Covalent control of polypeptide folding. Induction of helix-loop-helix motifs by bridging

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A *de novo* designed helix-loop-helix polypeptide motif is induced by introduction of an interhelical carbon bridge by a site selective reaction. The proposed structure of the bridged polypeptide is based on CD spectroscopy and previous structural studies of polypeptides with similar amino acid sequences. The polypeptide used for the bridging experiments is PE42Dcap, a *de novo* designed polypeptide with 42 amino acid residues that has been designed to fold into a helix-loop-helix motif and to dimerise to form four-helix bundles in aqueous solution. PE42Dcap is engineered to perform acyl transfer reactions with bifunctional esters yielding an interhelical, covalent bridge between two lysine residues. The active residues in PE42Dcap are His7, His11 and Lys15 in helix I and His26, His30 and Lys34 in helix II. Reaction of PE42Dcap with disuccinimidyl glutarate at pH 4.1 in sodium acetate buffer and 5 vol% 2,2,2-trifluoroethanol gave the pure bridged polypeptide DSG-PE42Dcap in an isolated yield of 35%.

CD spectroscopy on the precursor polypeptide PE42Dcap gave the absolute mean residue ellipticity −4800 deg cm^2 $dmol^{-1}$ at 222 nm in aqueous solution at pH 5.1. This shows that the helical content in the polypeptide is low. The introduction of the interhelical carbon bridge increased the absolute mean residue ellipticity to −20100 deg cm^2 $dmol^{-1}$, showing that the bridge has dramatically increased the helical content and that DSG-PE42Dcap is mainly present as helix-loop-helix motifs. The mean residue ellipticity of DSG-PE42Dcap shows concentration dependence that indicates that this bridged polypeptide is mainly monomeric at micromolar concentrations and dimeric above 100 μM.

Introduction

Large efforts are being made to create *de novo* designed polypeptides for catalysis, *e.g.* catalytic four-helix bundles have recently been developed.[1–5] In the present work we have made use of a recently discovered site selective acylation reaction to introduce a covalent carbon bridge between the helices in a *de novo* designed helix-loop-helix polypeptide motif. As a result it is found that the bridge promotes helical structures strongly.

The main driving force for peptide folding is hydrophobic interactions. Folded coiled-coils are stabilised by both intra- and interchain interactions, such as van der Waals, hydrophobic, ionic, electrostatic and sometimes covalent interactions. The structure of a designed coiled-coil can be strongly stabilised by optimising the packing of hydrophobic side chains or by introducing metal ions, salt bridges and/or disulfide bridges.[6]

In solution, most four-helix bundles contain coiled-coil structures, in which two helices are wrapped around each other to form a super coil.[7,8] Natural coiled-coils, as well as designed coiled-coils, usually have a repeating heptad of amino acid residues $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, where positions *a* and *d* are occupied by non-polar amino acids.[9–11]

The stability of designed coiled-coils can be increased by optimising the interhelical hydrophobic interactions, *e.g.* by incorporating leucine residues in position *d* and apolar β-branched residues in position *a*. DeGrado and co-workers reported α_2B, a peptide with a repeating heptad of amino acids that dimerises to form four-helix bundles.[12] The hydrophobic core of α_2B was solely made up by leucine residues and the peptide was found to be a typical molten globule in solution. The stability of the designed four-helix bundle was increased by replacing some of the leucine residues by aromatic or β-branched amino acids.[13] Further stability was achieved by introducing a metal binding site to create the polypeptide α_2D.[14,15] This polypeptide was found to assemble into a dimer with native-like structure even in the absence of metal ions.

Regan *et al*. redesigned the four-helix bundle protein ROP by systematically substituting the amino acids in the hydrophobic core and evaluated the changes in protein structure and stability.[16]

The effect of individual amino acid residues in different positions in the repeating heptad on the stability of coiled-coils has been thoroughly examined by Hodges and co-workers.[17]

Dolphin *et al*. have demonstrated how a designed aromatic ensemble between the helices can restrict dynamics in a helix-loop-helix polypeptide motif.[18]

There are a number of reports on the use of electrostatic salt bridges and covalent disulfide bridges to stabilise the three-dimensional structure of coiled-coils.[19–21] However, the use of salt bridges and disulfide bridges has some disadvantages. The stabilising effect of a disulfide bridge is very sensitive to the relative position of the bridge within the coiled-coil structure. A disulfide bridge has to be incorporated into the hydrophobic core without disrupting the packing of the amino acid side chains to be strongly stabilising.[22] Disulfide bridges may also be chemically unstable under desired reaction conditions, *e.g.* inside cells.[23]

Solvent exposed salt bridges have a minor effect on the overall stability of the peptide fold, while salt bridges within the hydrophobic core contribute more strongly to stability. The interaction is sensitive to ionic strength and pH, and salt bridges primarily affect the dimerisation process and not the overall stability of the folded polypeptide motif. Recently, Schneider and co-workers reported a heterodimeric coiled-coil with a designed buried salt bridge that significantly favoured the formation of heterodimers over homodimers.[20]

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Furthermore, salt and disulfide bridges contain only few atoms, and they are therefore short and inflexible. These bridges do not allow the possibility of introducing additional functional groups, that later may be used for catalysis, into the folded polypeptide motif.

Several attempts have also been made to stabilise the structure of coiled-coils by using template-assembled synthetic proteins (TASP),[24–26] lactam bridges[27] or metal binding.[28]

For many designed polypeptides it has proved difficult to achieve a specific three-dimensional fold using only non-covalent interactions, mainly due to the small number of amino acid residues.

In the present work we have investigated the possibility of stabilising the folded structure of a designed helix-loop-helix polypeptide motif by connecting the helices with a covalent carbon bridge.

Results and discussion

Design of the polypeptide for bridging

Recently Baltzer and co-workers reported RA42, a *de novo* designed polypeptide with 42 amino acid residues that folds into a helix-loop-helix motif and dimerises to form four-helix bundles.[29] RA42 was engineered to perform acyl transfer reactions with mono-*p*-nitrophenyl fumarate and the catalytic site was built up by His11, Orn15 and Orn34. It was found that RA42 became covalently modified when the polypeptide was reacted with the fumarate ester. The reaction was proved to be a self-catalysed acylation of the ornithine residue in position 15.[30] It proceeds through a rate limiting attack of the unprotonated histidine side chain on the carbonyl group of the ester. This yields an acyl intermediate under the release of *p*-nitrophenol. The second step is a fast intrapolypeptide transfer from the histidine side chain to the flanking ornithine residue, yielding the corresponding amide. The self-catalysed acylation reaction was shown to be highly site selective.[31] Only a lysine or ornithine residue in position $i + 4$ or $i - 3$ relative to a histidine in position i becomes modified. Acylation of the residue in position $i + 4$ is preferred over acylation of the residue in position $i - 3$. The site selective reactions have now been used to introduce sugars[32] and cofactors[33] into designed polypeptides.

The knowledge of the folded structure of the polypeptide RA42 and of the self-catalysed acylation reaction was used to develop the polypeptide PE42Dcap for bridging experiments. The design of RA42 has previously been described and made use of amino acids based on their intrinsic helix propensity, helix dipole stabilisation, a stabilising intrahelical salt bridge, and N- and C-terminal capping residues.[29] Hydrophobic interactions between the two amphiphilic helices were used to induce the hairpin motif and dimerisation to form four-helix bundles. The primary sequence of PE42Dcap was obtained from that of RA42 by computer modelling.[34] Only the positions of the amino acid side chains were energy minimised while the backbone structure of the polypeptide was held constant during the modelling. Into each helix in PE42Dcap, one lysine residue was introduced in position $i + 4$ (Lys15 in helix I and Lys34 in helix II) relative to a histidine in position i (His11 in helix I and His30 in helix II). It has previously been shown that a properly located protonated histidine residue catalyses the rate limiting imidazole acylation.[35,36] Therefore another two histidine residues (His7 and His26) were introduced to make the expected site selective acylations more competitive relative to the solvolysis of the bifunctional ester and hence to increase the yield of bridged polypeptide. To simplify future analysis of the bridged reaction product, all unwanted lysine and ornithine residues in the sequence of RA42 were changed into arginines in the PE42Dcap and the N-terminus was capped by an acetyl group. Bridging of the peptide converts two positively charged

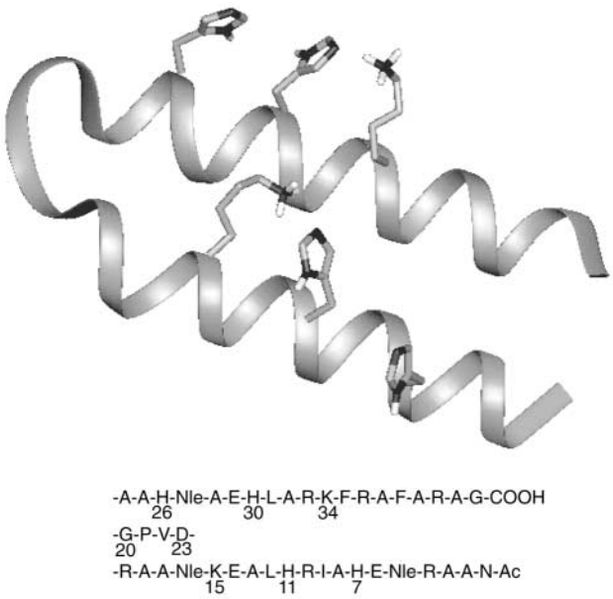


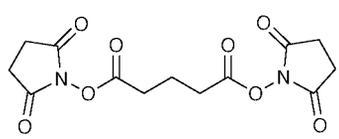
Fig. 1 The amino acid sequence and the modelled folded structure of PE42Dcap. For clarity the peptide is displayed as a monomer and only the histidine and lysine side chains are shown. The one-letter code for amino acids is used where A is Ala, D is Asp, E is Glu, F is Phe, G is Gly, H is His, I is Ile, K is Lys, L is Leu, N is Asn, P is Pro, R is Arg and V is Val. Nle is norleucine.

lysine residues into uncharged amides. To ensure solubility of the bridged peptide, two additional arginine residues (Arg4 and Arg36) were incorporated into the amino acid sequence. All Aib residues in RA42 were changed into alanines in PE42Dcap to simplify polypeptide synthesis.

The amino acid sequence and the structure of the intended folded monomer PE42Dcap is shown in Fig. 1. PE42Dcap was designed to fold into a helix-loop-helix motif and dimerise to form four-helix bundles, but CD spectroscopy shows that the peptide has a low helical content and probably is monomeric in aqueous solution.

Bridging

By reaction of the bifunctional ester disuccinimidyl glutarate (DSG) with PE42Dcap an interhelical 5-carbon bridge—a spacer of 7.7 Å—was expected to be introduced between the nitrogens of the lysine residues. DSG is an *N*-hydroxysuccin-



DSG (disuccinimidyl glutarate)

imidyl ester (NHS-ester), and these esters are commonly used as cross-linking reagents in protein chemistry.[37,38] They react with primary amines, such as lysine side chains, to yield stable amides.[39] Cross-linking of proteins is usually performed at pH 7–9 using a large molar excess of NHS-ester over protein, often as much as 10–50 equivalents.[39]

The bridging reaction was performed at slightly acidic conditions to make the self-catalysed, site selective acylations of the polypeptide more competitive for DSG compared with the solvolysis. The reaction of PE42Dcap was carried out at ambient temperature at pH 4.1 in 100 mM sodium acetate buffer containing 5 vol% 2,2,2-trifluoroethanol. 2,2,2-Trifluoroethanol was added to stabilise the helical structure of PE42Dcap and to solubilise DSG.

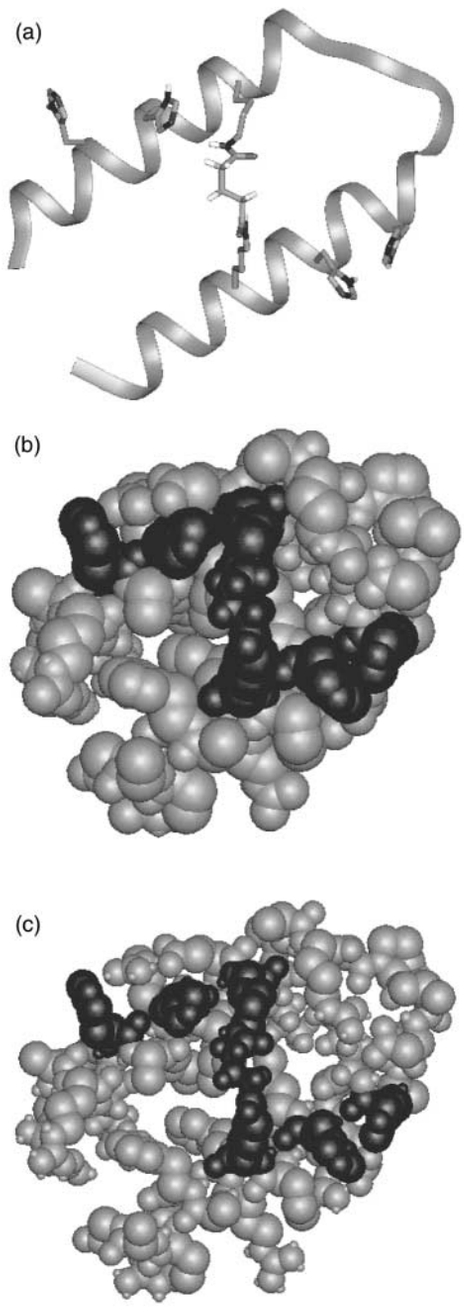


Fig. 2 (a) The modelled folded structure of bridged DSG-PE42Dcap. For clarity only the carbon bridge, the lysine and histidine side chains are detailed. The peptide is displayed as a monomer. (b) Space-filling model with 100% van der Waals radius of bridged DSG-PE42Dcap. The bridge, the lysine and histidine side chains are shown in black. (c) Space-filling model with 70% van der Waals radius of the bridged DSG-PE42Dcap. The bridge, the lysine and histidine side chains are shown in black.

The purified bridged DSG-PE42Dcap was lyophilised in water and identified by MALDI-TOF mass spectrometry. Analysis of the bridged peptide showed a single peptide component with a molecular mass of 4723.4 (Calculated: 4722.5). The total yield of pure, bridged DSG-PE42Dcap was 35%. The proposed structure of bridged DSG-PE42Dcap is shown in Fig. 2. The structure is based on CD spectroscopy and previous structural investigations on polypeptides with similar amino acid sequences.

The design principle of PE42Dcap using only two lysine residues and a capped N-terminus gives just one possibility for intramolecular incorporation of the bridge and ^{1}H NMR spectroscopy has confirmed the position of the bridge. At pH 3.1, the lysine ε protons in unbridged PE42Dcap give a

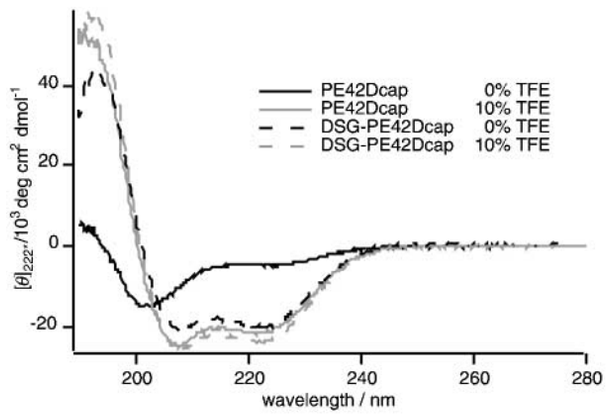


Fig. 3 CD spectra of unbridged PE42Dcap and bridged DSG-PE42Dcap in 100 mM sodium acetate buffer at pH 5.0 at different concentrations of 2,2,2-trifluoroethanol (TFE).

multiplet at 3.0 ppm, while proton resonances are missing in same region in the corresponding spectrum of bridged DSG-PE42Dcap. Therefore, no enzymatic digestion of the bridged peptide was performed to confirm the position of the bridge.

CD spectroscopy of bridged DSG-PE42Dcap shows a typical α-helix spectrum with double minima at 208 and 222 nm.[40] The mean residue ellipticity at 222 nm, $[\theta]_{222}$, is often used as a measure of the helical content in helical peptides. The mean residue ellipticity at 222 nm can be used to estimate the percentage of helicity using eqn. (1).

$$\% \text{ Helix} = 100 \times [\theta]_{222}/[\theta]_{\max,n} \quad (1)$$

The maximum value of the mean residue ellipticity is chain length dependent and can be calculated using eqn. (2),

$$[\theta]_{\max,n} = [\theta]_{\text{theor}}(1 - k/n) \quad (2)$$

where $[\theta]_{\text{theor}}$ is the maximum mean residue ellipticity for an indefinitely long helix. $[\theta]_{\text{theor}}$ is often set to −39500 deg cm^2 dmol^{-1} at 222 nm,[41] the factor k is 2.57, and n is the number of amino acid residues in the polypeptide.

The mean residue ellipticity is −20100 deg cm^2 dmol^{-1} (55% helix) for bridged DSG-PE42Dcap (0.6 mM) in 100 mM sodium acetate buffer at pH 5.1 (Fig. 3). The mean residue ellipticity obtained for unbridged PE42Dcap under the same conditions is only −4800 deg cm^2 dmol^{-1} (13% helix). Obviously, the bridge has a strong helix promoting effect.

Concentration dependency of the mean molar ellipticity indicates that bridged DSG-PE42Dcap is mainly monomeric at micromolar concentration and dimerises above 100 μM at pH 5.1 in sodium acetate buffer (Fig. 4). The ratio of the mean molar ellipticity at 222 nm and 208 nm, $[\theta]_{222}/[\theta]_{208}$, is close to 1 for concentrations above 20 μM, which corresponds well to reported values for coiled-coils.[42–44] The ratio decreases at lower peptide concentrations, which is indicative of monomer formation or non-interacting helices.[45] The $[\theta]_{222}/[\theta]_{208}$ ratio for unbridged PE42Dcap is much lower, 0.4, at 100 μM and close to 0.6 at 1000 μM. This indicates that unbridged PE42Dcap is mainly monomeric and forms non-interacting α-helices or even 3_{10}-helices[46] in aqueous solution and not the intended hairpin helix-loop-helix dimer.

Addition of 2,2,2-trifluoroethanol, a solvent known to promote helical structure of polypeptides, strongly affects the helical content of unbridged PE42Dcap (Fig. 5). An addition of 10 vol% 2,2,2-trifluoroethanol increases the absolute mean residue ellipticity at 222 nm from −4800 deg cm^2 dmol^{-1} (13% helix) to −22000 deg cm^2 dmol^{-1} (60% helix) (Fig. 3). At the same time the ratio $[\theta]_{222}/[\theta]_{208}$ increases to 0.9.

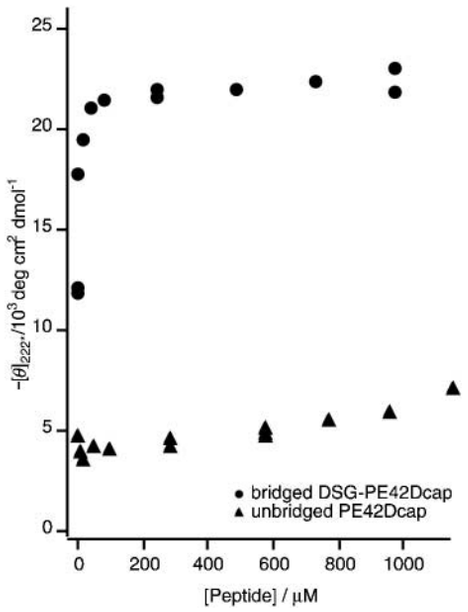


Fig. 4 The mean molar residue ellipticity at 222 nm as a function of peptide concentration at pH 5.1 in sodium acetate buffer and at ambient temperature.

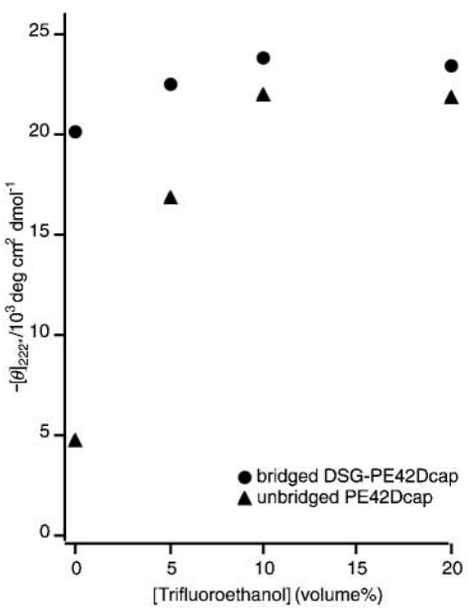


Fig. 5 The mean molar residue ellipticity at 222 nm as a function of vol% trifluoroethanol at pH 5.1 in sodium acetate buffer and at ambient temperature.

As expected, the structure of bridged DSG-PE42Dcap is only slightly affected by 2,2,2-trifluoroethanol. An addition of 10 vol% 2,2,2-trifluoroethanol increases the absolute mean residue ellipticity at 222 nm from −20100 deg cm^2 dmol^{-1} (55% helix) to −23800 deg cm^2 dmol^{-1} (65% helix) under the same conditions. The ratio $[\theta]_{222}/[\theta]_{208}$ decreases with increasing 2,2,2-trifluoroethanol concentration which is consistent with formation of monomers.

The helical content of both peptides decreases with decreasing pH, which is explained by the protonation of histidine, glutamic acid and aspartic acid side chains. The structure of unbridged PE42Dcap is very sensitive to pH changes. It reaches a maximum absolute mean residue ellipticity of −20000 deg cm^2 dmol^{-1} in aqueous solution at pH 8 (Fig. 6). Bridged DSG-PE42Dcap is less sensitive to pH variations and reaches a maximum absolute residue ellipticity of −25000 deg cm^2 dmol^{-1}. The ratio $[\theta]_{222}/[\theta]_{208}$ increases in the same manner as

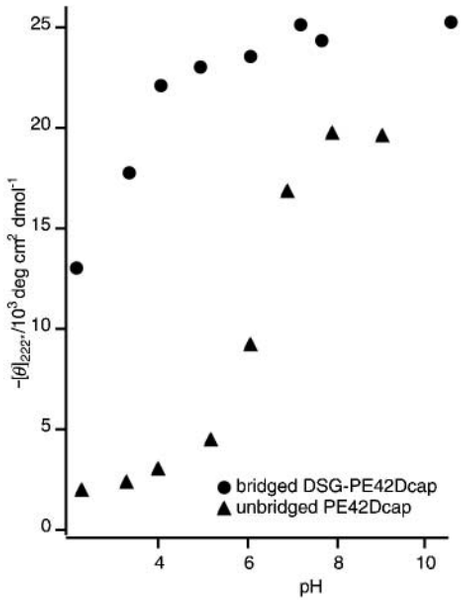


Fig. 6 The mean residue ellipticity at 222 nm as a function of pH in aqueous solution and at ambient temperature.

the absolute ellipticity for both peptides and the peptides are monomeric at low pH (pH 2).

Bridged polypeptides are now being explored as templates for novel catalysts. The bridging reaction may be of general applicability also for the introduction of bridges with chemically active groups, yielding polypeptides for selective catalysis and chiral recognition.

Experimental

Computer graphics and molecular modelling was performed on a Silicon Graphics INDIGO 2 using the Quanta 97 software package.

PE42Dcap was synthesised using a PerSeptive Biosystems Pioneer automated peptide synthesiser using standard Fmoc chemistry protocols. The N-terminus was capped by acetic anhydride. The peptide was cleaved from the resin (Fmoc-Gly-PEG-PS) using a mixture of trifluoroacetic acid (13.5 ml), thioanisole (750 μl), ethanedithiol (450 μl) and anisole (300 μl) for 2.5 hours at ambient temperature. PE42Dcap was purified by reversed phase HPLC on a semi-preparative C-8 HiCHROM Kromasil, 10 μm column, using a mobile phase with 36.0 vol% isopropanol and 0.1 vol% trifluoroacetic acid in water, flow rate of 10.0 ml min^{-1} and UV detection at 229 nm. The purified peptide was repeatedly lyophilised in distilled and filtered water.

The purity was checked by analytical HPLC on an analytical C-8 HiCHROM Kromasil, 5 μm column, using a mobile phase with 37.0 vol% isopropanol and 0.1 vol% trifluoroacetic acid in water and a flow rate of 0.8 ml min^{-1}. The peptide was found to be more than 95% pure.

PE42Dcap was identified by electrospray MS (VG Analytical, ZabSpec) (Found: 4626.7, calculated: 4626.4) and by MALDI-TOF MS (Malditof VG TofSpec E) (Found: 4625.0, calculated: 4626.4).

The bridging reaction was performed with a peptide concentration of 0.50 mM at pH 4.1 in sodium acetate buffer with 5 vol% 2,2,2-trifluoroethanol and at room temperature. DSG was dissolved to a concentration of 0.025 M in sodium acetate buffer with 35 vol% 2,2,2-trifluoroethanol at pH 4.1. The DSG-solution was added to the peptide solution in portions at typically two hours interval. Each addition of DSG corresponded to 0.3 equivalents compared to the peptide. In total 4 equivalents of DSG compared to peptide was added and the total reaction time was 72 hours. The crude product was

lyophilised and purified by reversed phase HPLC on a semi-preparative C-8 HiCHROM Kromasil, 10 μm column using a mobile phase with 33.5 vol% isopropanol and 0.1 vol% 2,2,2-trifluoroacetic acid in water, flow rate of 8.0 ml min^{-1} and UV detection at 229 nm. Bridged DSG-PE42Dcap eluted as a single peak after 10 minutes. By a five minutes gradient the isopropanol content was raised to 50 vol% and unbridged polypeptide by-products were eluted after a total of 23 minutes. The retention time for bridged DSG-PE42Dcap was significantly lower than for unbridged PE42Dcap and this is in agreement with what has earlier been seen for coiled-coils that are stabilised by interhelical disulfide bridges.[47] The purified peptide was repeatedly lyophilised in distilled and filtered water.

Bridged DSG-PE42Dcap was found to be more than 95% pure as determined by reversed-phase HPLC on an analytical C-8 HiCHROM Kromasil, 5 μm column, using a mobile phase with 33.5 vol% isopropanol and 0.1 vol% trifluoroacetic acid in water and a flow rate of 0.8 ml min^{-1}.

Bridged DSG-PE42Dcap was identified by electrospray MS (Found: 4723.0, calculated: 4722.5) and by MALDI-TOF MS (Found: 4723.4, calculated: 4722.5).

1D NMR spectra were recorded on a Varian Unity 400 MHz NMR Spectrometer. The peptides were dissolved in 5 vol% d_3-2,2,2-trifluoroethanol in D_2O and pH was adjusted with NaOD–D_2O (1 : 99) and DCl–D_2O (10 : 100).

CD spectra were recorded at ambient temperature on a JASCO J-715 spectrometer, routinely calibrated with (+)-camphor-10-sulfonic acid. CD spectra in the wavelength interval 280–190 nm were recorded in 0.1, 0.5 and 1.0 mm cells. Sodium acetate buffer was used for peptide solutions below pH 5.8 and Bis-tris buffer at pH above 5.8, except for in the pH titrations when no buffer was used. Peptide samples were prepared by diluting stock solutions by pipetting. The peptide concentrations of the stock solutions were determined by quantitative amino acid analysis.

Acknowledgements

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