Interhelical carbon bridging of catalytic helix–loop–helix polypeptide motifs †

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Interhelically bridged polypeptides with helix–loop–helix motifs have been synthesised by self-catalysed, site-selective acylation reactions. Lysine residues in the helices have been bridged using the bifunctional *N*-hydroxysuccinimide ester disuccinimidyl glutarate (DSG). The helical content of the polypeptides increases upon bridging and the structures of the bridged peptides have been shown to have increased thermal stability. The bridging also causes increased polypeptide catalytic activity in ester hydrolysis in aqueous solution. The catalysis of the site-selective reaction has been investigated in detail using a number of designed helix–loop–helix polypeptide motifs that have been reacted with DSG and the longer *N*-hydroxysuccinimide ester bis(sulfosuccinimidyl) suberate (BS^3). The bridging of the polypeptide PE42Dcap with DSG has been optimised and the best yield was given at pH 4.1 in 5 vol% 2,2,2-trifluoroethanol (TFE).

Introduction

Large efforts have been made to create novel catalysts using *de novo* designed polypeptides,[1–9] but it still remains a challenging problem. It is difficult to obtain a specific three-dimensional structure for the designed polypeptide catalysts with a small number of amino acid residues and to obtain stabilising multiple interactions in the catalytic transition state.

The helix–loop–helix polypeptide motif is naturally occurring and can be used as a template structure in the design of novel catalysts. It consists of two amphiphilic α-helices connected by a short hairpin loop,[10] and has often been shown to dimerise into four-helix bundles.[11–13] Recently, several designed four-helix bundles with catalytic activity have been developed.[2–4] In solution, many four-helix bundles have been shown to have coiled-coil structures,[14–18] in which the helices are wrapped around each other to form a super coil.[19,20] The structures of coiled-coil polypeptides are stabilised by both intra- and interchain interactions. The main driving force for folding is hydrophobic interactions, but other van der Waals, ionic and electrostatic interactions are also important. Sometimes, the folded structure is further stabilised by salt bridges or covalent interactions, *e.g.* disulfide bridges or lactam bridges.[13,21–25]

The use of disulfide bridges and salt bridges in designed coiled-coil polypeptides is complicated, since the stabilising effect is dependent on the position of the bridge within the structure. The disulfide bridge or the salt bridge has to be introduced inside the hydrophobic core without disrupting the packing of the hydrophobic side chains in order to be strongly stabilising.[26–28] The strength of a salt bridge is dependent on pH and the ionic strength, and salt bridges have been shown to primarily influence the aggregation process and not the overall stability of the polypeptide fold.[21] Furthermore, disulfide bridges may be chemically unstable, *e.g.* inside cells,[29] and they are short and inflexible. Neither salt bridges nor disulfide bridges allow the possibility of introducing additional functional groups into a folded polypeptide motif.

In 1996, Baltzer and co-workers reported the *de novo* designed polypeptide RA-42, which consists of 42 amino acid residues.[30,31] It was shown to fold into a helix–loop–helix motif and to dimerise into four-helix bundles. RA-42 was engineered to catalyse acyl transfer reactions with mono(*p*-nitrophenyl) fumarate and the active amino acid residues were His11 and Orn15 in helix 1 and Orn34 in helix 2.

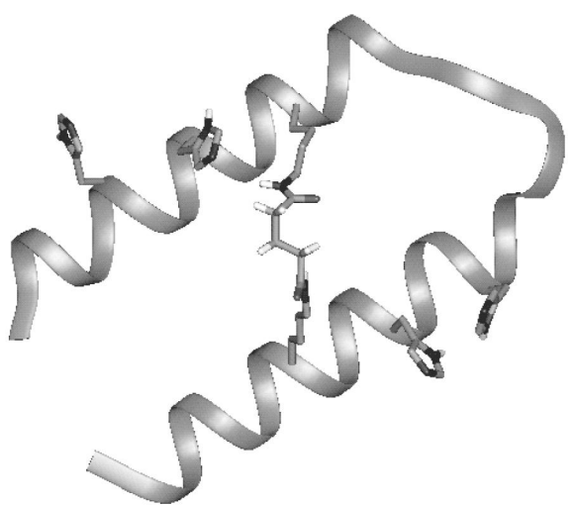

Fig. 1 The modelled folded structure of the bridged polypeptide DSG-PE42Dcap. For clarity, the polypeptide is displayed as a monomer and only the bridge and the side chains of the histidine and the lysine residues are detailed.

The ornithine residue in position 15 was shown to be acylated during the reaction of RA-42 with mono(*p*-nitrophenyl) fumarate.[31] This acylation reaction was later proved to be both self-catalysed with a rate-limiting attack of the unprotonated histidine residue on the carbonyl group of the ester[32] and site-selective, since only a lysine or ornithine residue in position $i + 4$ or $i - 3$ relative to the histidine in position i becomes modified.[33] The acylation of the residue in position $i + 4$ was proved to be preferred over acylation of the residue in position $i - 3$. The self-catalysed, site-selective reaction has now been used to introduce sugars[34,35] and cofactors[36] into designed polypeptides.

Recently, we reported the interhelically bridged polypeptide DSG-PE42Dcap (Fig. 1), which was obtained in the reaction

† Figs. S1–S5 and Table S1 are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p2/b0/b003270k/

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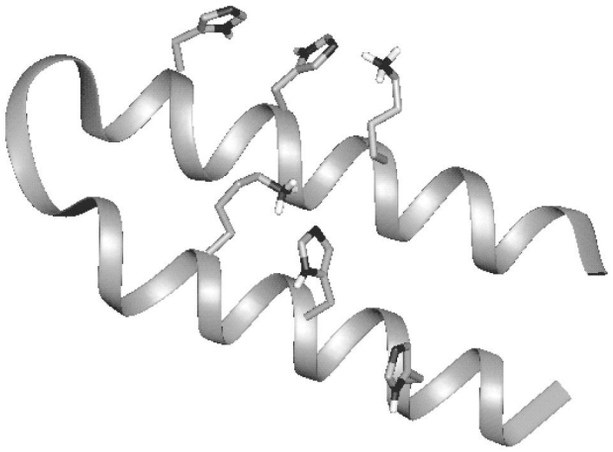
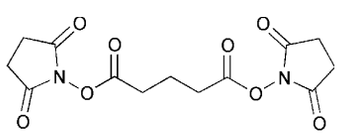


Fig. 2 The amino acid sequence and the modelled folded structure of the polypeptide PE42Dcap. For clarity, the polypeptide is displayed as a monomer and only the side chains of the histidine and the lysine residues are shown. The one-letter code for amino acids is used where A is Ala, D is Asp, E is 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.



of the polypeptide PE42Dcap (Fig. 2) and the bifunctional *N*-hydroxysuccinimide ester disuccinimidyl glutarate (DSG).[37] The polypeptide PE42Dcap was designed using knowledge of the self-catalysed, site-selective acylation reaction and of the primary sequence of RA-42. PE42Dcap contained one His(i − 4)–His(i)–Lys(i + 4) triad in each helix, and the interhelical bridge was introduced between Lys15 in helix 1 and Lys34 in helix 2 using the histidine residues in positions 11 and 30. The isolated yield of the bridged polypeptide DSG-PE42Dcap was 35% at pH 4.1 in 5 vol% 2,2,2-trifluoroethanol (TFE). The structure of the folded polypeptide motif was shown by CD spectroscopy to be dramatically stabilised by the interhelical bridge and DSG-PE42Dcap was shown to fold into the intended four-helix bundle structure in aqueous solution at concentrations above 100 μM.[37]

In this paper we report the higher thermal stability and increased catalytic activity of DSG-PE42Dcap compared to PE42Dcap in aqueous solution. We have also investigated the bridging reaction in detail using a number of designed helix–loop–helix polypeptide motifs. The bridging reaction has been shown to be of general use and, maybe, it can be used for the purpose of creating enzyme-like binding cavities in designed polypeptide catalysts.

Results and discussion

Structure and pK_a-values

The increased helical content of the interhelically bridged polypeptide has previously been shown by CD spectroscopy,[37] and now, we have also studied the effect of temperature on the helical content of the polypeptide. The effect of temperature on the helical content of DSG-PE42Dcap was shown to be weak. The thermal denaturation curve of the bridged polypeptide does not show a sharp melting point (see Fig. S1), and the mean molar residue ellipticity at 222 nm, $[\theta]_{222}$, at 323 K is only 15% less than that at 293 K, indicating a high thermal stability. The

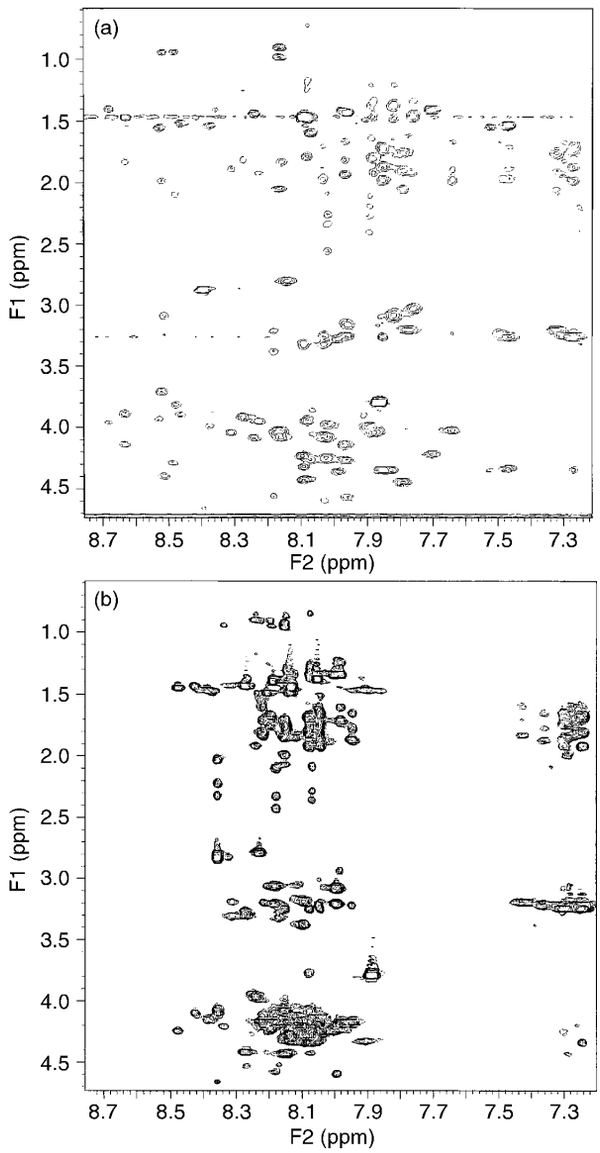


Fig. 3 Part of the 600 MHz TOCSY spectrum of (a) the bridged polypeptide DSG-PE42Dcap and (b) the unbridged polypeptide PE42Dcap, recorded at pH 5.1 in 5 vol% [D_3] TFE in D_2O–H_2O (10 : 90) at 308 K. The cross-peaks are considerably weaker for PE42Dcap than for DSG-PE42Dcap, and therefore, the threshold has been lowered in the spectra of PE42Dcap.

measured CD spectra have an isodichroic point at 203 nm, indicating a two-state transition between a dimeric and monomeric state.[38–44] Similarly, the unbridged polypeptide PE42Dcap does not show a sharp melting point (see Fig. S1) and the absolute value of $[\theta]_{222}$ is lowered by 25% when the temperature is raised from 293 to 323 K. The noise at low wavelengths in the CD spectra of PE42Dcap is very high and an isodichroic point could not be found.

The 2D NMR TOCSY spectrum of DSG-PE42Dcap at pH 5.1 in 5 vol% [D_3] TFE showed a large chemical shift dispersion and many cross-peaks [Fig. 3(a)], which is an indication of a stable polypeptide structure. The unbridged polypeptide PE42Dcap had a poorer chemical shift dispersion [Fig. 3(b)], showing once again that the polypeptide structure is strongly stabilised by the interhelical carbon bridge. The NOESY spectrum of the bridged polypeptide also showed a larger number of cross-peaks indicating a larger number of through-space interactions compared to the unbridged polypeptide. The NOESY and the TOCSY spectra of PE42Dcap and DSG-PE42Dcap have not been assigned, and have therefore not been used for determination of the three-dimensional structures of the polypeptides. However, the larger number of cross-peaks

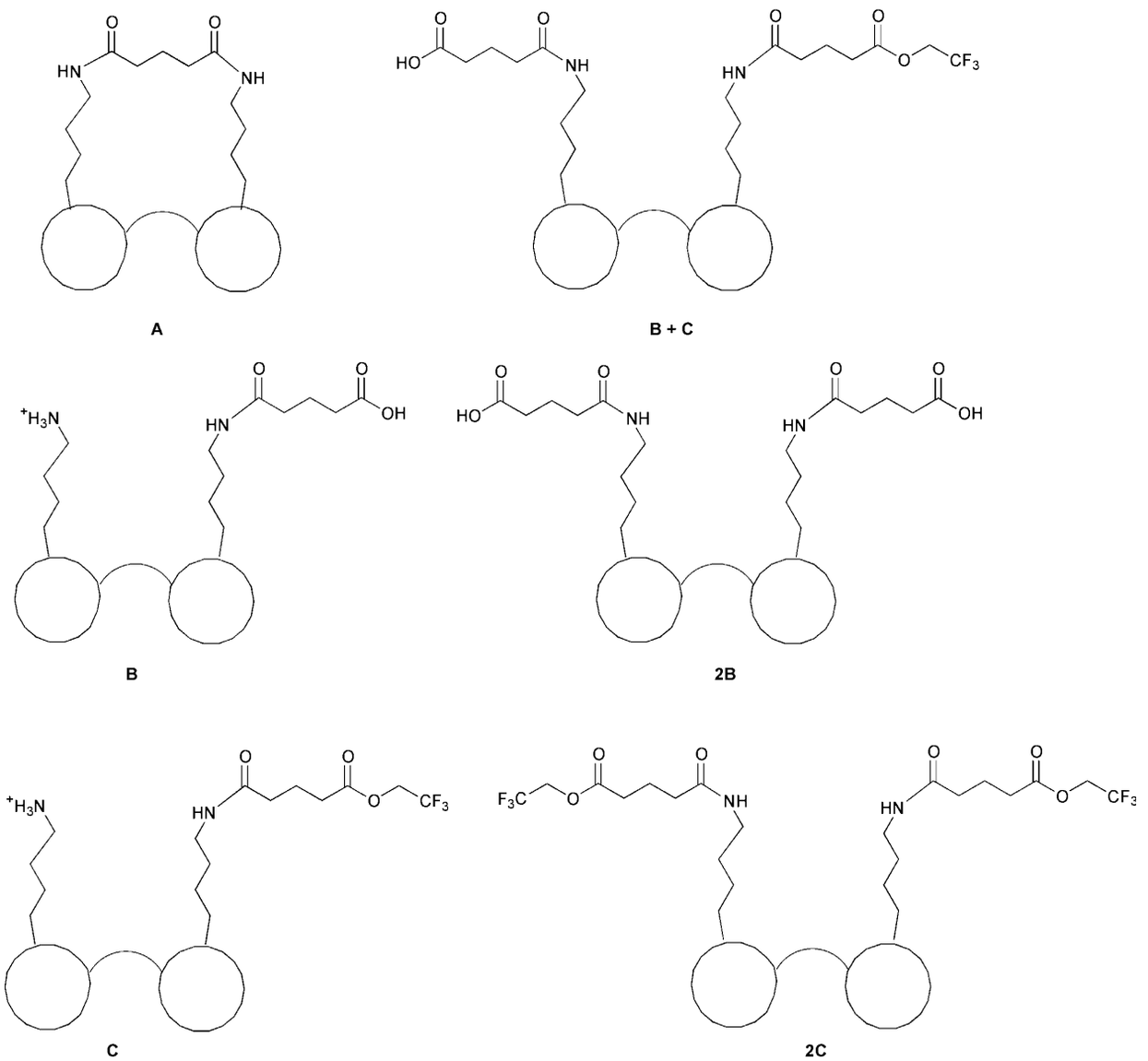


Fig. 4 Possible adducts in the reaction of DSG and a helix–loop–helix polypeptide with one lysine residue in each helix. The helix–loop–helix motif is shown end-on and only the lysine residues are detailed.

and the larger chemical shift dispersion in the 2D NMR spectra of DSG-PE42Dcap strongly support the four-helix bundle structure that has been proposed from the CD spectroscopic investigations.

The pK_a-values of the catalytic histidine side chains in PE42Dcap and DSG-PE42Dcap were determined by ^{1}H NMR spectroscopy in 5 vol% [D_3] TFE in D_2O. The measured pK_a-values were essentially unaffected by the interhelical bridge, but it should be noted that the pK_a-values have not been assigned to the individual histidine residues in either of the polypeptides. The observed pK_a-values of the four protonated histidine residues in PE42Dcap are 6.4, 6.0, 5.7 and 5.4, and the corresponding values in the bridged polypeptide DSG-PE42Dcap are 6.4, 6.2, 5.7 and 5.4.

Optimisation of the bridging of PE42Dcap with DSG

The reaction of PE42Dcap with DSG can give several different types of polypeptide structures. The bridged polypeptide **A** is obtained when two lysine residues in the polypeptide displace both *N*-hydroxysuccinimide (NHS) groups in the same DSG molecule (corresponding to an increase of the polypeptide molecular weight by 96.1 Da) (Fig. 4). The non-bridged polypeptide adducts are produced when one of the NHS groups is displaced by a lysine residue and the other one by water (**B**) or by TFE (**C**). In total, there are six possible types of adducts in the bridging reaction of PE42Dcap with DSG (**A**, **B**, **2B**, **B** + **C**, **C** and **2C**, Fig. 4).

In the basic experiment, PE42Dcap was reacted with DSG at pH 4.1 in 5 vol% TFE. The peptide components in the crude product mixture were separated by gradient analytical reversed phase HPLC and lyophilised as individual fractions (Fig. 5). The fractions were identified by electrospray mass spectrometry as the bridged polypeptide DSG-PE42Dcap (**A**), the unmodified peptide PE42Dcap or as one of the possible non-bridged adducts **B**, **2B**, **B+C**, **C** or **2C** (Fig. 4). In theory, it would also be possible to get covalently bridged polypeptide dimers, if the NHS groups of the DSG molecule were replaced by lysine residues in different helix–loop–helix monomers. This would have given additional peaks corresponding to ions with an odd number of charges in the mass spectrum, and this was not observed for any of the polypeptide–NHS ester mixtures. As shown in Fig. 5, the bridged polypeptide DSG-PE42Dcap eluted as a separate peak, followed by the unmodified PE42Dcap and the different non-bridged adducts.

The bridging of PE42Dcap with DSG was repeated at different reaction conditions and the results were evaluated by HPLC. The effect of peptide concentration, pH and the fraction of TFE on the yield of DSG-PE42Dcap was investigated. The fractions of DSG-PE42Dcap and unmodified PE42Dcap were

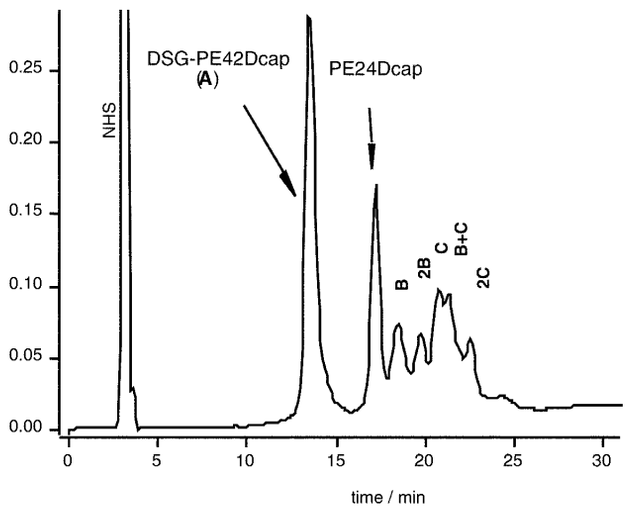


Fig. 5 An analytical HPLC chromatogram of the crude product mixture from the reaction of PE42Dcap with DSG in 5 vol% TFE in 100 mM sodium acetate buffer at pH 4.1. The chromatogram was recorded at 229 nm using a Varian 9065 Polychrom®/Polyview™ Diode Array Detector.

estimated from the peak integrals in the analytical HPLC chromatograms of the crude product mixtures. The absolute yield of bridged peptide was calculated by

$$\text{Absolute yield (\%)} = \frac{I(\text{bridged peptide})}{\underset{\text{peptide fractions}}{\Sigma I}} \times 100$$

whereas the relative yield of bridged peptide was calculated as

$$\text{Relative yield (\%)} = \frac{I(\text{bridged peptide})}{\underset{\text{peptide fractions}}{\Sigma I - I(\text{unmodified peptide})}} \times 100$$

where I is the integral of the respective peak in the chromatogram. The absolute yield of the bridged polypeptide DSG-PE42Dcap was shown to increase with increasing concentration of PE42Dcap (see Fig. S2), whereas the relative yield was higher at low peptide concentrations. This study was performed at pH 4.1 in 5 vol% TFE, and the best bridging result was obtained at peptide concentrations above 0.5 mM.

The reaction of PE42Dcap with DSG was shown to be very slow at pH ≤ 4 (see Fig. S3), most likely because the histidine and the lysine residues are almost fully protonated in the acidic solution. In the reaction mixtures formed at pH < 3.5, no bridged product was obtained even after extended reaction times. A maximum absolute yield of DSG-PE42Dcap was achieved at pH 4, and at higher pH the yield decreased with increasing pH. No unmodified PE42Dcap was found in the reaction mixtures at pH > 5, probably due to the fast catalysis by the unprotonated histidine residues followed by bridging or by hydrolysis/esterification of the remaining NHS group through the background reactions. At pH > 7, the absolute yield of bridged peptide was again increased, presumably due to direct acylation of unprotonated lysine side chains. The relative yield of DSG-PE42Dcap was slightly higher at pH 4 than at pH 9. The pH-dependence of the bridging reaction was examined with a peptide concentration of *ca.* 0.5 mM in 5 vol% TFE.

The bridging reaction of PE42Dcap with DSG was also performed with different fractions of the helix-stabilising solvent TFE at pH 4.1 (see Fig. S4). The helical content of PE42Dcap in 0% TFE is low, and a small addition of TFE is expected to increase the yield of the bridged polypeptide. A maximum yield of DSG-PE42Dcap was given at 5 vol% TFE, whereas the best relative yield was obtained in 0% TFE. A larger fraction of TFE increases the helical content of PE42Dcap even more, but is at the same time known to cause separation of the helices in the hairpin formation to yield non-interacting helices. In combination with the higher background reaction rates with larger fractions of TFE, the yield of the non-bridged adducts **C**, **2C** and **B+C** is increased, and the fractions of bridged and unmodified peptide are decreased.

Table 1 The measured mean molar residue ellipticities[a] at 222 nm of the unbridged polypeptides PE42Dcap, PE42Hcap and the bridged polypeptides DSG-PE42Dcap, BS^3-PE42Dcap and DSG-PE42Hcap

Peptide	TFE/ vol%	$[\theta]_{222}$/deg cm^2 dmol^{-1}	TFE/ vol%	$[\theta]_{222}$/deg cm^2 dmol^{-1}
PE42Dcap	0	−6500	10	−19500
PE42Hcap	0	−8400	10	−22600
DSG-PE42Dcap	0	−21100	10	−22300
BS^3-PE42Dcap	0	−22200	10	−24400
DSG-PE42Hcap	0	−19100	10	−23700

[a] The peptide concentrations (*ca.* 0.3–0.4 mM) were determined by quantitative amino acid analysis.

Catalytic activity of the bridged polypeptides

Broo and co-workers have previously shown that a properly placed arginine residue can lower the pK_a of a histidine residue and thereby increase its activity as a nucleophilic catalyst (at pH < pK_a).[45] In an attempt to increase the catalytic activity of the polypeptide PE42Dcap (and consequently also for DSG-PE42Dcap), four amino acid residues were altered in the amino acid sequence. In the new polypeptide PE42Hcap, Asp4 and Glu36 replaced the arginine residues in PE42Dcap and Arg14 and Arg29 replaced glutamic acid residues. The catalytic triads were not altered.

PE42Hcap

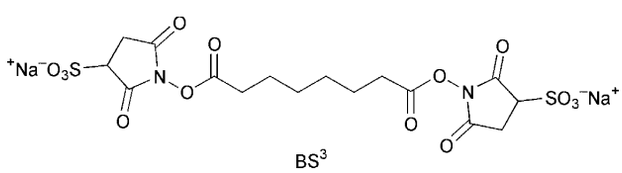
CH_3CO-N^1-A-A-D-Nle-E-**H**^{7}-A-I-R-**H**^{11}-L-A-R-**K**^{15}-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-**H**^{26}-Nle-A-R-**H**^{30}-L-A-R-**K**^{34}-F-E-A-F-A-R-A-G^{42}-COOH

PE42Dcap

CH_3CO-N^1-A-A-R-Nle-E-**H**^{7}-A-I-R-**H**^{11}-L-A-E-**K**^{15}-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-**H**^{26}-Nle-A-E-**H**^{30}-L-A-R-**K**^{34}-F-R-A-F-A-R-A-G^{42}-COOH

The polypeptide PE42Hcap was bridged by DSG and the inter-helical bridge was shown by CD spectroscopy to be strongly helix-stabilising also in this polypeptide (Table 1).

The polypeptide PE42Dcap was also bridged by a longer NHS ester, the sodium salt of bis(sulfosuccinimidyl) suberate (BS^3). The bridging reagent DSG has a non-cleavable five-carbon spacer of 7.7 Å and BS^3 has a non-cleavable eight-carbon spacer of 11.4 Å. The resulting bridged polypeptide BS^3-PE42Dcap was purified and identified in the usual manner and BS^3-PE42Dcap was shown to adopt a helical structure in aqueous solution (Table 1).



BS^3

The catalytic activities of the polypeptides PE42Dcap and PE42Hcap were determined before and after bridging using the hydrolysis reaction of mono(*p*-nitrophenyl) fumarate at pH 5.9 in 50 mM Bis-tris buffer (Table 2). As expected, the second-order catalytic rate constant of the polypeptide PE42Hcap (0.22 M^{-1} s^{-1}) was larger than that of PE42Dcap (0.15 M^{-1} s^{-1}).

Table 2 Second-order rate constants for the reaction of the unbridged polypeptides PE42Dcap, PE42Hcap and the bridged polypeptides DSG-PE42Dcap, BS^3-PE42Dcap and DSG-PE42Hcap with mono-(*p*-nitrophenyl) fumarate at 290 K at pH 5.9 in 50 mM Bis-tris buffer. The catalytic rate constant of 4-methylimidazole (4-MeIm) is included as a reference

Active species	$10^3\ k_2/M^{-1}\ s^{-1}$	k_2/k_2 (4-MeIm)	$10^3\ k_0/s^{-1}$
PE42Dcap[a]	150	15	
PE42Hcap[a]	220	22	
DSG-PE42Dcap[a,b]	220	22	
BS^3-PE42Dcap[a,b]	190	19	
DSG-PE42Hcap[a,b]	280	28	
4-MeIm	10	1	
Background			0.01

[a] The concentrations of the batch peptide solutions were determined by quantitative amino acid analysis. [b] The rate constants were determined using only one peptide concentration.

Both polypeptides showed enhanced catalytic activity after bridging with DSG or BS^3 (Table 2), probably as a result of the stabilised structure.

Exploring the generality of the bridging reaction

The site-selectivity of the self-catalysed acylation reaction had previously been determined using a number of polypeptides with 20 and 42 amino acid residues respectively.[33] The results showed that a lysine residue in position $i + 4$ or $i - 3$ relative to a histidine in position i can be covalently modified in the acylation reaction. This was used to explore the generality of the bridging reaction by designing a number of helix–loop–helix polypeptide motifs with lysine and histidine residues in varying positions.

The polypeptides PE42A and PE42B (Fig. 6) were designed from the amino acid sequence of RA-42. A His($i - 4$)–His(i)–Lys($i + 4$) triad was introduced into helix 1 and a Lys($i - 3$)–His(i)–His($i + 4$) triad into helix 2 to create the polypeptide PE42A. The reaction of PE42A with DSG was supposed to give an interhelical bridge near the loop of the polypeptide (*i.e.* between Lys15 and Lys27). The polypeptide PE42B was designed with a Lys($i - 3$)–His(i)–His($i + 4$) triad in helix 1 and a His($i - 4$)–His(i)–Lys($i + 4$) triad in helix 2. In this peptide, the bridge was intended to form between Lys8 and Lys34, *i.e.* closer to the helix termini. To simplify future analysis of the bridged reaction product, all unwanted lysine and ornithine residues in the sequence of RA-42 were changed into arginines in the polypeptides PE42A and PE42B. All Aib residues in RA-42 were changed into alanines to simplify peptide synthesis.

PE42A

H_2N-N^{1}-A-A-D-Nle-E-**H^{7}**-A-I-R-**H^{11}**-L-A-E-**K^{15}**-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-Q-**K^{27}**-A-E-**H^{30}**-L-A-R-**H^{34}**-F-E-A-F-A-R-A-G^{42}-COOH

PE42B

H_2N-N^{1}-A-A-D-Nle-E-A-**K^{8}**-I-R-**H^{11}**-L-A-E-**H^{15}**-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-**H^{26}**-Nle-A-E-**H^{30}**-L-A-R-**K^{34}**-F-E-A-F-A-R-A-G^{42}-COOH

RA-42

H_2N-N^{1}-Aib-A-D-Nle-E-A-A-I-K-**H^{11}**-L-A-E-**Orn^{15}**-Nle-Aib-A-K^{19}-G^{20}-P-V-D^{23}-Aib^{24}-A-Q-Nle-A-E-Q-L-A-K-**Orn^{34}**-F-E-A-F-A-R-Aib-G^{42}-COOH

The polypeptides PE42A and PE42B have uncapped N-termini, which may become acylated by DSG. The many possible adducts (**A**, **A+B**, **A+C**, **B**, **2B**, **3B**, **C**, **2C**, **3C**, **B+C**, **2B+C** and **B+2C**) in the bridging reaction made mass spectrometric analysis of the product mixture difficult. Therefore, the N-terminus of PE42B was capped by an acetyl group, yielding the polypeptide PE42Bcap.

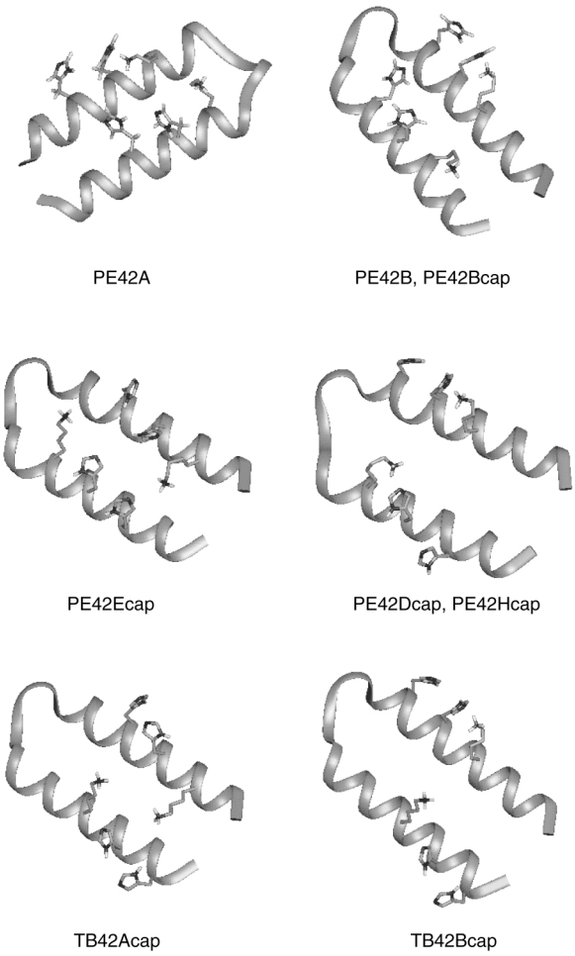


Fig. 6 The modelled folded structures of the polypeptides PE42A, PE42B/PE42Bcap, PE42Dcap/PE42Hcap, PE42Ecap, TB42Acap and TB42Bcap. For clarity, the polypeptides are displayed as monomers and only the side chains of the lysine and the histidine residues are shown.

The polypeptides PE42Ecap, TB42Acap and TB42Bcap (Fig. 6) were designed by modifying the primary sequence of the polypeptide PE42Dcap. These polypeptides were used to determine the effect of distance between the lysine residues in the two helices. The His($i - 4$)–His(i)–Lys($i + 4$) triad was moved one helical turn towards the terminus in one or both of the helices. No attempt was made to move the triads closer to the loop region, since such a move was expected to disrupt the helix–loop–helix hairpin motif.

In the polypeptide TB42Acap, the His($i - 4$)–His(i)–Lys($i + 4$) triads had been moved one turn towards the terminus in both helices. The active residues in TB42Acap are His3, His7 and Lys11 in helix 1 and His30, His34 and Lys38 in helix 2. In TB42Bcap, the triad was moved one turn towards the N-terminus in helix 1, whereas helix 2 was left unchanged compared to PE42Dcap. In PE42Ecap, the triad was moved one turn towards the C-terminus in helix 2 relative to PE42Dcap, whereas the position of the triad in helix 1 was unchanged.

TB42Acap

CH_3CO-N^{1}-A-**H^{3}**-D-Nle-E-**H^{7}**-A-I-R-**K^{11}**-L-A-E-R-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-Q-Nle-A-E-**H^{30}**-L-A-R-**H^{34}**-F-E-A-**K^{38}**-A-R-A-G^{42}-COOH

Table 3 A summary of the key amino acid residues in the polypeptides used for bridging experiments. For clarity, only the positions of the lysine and the histidine residues are reported

	Position									
Peptide	3	7	8	11	15	26	27	30	34	38
PE42A	—	H	—	H	K	—	K	H	H	—
PE42B	—	—	K	H	H	H	—	H	K	—
PE42Bcap	—	—	K	H	H	H	—	H	K	—
PE42Dcap	—	H	—	H	K	H	—	H	K	—
PE42Ecap	—	H	—	H	K	—	—	H	H	K
PE42Fcap	—	H	—	—	K	H	—	—	K	—
PE42Gcap	—	—	—	—	K	—	—	—	K	—
PE42Hcap	—	H	—	H	K	H	—	H	K	—
PE42Icap	—	H	—	H	K	—	—	—	K	—
PE42Kcap	—	—	—	H	K	—	—	H	K	—
PE42Lcap	—	—	—	—	K	H	—	H	K	—
TB42Acap	H	H	—	K	—	—	—	H	H	K
TB42Bcap	H	H	—	K	—	H	—	H	K	—

TB42Bcap

CH_3CO-N[1]-A-**H**[3]-D-Nle-E-**H**[7]-A-I-R-**K**[11]-L-A-E-R-Nle-A-A-R[19]-G[20]-P-V-D[23]-A[24]-A-**H**[26]-Nle-A-E-**H**[30]-L-A-R-**K**[34]-F-E-A-F-A-R-A-G[42]-COOH

PE42Ecap

CH_3CO-N[1]-A-A-R-Nle-E-**H**[7]-A-I-R-**H**[11]-L-A-E-**K**[15]-Nle-A-A-R[19]-G[20]-P-V-D[23]-A[24]-A-Q-Nle-A-E-**H**[30]-L-A-R-**H**[34]-F-R-A-**K**[38]-A-R-A-G[42]-COOH

PE42Dcap

CH_3CO-N[1]-A-A-R-Nle-E-**H**[7]-A-I-R-**H**[11]-L-A-E-**K**[15]-Nle-A-A-R[19]-G[20]-P-V-D[23]-A[24]-A-**H**[26]-Nle-A-E-**H**[30]-L-A-R-**K**[34]-F-R-A-F-A-R-A-G[42]-COOH

The key amino acid residues of the polypeptides used for bridging experiments are summarised in Table 3. The polypeptides were synthesised, purified and reacted with DSG or BS^3 in the same manner as PE42Dcap, and the bridging results were evaluated by analytical HPLC and mass spectrometry. The same HPLC gradient as for DSG-PE42Dcap was used to assure similar behaviour for all peptides and the collected fractions were identified by mass spectrometry, showing that the inherent elution order was not to change for the different peptides.

The reaction of PE42Dcap with DSG yielded the bridged peptide DSG-PE42Dcap as the major product (*ca.* 37%), and approximately 13% of the peptide was unmodified after the reaction. The corresponding reaction of PE42Dcap with BS^3 gave *ca.* 36% bridged peptide and no unmodified peptide. The reaction mixture of PE42Hcap and DSG produced *ca.* 31% bridged peptide and *ca.* 13% unmodified peptide. The presence of the bridged polypeptides was confirmed by mass spectrometry.

No bridged polypeptide could be observed in the reaction of PE42A with DSG, and the major component was the unmodified peptide PE42A as determined by analytical HPLC. The reaction of the polypeptide PE42B with DSG yielded a small fraction (*ca.* 8%) of two different bridged peptides, which is possibly due to the uncapped N-terminus of PE42B, but the major component was the unmodified peptide. The presence of bridged polypeptide was confirmed by mass spectrometry.

The N-terminus of the polypeptide PE42Bcap was capped by an acetyl group during solid phase peptide synthesis to prevent the reaction between DSG and the terminal amino group. The fraction of the bridged polypeptide DSG-PE42Bcap was estimated to be *ca.* 7% and the non-bridged polypeptide adducts were shown to be the major components in the reaction mixture of PE42Bcap and DSG. The presence of the bridged peptide was confirmed by mass spectrometry.

Comparison of the results from these experiments with the bridging result of PE42Dcap shows that it is not only important to use a protected N-terminal amino group during the bridging reaction, but also that the bridging reaction may not be as effective between a His(*i*)–Lys(*i* − *3*) pair as it is between a His(*i*)–Lys(*i* + *4*) pair.

The reaction of PE42Ecap with DSG yielded *ca.* 14% bridged peptide and the major component was found to be the unmodified peptide (*ca.* 25%). Analysis of the reaction mixture of TB42Acap and DSG showed *ca.* 9% bridged peptide and *ca.* 26% unmodified peptide. Finally, the reaction mixture of TB42Bcap and DSG contained *ca.* 16% bridged peptide and *ca.* 15% unmodified peptide. The formation of DSG-PE42Ecap and DSG-TB42Bcap was confirmed by mass spectrometry, but DSG-TB42Acap could not be observed in the mass spectrum, presumably due to the low fraction of the bridged species.

The polypeptides PE42Ecap, TB42Acap and TB42Bcap were also reacted with the longer bridging reagent BS^3 using the same reaction conditions. The reaction of BS^3 with PE42Ecap yielded *ca*. 18% of the bridged polypeptide, with TB42Acap *ca.* 11% bridged peptide and with TB42Bcap *ca.* 11% bridged peptide. The fractions of unmodified peptide in the reactions of the polypeptides with BS^3 were very low (≈1%). It was not possible to confirm the formation of BS^3-TB42Acap and BS^3-TB42Bcap by mass spectrometry, probably due to the low fraction of the bridged polypeptides.

The chromatographic results from these experiments indicate that the distance between the lysine residues and the length of the bridging reagent are important in the bridging reaction. The bridging reaction of the polypeptide PE42Ecap is more effective with the longer NHS reagent BS^3 than with DSG. The polypeptide TB42Bcap is more efficiently bridged with DSG than with BS^3. The bridging result for the polypeptide TB42Acap is almost the same for both NHS esters. This is in agreement with the distances between the lysine residues in the polypeptides PE42Ecap, TB42Acap and TB42Bcap.

Exploring the importance of the histidine residues

The polypeptide PE42Dcap has two histidine residues in each helix that can perform catalysis and assist in the incorporation of the bridge. In an attempt to elucidate the importance of the individual histidine residues in the bridging reaction, a number of new polypeptides were designed. In these polypeptides, two or four of the histidine residues were replaced by arginines in order to retain the solubility of the polypeptides after bridging. All the polypeptides used in this experiment had lysine residues in positions 15 and 34 (Fig. 7).

All four histidine residues in PE42Dcap were replaced by arginine residues to yield the polypeptide PE42Gcap. In the polypeptide PE42Fcap, the histidine residues in positions 11 and 30 of PE42Dcap were replaced by arginine residues. The design of the polypeptides PE42Icap, PE42Kcap and PE42Lcap were based on the amino acid sequence of the catalytically more active polypeptide PE42Hcap. Two of the histidine residues in PE42Hcap were replaced by arginine residues in each of the new polypeptides. The polypeptide PE42Icap has a His(*i* − *4*)–His(*i*)–Lys(*i* + *4*) triad in helix 1, but lacks histidine residues in helix 2. The polypeptide PE42Lcap has a His(*i* − *4*)–His(*i*)–Lys(*i* + *4*) triad in helix 2, but contains no histidine residues in helix 1. Finally, the polypeptide PE42Kcap has a His(*i*)–Lys(*i* + *4*) pair in each helix, but lacks additional flanking histidine residues.

PE42Fcap

CH_3CO-N[1]-A-A-R-Nle-E-**H**[7]-A-I-R-R-L-A-E-**K**[15]-Nle-A-A-R[19]-G[20]-P-V-D[23]-A[24]-A-**H**[26]-Nle-A-E-R-L-A-R-**K**[34]-F-R-A-F-A-R-A-G[42]-COOH

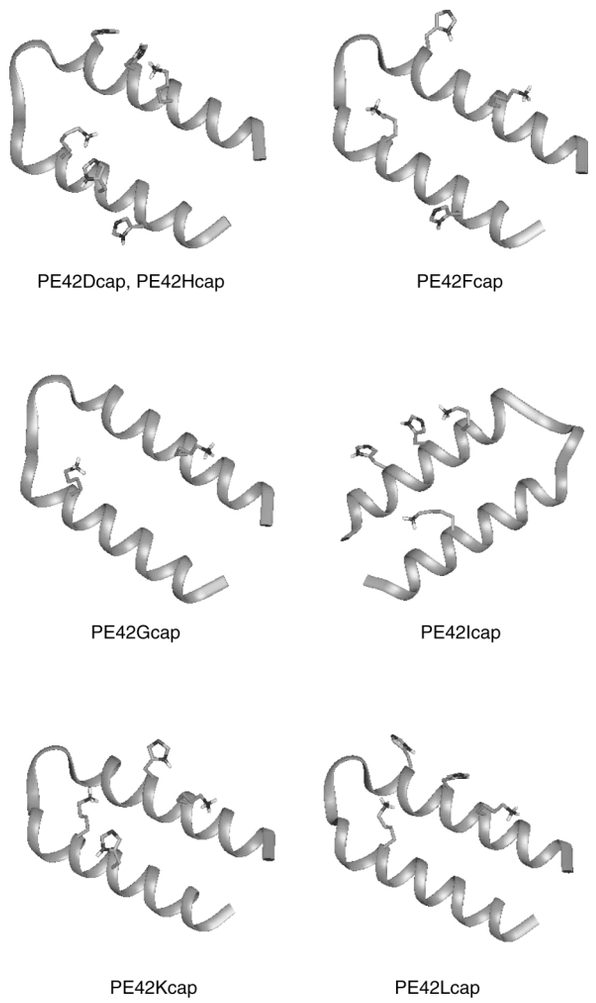


Fig. 7 The modelled folded structures of the polypeptides PE42Dcap/PE42Hcap, PE42Fcap, PE42Gcap, PE42Icap, PE42Kcap and PE42Lcap. For clarity, the polypeptides are displayed as monomers and only the side chains of the lysine and the histidine residues are shown.

PE42Gcap

CH_3CO-N^{1}-A-A-R-Nle-E-R-A-I-R-R-L-A-E-**K**^{15}-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-R-Nle-A-E-R-L-A-R-**K**^{34}-F-R-A-F-A-R-A-G^{42}-COOH

PE42Dcap

CH_3CO-N^{1}-A-A-R-Nle-E-**H**^{7}-A-I-R-**H**^{11}-L-A-E-**K**^{15}-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-**H**^{26}-Nle-A-E-**H**^{30}-L-A-R-**K**^{34}-F-R-A-F-A-R-A-G^{42}-COOH

PE42Icap

CH_3CO-N^{1}-A-A-D-Nle-E-**H**^{7}-A-I-R-**H**^{11}-L-A-R-**K**^{15}-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-R-Nle-A-R-R-L-A-R-**K**^{34}-F-E-A-F-A-R-A-G^{42}-COOH

PE42Kcap

CH_3CO-N^{1}-A-A-D-Nle-E-R-A-I-R-**H**^{11}-L-A-R-**K**^{15}-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-R-Nle-A-R-**H**^{30}-L-A-R-**K**^{34}-F-E-A-F-A-R-A-G^{42}-COOH

PE42Lcap

CH_3CO-N^{1}-A-A-D-Nle-E-R-A-I-R-R-L-A-R-**K**^{15}-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-**H**^{26}-Nle-A-R-**H**^{30}-L-A-R-**K**^{34}-F-E-A-F-A-R-A-G^{42}-COOH

Table 4 The fractions of bridged and unmodified polypeptide after reaction of the polypeptide with DSG at pH 4.1 in 5 vol% TFE

Peptide	Bridged peptide (%)	Unmodifed peptide (%)
PE42Fcap[a]	18	49
PE42Gcap[a]	21	40
PE42Icap	9	12
PE42Kcap	26	5
PE42Lcap	16	13
PE42Dcap	37	13

[a] The reaction time was approximately doubled for PE42Fcap and tripled for PE42Gcap to compensate for the lower activity of the polypeptides.

PE42Hcap

CH_3CO-N^{1}-A-A-D-Nle-E-**H**^{7}-A-I-R-**H**^{11}-L-A-R-**K**^{15}-Nle-A-A-R^{19}-G^{20}-P-V-D^{23}-A^{24}-A-**H**^{26}-Nle-A-R-**H**^{30}-L-A-R-**K**^{34}-F-E-A-F-A-R-A-G^{42}-COOH

The polypeptides were synthesised, purified and bridged in the usual way, and the key amino acid residues are summarised in Table 3.

The results from the bridging reactions of these polypeptides with DSG are summarised in Table 4. The experiments show that the bridging reaction can proceed even in the absence of flanking histidine residues. The polypeptides PE42Fcap and PE42Gcap lack histidine residues in position *i* relative to the lysine residues in position *i* + *4*, but the peptides are still bridged in the reaction with DSG after extended reaction times. This may be explained by the direct modification of the (unprotonated) lysine residues, which is a slow reaction in acidic media. The fraction of unprotonated lysine residues will increase with increasing pH, but so will the background reactions, leading to an increased yield of non-bridged adducts.

The bridging of the polypeptide PE42Kcap is more effective than the bridging of PE42Icap and PE42Lcap, indicating that the best bridging result is obtained when the lysine residue in position *i* + *4* is flanked by a histidine residue in position *i* in both helices. Comparison of the values for PE42Kcap, PE42Hcap and PE42Dcap shows that the bridging reaction can be further assisted by additional flanking histidine residues in position *i* + *4*. The reaction of the polypeptides PE42Icap and PE42Lcap yielded larger amounts of the non-bridged adducts (**B**, **2B**, **C**, **2C** and **B+C**) compared to the reactions of the polypeptides PE42Fcap and PE42Gcap. This result can be explained by the additional histidine residues in the former polypeptides. The lysine residue in position 15 of PE42Icap is easily modified by DSG using the histidine residue in position 11. The lysine residue in position 34 does not have a flanking histidine residue. To complete the bridging of the polypeptide, this lysine has to compete for the second NHS group of DSG with the background reactions, which are assisted by the histidine residues in helix 1. A similar situation is true for the polypeptide PE42Lcap. The polypeptide PE42Gcap lacks histidine residues and the lysine residues are now only competing with the unassisted background reactions.

Conclusions

The interhelically bridged polypeptide DSG-PE42Dcap has been shown to have an increased catalytic activity and a higher thermal stability than the precursor polypeptide PE42Dcap. The bridging of PE42Dcap with DSG has been optimised by varying the reaction conditions. Furthermore, the novel bridging reaction has been investigated using several designed polypeptides, showing that helical structures can be bridged by NHS esters with varying spacer length. We have also shown that bridging of helix–loop–helix polypeptide motifs is

most efficient using a histidine-catalysed reaction in acidic solution.

The bridging reaction may be a step towards *de novo* designed polypeptides with more pronounced cavities. It may be possible to introduce two bridges into the same helix–loop–helix polypeptide motif (using lysine residues in positions $i + 4$ and $i - 3$ relative to the histidine residues) or to introduce bridges between the two monomeric structures in the four-helix bundle (using bridging reagents with longer spacers). It may also be possible to introduce interhelical bridges with additional functional groups, which may be used for increased catalytic activity and/or stereoselectivity.

Experimental

Computer modelling, polypeptide synthesis, bridging, purification and identification were performed as previously described for PE42Dcap.[37] The bridging of polypeptides with DSG or BS^3 was performed for at least 72 hours. The crude product mixtures from the bridging reactions were lyophilised and analysed by analytical reversed phase HPLC, using an analytical C-8 HiCHROM Kromasil, 5 μm column and a flow rate of 0.8 ml min^{-1}. HPLC analysis was performed on a Varian 9012 Solvent Delivery System equipped with a Varian 9065 Polychrom®/PolyView™ Diode Array Detector and Varian LC Star Workstation software. A solvent gradient was used to separate the bridged polypeptide and the different non-bridged adducts, starting with 32 vol% propan-2-ol (IPA) in 0.1 vol% TFA in water. After five minutes, the IPA concentration was increased to 60 vol% during 30 minutes. The bridged polypeptide eluted as a separate peak prior to unmodified polypeptide and non-bridged adducts (Fig. 5).

Kinetics

The catalytic activity measurements were performed using Varian Cary 4 or Cary 5 Spectrophotometers, equipped with a Varian Temperature Controller and the Varian CARY Kinetics software. The absorbance of *p*-nitrophenol was recorded at 320 nm at constant temperature (290 or 298 K).

In a typical kinetic study, the lyophilised polypeptide was dissolved in buffer and pH was adjusted using 1 M NaOH and 1 M HCl in an appropriate fraction of TFE. The peptide solution (0.5 mM) was centrifuged and diluted by pipetting to 0.4 and 0.3 mM. The peptide solutions were centrifuged before 270 μl of each solution (0.5, 0.4 and 0.3 mM) were transferred to 1.0 mm cuvettes. The samples were equilibrated at the chosen temperature for at least 20 minutes before the substrate was added. The peptide concentration of the stock solution (0.5 mM) was determined by quantitative amino acid analysis.

The substrate mono(*p*-nitrophenyl) fumarate was weighed, dissolved to a concentration of 7.1 mM in 50 vol% MeCN in water and centrifuged. A volume of 5 μl substrate solution was transferred to each cuvette by pipetting.

The recorded kinetic data were evaluated by fitting the best single exponential curve to the experimental values using the Igor Pro Software. A linear regression analysis of the given pseudo-first-order rate constants *vs.* the peptide concentration yielded the second-order rate constant.

The kinetic measurements of the bridged polypeptides and mono(*p*-nitrophenyl) fumarate at pH 5.9 were performed using only one peptide concentration, due to shortage of the bridged polypeptides. The second order rate constant, k_2, was then calculated using

$$k_2 = \frac{k_{obs} - k_0}{[\text{peptide}]}$$

where k_{obs} is the observed pseudo-first-order rate constant for the peptide-catalysed reaction, and k_0 is the observed first-order rate constant for the reaction in the absence of peptide.

CD spectroscopy

CD spectra were recorded as previously described for PE42D-cap and DSG-PE42Dcap on a JASCO J-715 spectrometer.[37] The solution structure of all unbridged polypeptides was determined at pH 4.1 (see Table S1). The temperature-dependent CD spectra were recorded in 50 mM Bis-tris buffer in a water-jacketed 0.5 mm cell using a HETO thermostat. The samples were allowed to equilibrate at the chosen temperature for a minimum of 15 minutes before the measurement. The temperature-dependent CD spectra were not base-line corrected.

NMR spectroscopy and pK_a measurements

1D ^1H NMR spectra were recorded on a Varian Unity 400 MHz NMR Spectrophotometer equipped with a matrix shim system from Resonance Research Inc. The polypeptides were dissolved in 5 vol% [D_3] TFE in D_2O, centrifuged and transferred to the NMR tubes. The observed pH value of the deuterated solution (pH*) was adjusted with NaOD–D_2O (1:99) and DCl–D_2O (10:100) using a small diameter glass electrode located directly in the NMR tube. The pH meter was calibrated against buffer prior to each measurement. The read pH* values were used uncorrected under the usual assumption that the isotope effects cancel.[4] The spectra were recorded at 319 K and the samples were allowed to equilibrate for 15 minutes before each acquisition. The chemical shift scale was set relative to the signal of residual HDO. The chemical shifts of the ring protons of the histidine side chains were determined as a function of pH*. The histidine residues were assumed to titrate as simple monoprotic acids and independently of each other. The pK_a values were determined by fitting an equation describing the dissociation of a monoprotic acid to the plots of the chemical shifts of the histidine ring protons *versus* pH* using the Igor Pro software (see Fig. S5). The extreme data points were used since they provide the chemical shifts for the fully protonated and unprotonated species.

The TOCSY and the NOESY spectra were recorded on an INOVA 600 NMR spectrometer equipped with a 5 mm $^1H\{^{13}C,^{15}N\}$ triple resonance pulsed field gradient probe and Sun Ultra 1 workstation (Solaris 7) running Varian NMR software (Vnmr 6.1B). The lyophilised polypeptides were dissolved in H_2O–D_2O (10:90) to a concentration of approximately 1 mM. The pH was adjusted using NaOD and DCl in D_2O–H_2O (10:90) and 5 vol% [D_3] TFE was added using an autopipette. The polypeptide solutions were centrifuged and transferred to the NMR tubes. The tnNOESY and tnTOCSY experiments were performed at 308 K with 2 × 256 increments and 32 transients in each increment, using preirradiation of the water resonance. The NOESY mixing time was 200 ms and the TOCSY spin lock time was 80 ns.

Error estimation

The estimated error in the measured $[\theta]_{222}$ values is ±1000 deg cm^2 $dmol^{-1}$. If the concentration of the peptide solution is not determined by quantitative amino acid analysis, the estimated additional error is ±10%.

The estimated error in the second-order rate constants is ±10% for the constants that have been determined from three peptide concentrations using linear regression and ±15% for the second-order rate constants that have been determined from one peptide concentration.

The estimated error for the absolute yield of bridged polypeptide as determined by HPLC is ±10%, and the error in the relative yield is higher (approximately 20%, but it depends on the fraction of unmodified peptide).

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