et al. 2009). While the stability of the fluorinated variants substituted at the a-position correlates with steric size of the corresponding fluorinated amino acid, no such trend was observed for position d. In general, however, coiled-coil stability varies directly with hydrophobicity and steric size of the residues in these central hydrophobic core positions (Wagschal et al. 1999; Tripet et al. 2000). In this contribution, we show that CF_3-Bpg does not affect the overall structure of the α-helical coiled coil. However, despite the pronounced hydrophobicity of the new building block, the stability of coiled-coil interactions is reduced, presumably as a result of the stiff and bulky side chain geometry.

Materials and methods

Peptide synthesis and purification

Peptides were synthesized using a SyroXP-I peptide synthesiser (MultiSynTech GmbH, Witten, Germany) on a 0.05 mM scale applying standard Fmoc/tBu chemistry (Fields and Noble 1990) as described recently (Salwiczek et al. 2009). Coupling of CF_3-Bpg was achieved by activation of two equivalents of the Fmoc-protected analogue with DIC/HOAt (two equivalents each, 7 min pre-activation) without the addition of DIEA to prevent racemization (Carpino and El-Faham 1999). These couplings were performed manually until completion, as indicated by a negative Kaiser test (Kaiser et al. 1970). Purification was carried out by RP-HPLC (Phenomenex® Luna C8, 10 µm, 250 × 21.2 mm) and purity was confirmed by analytical HPLC (Phenomenex® Luna C8, 5 µm, 250 × 4.6 mm). Deionized water (MilliQ®-AdvantageA10®, Millipore) and acetonitrile (99.9%, HPLC gradient grade, Acros Organics) containing 0.1% TFA (Uvasol®, Merck) were used as eluents with a gradient from 5 to 70% acetonitrile over 30 min. All products were identified by high-resolution ESI-MS (Agilent 6210 ESI-TOF mass spectrometer; Agilent Technologies, Santa Clara, CA, USA): VPK(CF_3Bpg16), 24 mg, calcd *m/z* for $[M+4H]^{4+}$was 970.7974, found: 970.7950;

VPK(CF_3Bpg19), 21 mg, calcd *m/z* for $[M+4H]^{4+}$ was 967.2934, found: 967.2940. VPE and VPK(Leu16) were taken from our in-house library (Salwiczek et al. 2009).

Circular dichroism (CD) spectroscopy

CD spectra were recorded on a Jasco J-715 spectropolarimeter at 20°C (Jasco PTC-348WI peltier thermostat). Overall peptide concentrations were 20 μM (10 μM VPE and 10 μM VPK) at pH 7.4 (100 mM phosphate buffer). CD spectra were obtained in the far-UV range (190–240 nm) using 0.1 cm Quartz Suprasil® cuvettes (Hellma) equipped with a stopper. The nitrogen flow rate was set to 3 L/min. Ellipticity was normalized to concentration (c/mol L^{-1}), number of residues ($n = 35$, including the N-terminal label), and path length ($l = 1$ cm) using Eq. 1:

$$[\theta] = \theta_{obs}/(10000lcn) \quad (1)$$

where θ_{obs} is the measured ellipticity in millidegrees and $[\theta]$ the mean residual ellipticity in 10^3 deg cm^2 $dmol^{-1}$ $residue^{-1}$. Melting curves were recorded using the signal at 222 nm applying a heating rate of 3 K/min from 20 to 100°C. Each experiment was carried out in triplicate, and both the baseline corrected spectra and the melting curves were averaged. The melting curves were fitted assuming two-state unfolding, and the thermodynamic parameters of folding were extracted applying our recently described protocol (Salwiczek et al. 2009).

Fluorescence spectroscopy (FRET)

The FRET assay to probe the orientation of the coiled-coil variants was performed according to previously published procedures (Pagel et al. 2005; Salwiczek et al. 2009). 2-Aminobenzoic acid (Abz, Bachem, $\lambda_{ex} = 320$ nm, $\lambda_{em} = 420$ nm) served as the fluorescence donor attached to the N-terminus of the VPK-variants while 3-nitrotyrosine (Bachem, $\lambda_{abs} = 420$ nm) served as the acceptor attached to the N-terminus of VPE (VPE-NYNO$_2$). Fluorescence spectra were recorded on a luminescence spectrometer LS 50B (Perkin Elmer) at room temperature using a Quartz Suprasil® cuvette (Hellma) with a path length of 1 cm. The concentration of the VPK-variants was kept constant at 20 μM and the concentration of VPE-NYNO$_2$ varied from 0 to 40 μM. Three scans from 350 to 550 nm were performed and the averaged spectra were normalized to maximum fluorescence (see Salwiczek et al. 2009 for further details).

Estimation of side chain volume and hydrophobicity

Side chain volumes were calculated starting from the β-carbon using atomic increments (Zhao et al. 2003). The hydrophobicity of CF_3-Bpg was determined according to a recently published procedure using the Fmoc-protected analogue (Samsonov et al. 2009). In short, approximately 10 μmol of the Fmoc-derivative were dissolved in 5 mL of a mixture of 40% acetonitrile (99.9%, HPLC gradient grade, Acros Organics) and 60% deionized water (MilliQ®-AdvantageA10®, Millipore) containing 0.1% TFA (Uvasol®, Merck) and filtered over 0.2 μm (13 mm PTFE syringe filters, VWR). The retention times on a C18 column (Capcell PAK, 5 μm, Shiseido) were determined in triplicate at room temperature.

Results and discussion

Compared to valine and leucine, the side chain of CF_3-Bpg is significantly larger and more hydrophobic (Table 1). Its steric geometry is not comparable to the conformationally flexible isopropyl and isobutyl side chains of the valine and leucine residues, which are typically found in the a- and d-positions of the heptad repeat within the hydrophobic core of coiled coils, where they engage in optimal packing interactions (Mason and Arndt 2004).

CD spectroscopy (Fig. 1b; Supplementary Material) and FRET (Fig. 2; Salwiczek et al. 2009) show that the substitution of CF_3-Bpg for Val16 or Leu19 has no apparent effect on the overall structural features of the coiled coil

Table 1 Side chain volume and retention time of Fmoc-CF_3-Bpg, as well as free energies of unfolding of the peptides modified either at position a16 or d19 in comparison to the parental sequence carrying Val and Leu at these positions, respectively

Amino acid	vdW Volume[a] (Å^3)	rt[b] (min)	$\Delta G^{\ominus}_{a16}$ (kcal/mol)	$\Delta G^{\ominus}_{d19}$ (kcal/mol)
Val	59.1	13.2 ± 0.1[c]	11.7 ± 0.2[d]	n.d.
Leu	76.4	15.6 ± 0.1[c]	13.8 ± 0.2[d]	11.7 ± 0.1[d]
CF_3-Bpg	101.9	18.7 ± 0.1	11.2 ± 0.2	9.2 ± 0.1

[a] Corresponding to the side chain starting at Cβ

[b] Retention time of the Fmoc-derivative

[c] See Samsonov et al. (2009)

[d] See Salwiczek et al. (2009)

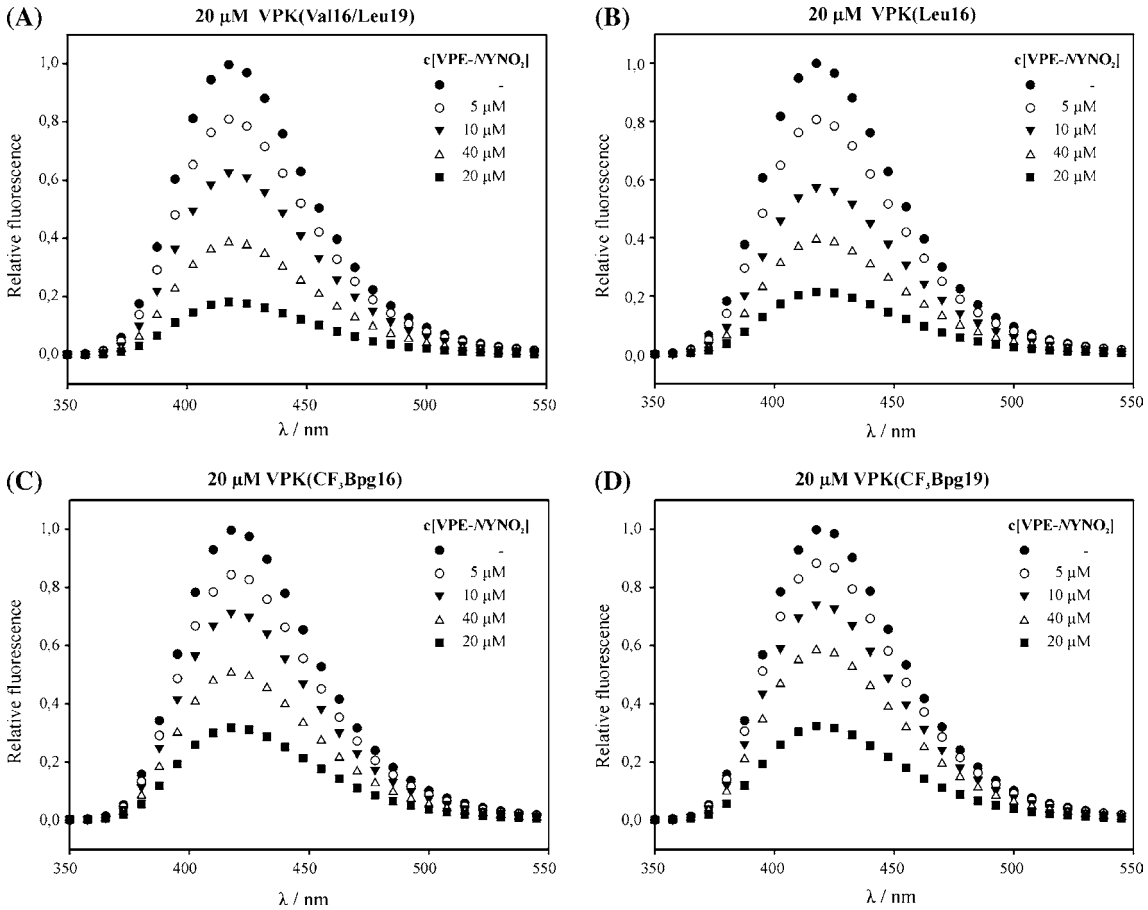

Fig. 2 Fluorescence spectra of VPK (parental sequence containing Val at position a16 and Leu at position d19) (**a**), its analogue containing Leu at position a16 (**b**), and the variants with CF3-Bpg at position a16 (**c**) or d19 (**d**) at increasing concentrations of the quencher VPE-*N*YNO$_2$ at pH 7.4 (100 mM phosphate buffer)

when compared to the VPE-VPK parent dimer. The CD spectra of equimolar mixtures of VPE with the VPK-variants display the typical minima at 208 and 222 nm with equal intensities. Also, the CF_3-Bpg16 analogue is structurally equivalent to a variant that carries Leu at position a16 of VPK. These results are in good agreement with earlier findings for α-helical peptides (Mikhailiuk et al. 2006; Afonin et al. 2007) or polyproline II helices (Mikhailiuk et al. 2008). The FRET assay that measures the fluorescence of N-terminally Abz labelled VPK-variants in the presence of increasing concentrations of VPE labelled with the fluorescence acceptor YNO_2 indicates that all VPK-variants associate with VPE in a parallel manner. This is shown by the progressive decrease in Abz fluorescence intensity as the concentration of the quencher (VPE-*N*YNO$_2$) increases (Fig. 2). Thus, CF_3-Bpg does also not affect the relative orientation of the helices.

The thermodynamic analysis of temperature-induced unfolding monitored by CD spectroscopy (Fig. 1c), however, reveals that the interactions of CF_3-Bpg within the hydrophobic core reduce the stability of the assembly. Despite its increased hydrophobicity compared to valine, its substitution at position a16 does not, as one may expect, stabilize the interaction of VPE and VPK. Instead, the effect, albeit marginal, is opposite ($\Delta\Delta G^{\ominus} = -0.5$ kcal/mol). The effect is even stronger when CF_3-Bpg is compared to leucine at position d19. Although the CF_3-Bpg side chain is closer to leucine in terms of size and hydrophobicity than it is to valine (Table 1), the replacement of Leu19 results in 2.5 kcal/mol destabilization. The same is true when comparing the CF_3-Bpg16 variant to a peptide carrying a leucine (instead of valine) at position a16. In this case, the coiled coil loses 2.6 kcal/mol in stability. Apparently, the bicyclic structure that imparts rigidity to the side chain is sterically unfavourable in terms of packing within the hydrophobic core. Thus, although more hydrophobic, CF_3-Bpg does not improve stability at position a16 compared to Val, and the substitution for leucine at either position a or d is thermodynamically less favourable.

In summary, we have shown that CF_3-Bpg as a residue in the environment of a coiled-coil hydrophobic core seems to be a conservative substitution in terms of secondary

structure. However, a notable loss in structural stability has been detected, especially when compared to leucine. Thus, when using this new building block as a ^{19}F label, one may assume that the structure of the peptide considered is preserved and that helix–helix interactions can be safely addressed by anisotropic NMR parameters as well as distance measurements. However, one should be aware of the destabilizing effect on helix–helix interactions; hence association equilibria are likely to be influenced. The effects of CF_3-Bpg on the function of proteins remain to be evaluated.

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