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De novo design of a stable N-terminal helical foldamer†

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A peptide NTH-18 was synthesised in which a N-terminal helix is stabilised by two crossed disulfide bonds to a C-terminal extension. The design was inspired by the structure of the neurotoxic peptide apamin, which has previously been used to stabilise helices in miniature enzymes. CD- and NMR-spectroscopy indicated that NTH-18 adopted a fold similar to that found in apamin. However, the arrangement of the elements of secondary structures was inverted relative to apamin; a N-terminal α-helix was connected by a reverse turn to a C-terminal extension of non-canonical secondary structure. NTH-18 displayed significant stability to heat and changes of pH. The high definition of the N-terminal end of the α-helix of NTH-18 should make this peptide a useful vehicle to stabilise α-helices in proteins with applications in protein engineering and molecular recognition.

Introduction

The generation of functional miniature proteins in which amino acid residues are held in reactive positions through the incorporation into small peptides has recently received much attention. The methods used to generate the functional part of the miniature protein vary from rational design[1–14] to library based screening.[15–18] However, they all rely on stable structural motifs for the display of the functional groups. Much effort has been put into the design of stably folded α-helical miniature proteins. Several synthetic approaches have been developed for the stabilisation of α-helices such as metal binding,[19–22] lactam bridging,[23] introduction of non-proteinogenic amino acids[24] or hydrazone linkers,[25] but the most robust scaffolds are based on natural peptides such as the dimeric pancreatic polypeptides[2–4,6] and the neurotoxic peptide apamin.[26,27] The bee venom peptide apamin is a component of the honeybee toxin with the ability to cross the blood brain barrier.[28,29] This 18-residue peptide forms a remarkably stable three-dimensional structure comprising a nine residue C-terminal α-helix connected through a 3-residue reverse turn to an extended N-terminal region (Fig. 1).[30] The stability of this monomeric peptide relies on the formation of two disulfide bonds between two cysteines (residues 1 and 3) in the extended region and cysteines 11 and 15 in the α-helix. This crossed disulfide pattern confers high stability on apamin which undergoes only small conformational changes on exposure to 6 M guanidinium chloride, temperatures as high as 70 °C and pH values as low as 2 with the α-helix staying largely intact.[31,32] The elements of the sequence responsible for the exclusive formation of the crossed disulfide pattern observed in apamin seems to reside in the N-terminal extension.[33] Residues 9–18 of apamin can be changed, with the exception of the two cysteine residues, while retaining the folding geometry and the disulfide arrangements. An apamin mutant, in which Lys 4 was deleted, showed exclusively the parallel disulfide pattern found in the vasoconstrictor peptide endothelin.[33] The helical structure of this deletion mutant resembled that found in apamin rather than in endothelin.

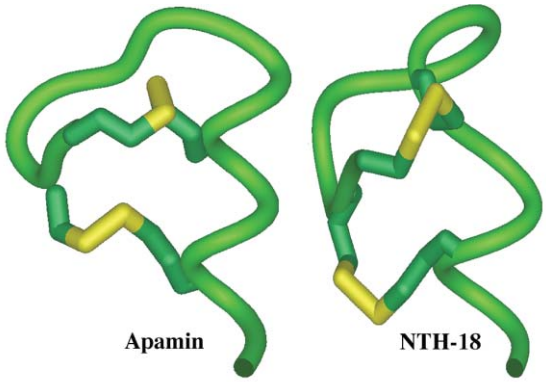

Fig. 1 Comparison of the averaged solution structures of apamin and NTH-18. The apamin structure is from its solution NMR structure,[30] while the structure of crossed NTH-18 was calculated from simulated annealing using NOE distance constraints. A sequence alignment of apamin, reversed apamin and the designed crossed NTH-18 is also indicated. The disulfide patterns are shown in yellow and the amino acid substitutions to convert reverse apamin to NTH-18 are shown in red.

The structural robustness together with its small size and high stability make apamin an attractive scaffold for the generation of miniature proteins. A hybrid between apamin and S-peptide of RNaseA complemented S-protein to generate an active ribonuclease,[27] while the introduction of three lysine residues into the solvent exposed face of apamin produced a miniature oxaloacetate decarboxylase of high stability against changes in pH, temperature and chemical denaturants.[26] The addition of an apamin-like peptide sequence to the N-terminal end of the DNA recognition helix of the basic helix–loop–helix domain of MyoD led to significant helix propagation and DNA binding specificity.[34] The hybrid protein was produced by conventional recombinant expression methods followed by air oxidation

† Electronic supplementary information (ESI) available: Validation of the modelling procedures and summary of conformational constraints and statistics of the NMR analysis of NTH-18. See DOI: 10.1039/b513891d. Coordinates for the structure of NTH-18 have been deposited in the Brookhaven Protein Data Bank.

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to produce the apamin-like extension. This disulfide-stabilised helix served as a nucleus for an α-helix that extended for a further ten residues leading to increased stability and specificity of the DNA complexes of the apamin stabilised protein. The DNA binding specificity, which was dependent on the redox state of the hybrid protein was increased 10-fold when compared to the bHLH domain of MyoD.

The availability of an apamin-like motif, in which a helix at the N-terminus is stabilised by two crossed disulfide bonds from a C-terminal extension, would be a useful tool with applications in molecular recognition and protein engineering. In particular, it would be possible to add the sequence for the motif to the C-terminus of a protein. This would enable helix stabilization of the C-terminus of a protein in a manner analogous to how apamin can be used at the N-terminal end[27,34] (indeed it might be possible to use both extensions at the same time). To date no such motif has been found to occur naturally.[35] The closest candidates are the long chain scorpion toxins such as AaH II where an α-helix near the N-terminus is stabilised through crossed disulfides with cysteines in an extended region.[36,37] However, this helix is preceded by a number of residues with a cysteine residue participating in a third disulfide bond. In addition, scorpion toxins are structurally much more complicated than apamin in that they contain a three-stranded anti-parallel β-sheet. Therefore the design of a peptide with the simplicity of apamin would provide a useful tool and test our ability to create *de novo* small peptides of defined structure.

We report here the design, synthesis and characterisation of NTH-18, a peptide of high stability in which a N-terminal helix is stabilised by two crossed disulfide bonds to a C-terminal extension. The structure of this N-terminal helical foldamer was solved by NMR-spectroscopy. CD spectroscopy showed that the helical structure remained stable at temperatures up to 95 °C.

Experimental

Computer modelling

The entire sequence of NTH-18 was built as an α-helix using the Sybyl 6.7 package (Tripos). The predicted helical residues were then heavily restrained during energy minimisation (100 kcal mol^{-1} $Å^{-2}$) to prevent unravelling during the extensive energy minimisation for the generation of a suitable starting structure. Distance restraints were applied to align the disulfides into the correct disulfide arrangement and to define the turn and loop region. The energy of the system was minimised using the AMBER package implementing the Amber force field and parm99 parameter set. A dual minimisation procedure was used. 150 steps of steepest descent minimisation to rapidly unfold residues not inherently helical and to reduce the worst steric clashes were followed by a further 2850 steps of conjugate gradient minimisation to refine and identify a low potential energy conformation. In a subsequent minimisation the distance restraints of the disulfides were removed and the disulfide bonds created. This minimisation was composed of 100 steps of steepest descent followed by 900 steps of conjugate gradient minimisation. The helical restraints were then removed and a final 1000 step minimisation generated a suitable starting structure, which was analysed to determine the presence or absence of helical residues. The starting structure was then subjected to simulated annealing to determine the stability of the helical residues with greater sampling of conformational space. The simulated annealing protocol was composed of a 1 ps rapid heat to 700 K followed by a 14 ps slow cool. At high temperatures the helical residues were restrained using harmonic restraints (25 kcal mol^{-1} $Å^{-2}$) but these were gradually reduced as the temperature was lowered until no restraints were applied. The resulting structure was re-minimised to generate a low potential energy structure and the helical content analysed with the program VMD. The procedure was validated using the NMR structure of tertiapin[38] and the X-ray structure of endothelin.[39]

The data base searching for protein sequences with similarity to NTH-18 was preformed at the Swiss Institute of Bioinformatics using the BLAST network service.[40]

Peptide synthesis

NTH-18 was synthesised using Pioneer Perceptive Biosystems or Applied Biosystems 433A automated peptide synthesisers and standard Fmoc protocols. The Fmoc and side-chain protected amino acids (Asn, Cys, Gln, His (trityl); Asp, Glu (OtBu); Phe, Ser, Tyr, Thr (tBu); Lys (*tert*-butyloxycarbonyl) and Arg (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)) were coupled to Fmoc-5-(4-aminomethyl-3,5-dimethoxyphenoxy) valeric acid on a polyethylene glycol support. Prior to cleavage and deprotection with 10 ml 2,2,2-trifluoroacetic acid (TFA) : water : phenol : triisopropylsilane (88 : 5 : 5 : 2; v/v) per gram of resin for two hours at room temperature, the peptide was acetylated with acetic anhydride (0.5 M), diisopropylethylamine (125 mM), *N*-hydroxybenzotriazole (15 mM) in *N*-methyl-pyrrolidone using 2-(1*H*-benzotriazole-1-yl)-1,3,3,3-tetramethyluronium hexafluorophosphate for activation. The resin was removed by filtration and the soluble products concentrated *in vacuo* and precipitated by washing with 3 × 5 ml of ice-cold diethyl ether. The peptide was dissolved in 50 ml of 10% acetic acid followed by lyophilisation. The peptide was then redissolved in 10 ml 0.05% TFA and purified by reversed-phase HPLC on a LUNA 10 μ C_{18} column (250 × 21.2 mm). The peptide eluted in 25% acetonitrile in water (0.05% TFA). Solvent was removed *in vacuo* followed by lyophilisation. NTH-18 was identified by ESI-TOF mass spectrometry. The experimentally determined mass was 1964.4, which agreed well with the theoretical mass of 1964.8.

Air oxidation

NTH-18 was dissolved in potassium phosphate buffer (pH 8) to a concentration of 0.5 mg ml^{-1} and stirred vigorously for two hours in the presence of 2 equivalents of TCEP to ensure that the cysteines were fully reduced. 10% (v/v) DMSO was added to facilitate oxidation and the solution left for 24 hours to allow complete oxidation, after which water was removed *in vacuo* with gentle heating. Two products were identified and separated by semi-preparative HPLC using a LUNA 10 μ C_{18} column (250 × 10 mm). HPLC analysis on an analytical LUNA 10 μ C_{18} column (250 × 4.6 mm) revealed the two isomers had been fully separated. MALDI-TOF-MS revealed a mass of 1960.0 for both peaks.

Orthogonal peptide synthesis

Orthogonally protected peptides were synthesised using trityl (Trt) and *S*-acetamidomethyl aminoacetal (Acm) protection for the thiol groups (crossed: Trt for Cys 4 and Cys 16, Acm for Cys 8 and Cys 18; parallel; Trt for Cys 8 and Cys 16, Acm for Cys 4 and Cys 18). Standard protecting groups were used for all other amino acids. After capping of the N-terminal amino group with acetic anhydride, the peptides were cleaved from the resin using 10 ml TFA : water : phenol : triisopropylsilane (88 : 5 : 5 : 2; v/v) per gram of resin for two hours at room temperature. This treatment removes the trityl groups, but leaves the Acm protected cysteines intact. The peptides were then precipitated and washed with ice-cold diethyl ether (3 × 15 ml). The first disulfide was formed by dissolving the peptide in potassium phosphate buffer (5 mM, pH 8) at a concentration of 0.5 mg ml^{-1}. Oxidation occurred overnight at room temperature. After concentration *in vacuo* with gentle heating, the peptides were purified by reversed-phase HPLC on a LUNA 10 μ C_{18} column (250 × 21.2 mm) with linear gradients of water (0.05% TFA) and acetonitrile (60%) in water (0.05% TFA) over 1 hour

with a flow rate of 5 ml min^{-1}. The Acm protecting groups were removed with silver triflate (10 peptide equivalents) in 5 ml TFA for 1 hour at 4 °C. TFA was removed *in vacuo* with gentle heating and the peptide precipitated and washed with ice-cold diethyl ether (3 × 15 ml). The peptides were purified by reversed-phase HPLC on a LUNA 10 μ C_{18} column (250 × 21.2 mm) with linear gradients from water containing 0.05% TFA to acetonitrile (30%) in water (0.05% TFA) over 1 hour with flow rates of 5 ml min^{-1}. The second disulfide bond was then formed by oxidation (*vide supra*) and the final product isolated by HPLC on a LUNA 10 μ C_{18} column (250 × 21.2 mm) with linear gradients of water (0.05% TFA) to acetonitrile (30%) in water (0.05% TFA) over 1 hour with flow rates of 5 ml min^{-1}. The identity of the products was confirmed by MALDI-TOF-MS, which revealed masses of 1960.4 and 1960.3 for the crossed and parallel versions, respectively, in good agreement with the theoretical mass of 1960.8.

Coinjection of air oxidised NTH-18 with orthogonally synthesised standards

HPLC analysis was performed on an analytical LUNA 10 μ C_{18} column (250 × 4.6 mm) with a linear gradient of acetonitrile (12%) in water (0.05% TFA) to acetonitrile (45%) in water over 40 minutes with a flow rate of 1 ml min^{-1}.

Circular dichroism spectroscopy

CD experiments were performed using a Jasco J-810 spectropolarimeter in 5 mm cells with an N_2 flow rate of 10 l min^{-1}. The temperature dependence of the CD-spectra of NTH-18 were studied with 10 μM NTH-18 between 10 and 95 °C (5 mM potassium phosphate, pH 7). pH experiments were performed with 10 μM NTH-18 in potassium phosphate (pH 7) or unbuffered (pH 2). All spectroscopic analyses were performed with HPLC-purified crossed and parallel NTH-18.

The α-helical content of peptides was estimated from the measured mean residue ellipticity at 222 nm and the predicted $[\theta]_{R,222}$ for 100% helix:

$[\theta]_{R,\,100\%\,helix} = -(40\,000(n-4))/n$.[26]

NMR Spectroscopy of crossed NTH-18

NMR spectroscopy was used to obtain almost complete assignments of pure crossed NTH-18 at pH 4.6. A 0.8 mM sample in 90% H_2O–10% of D_2O was used for all NMR experiments.

All NMR spectra were recorded on a 800 MHz Varian INOVA spectrometer using a 0.8 mM sample at pH 4.6 in a solution of 90% H_2O–10% of D_2O and 5 mM NaOAc. TOCSY and NOESY spectra were recorded with 2048 points in the fast dimension and 1024 points in the incremented dimension, both with excitation sculpting water suppression.[41] Buildup curves of NTH-18 at 30 °C for mixing times between 150 and 600 ms showed hardly any spin diffusion. The NOESY spectrum recorded at 30 °C with a mixing time of 600 ms gave the most comprehensive peak list and was therefore used for structure determination. In the TOCSY spectrum a DIPSI-2rc mixing sequence was used with a mixing time of 60 ms. At lower temperatures a similar NOESY spectrum could be obtained while the TOCSY spectrum showed only part of the resonances.

All data were processed in NMRLab[42] and ARIA[43] was used for automated NOESY assignment and structure determination. NOESY spectra were analysed using the CCPNMR analysis software.[44] NOESY signals were assigned and structures were simultaneously calculated using the ARIA software.[43]

The initial NOE table containing 164 cross peaks with multiple assignment possibilities was refined using ARIA in 8 iterations of iterative NOE assignment and structure calculations. In each iteration, 20 structures were calculated and the energetically best 7 models were used to interpret the NOEs in the following cycle. ARIA calculations and some manual assignments led to 96 unique and unambiguous NOE assignments and 12 ambiguous distance restraints.

Structures were calculated for the 2003 ARIA topology for disulfide dihedral angles using a composite cosine series with a large energy maximum corresponding to the C_β–S–S–C_β *cis* conformation and much smaller maxima at ±120°.[45] Non-specifically assigned prochiral groups were treated with the floating chirality approach.[46]

The NOE assignment table together with the list of rejected NOEs (due to the internal violation analysis scheme of ARIA) were manually checked and corrected. The resulting list of NOE distance restraints was subject to a final run of structure calculations. Finally 200 structures were calculated using the simulated annealing protocol of ARIA. The best 20 models were finally subject to simulated annealing.

The solution structure of apamin was mainly calculated using sequential NOEs, with only 13 mid-range and long-range NOEs identified. However, comparisons can be made about the overall fold.

HPLC-analysis after the completion of the NMR-experiments with crossed NTH-18 indicated that the peptide was stable and that no exchange between the two disulfide patterns had occurred.

Results and discussion

Design of NTH-18

The starting point for the design of NTH-18 was a simple reversal of the sequence of apamin (Fig. 1). Molecular modelling studies using standard simulated annealing procedures suggested that the α-helix of this peptide would be unstable.[47] Several amino acid replacements were therefore introduced into the sequence of reversed apamin. Cysteine residues were maintained in positions 4, 8, 16 and 18 (Fig. 1). This arrangement was observed in scorpion toxins[36,37] and should therefore allow the formation of the desired crossed pattern of disulfide bonds between cysteines 4 and 16 and 8 and 18, respectively. In order to remove the unfavourable effects from a positively charged amino terminus His 1 was changed to Asp and the N-terminus was acetylated. NMR-experiments had suggested that the three C-terminal amino acids of apamin did not adopt an α-helical conformation.[30] To ensure that the C-terminal end of the helix in NTH-18 would be stable, Thr 11 was replaced by a glycine to allow the formation of a reverse turn at the C-terminal end of the helix. Ala 10 was left in its reverse apamin position because of its high α-helical propensity[48] and Arg 9 was included to cap the C-terminal end of the helix. Positively charged residues at the C-terminal end of helices have been shown to stabilise the folded structure.[49] A leucine and an isoleucine residue were placed in positions 2 and 6 because of their high helical propensity.[48] The remaining residues (Arg-Arg-Ala) in the α-helix of reversed apamin were reorganised to avoid charge repulsion between positively charged side chains. The final NTH-18 sequence differed from reverse apamin in six out of the possible eight residues in the helical region that were not required to be cysteines. Molecular modelling by a combination of energy minimisations using the AMBER force field and simulated annealing procedures suggested that, unlike reverse apamin, the designed NTH-18 was stable (Fig. 2) (for details see Experimental). An extensive search of the protein data bank revealed no significant matches between the NTH-18 sequence and any known protein.

Solid phase peptide synthesis of NTH-18

NTH-18 was produced by solid phase peptide synthesis using fluorenylmethoxycarbonyl (Fmoc) chemistry. The peptide was purified by HPLC to greater than 98% purity and its identity confirmed by ESI-MS. The experimentally determined mass was 1964.4, which agreed well with the theoretical mass of 1963.8 for

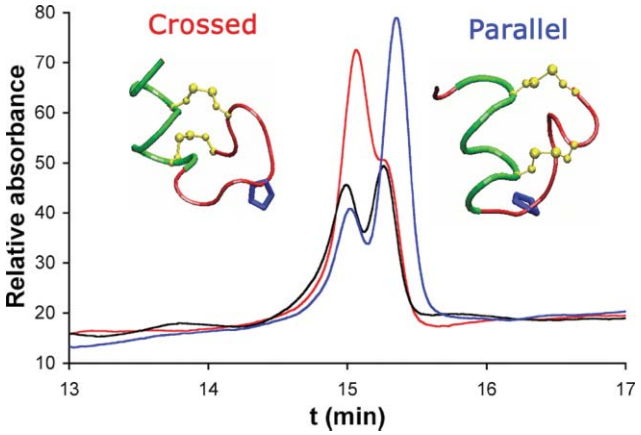

Fig. 2 HPLC traces of the unpurified "mixed" disulfide pattern (black) obtained from spontaneous air-oxidation of synthetic NTH-18 and co-injections with samples of "crossed" (red) and "parallel" (blue) NTH-18. Crossed and parallel NTH-18 were produced by polypeptide synthesis using an orthogonal protecting group strategy. The structures of crossed and parallel NTH-18 obtained from the molecular modelling are shown.

the fully reduced, protonated peptide. Air oxidation of NTH-18 in 10% DMSO at pH 8 yielded 2 products that could be separated by reversed phase HPLC (Fig. 2). MALDI-TOF MS revealed that both peptides had a molecular mass of 1960.0 suggesting that they were both oxidised forms of NTH-18.

Two versions of NTH-18, one with a crossed and the other with a parallel disulfide pattern, were synthesised on a solid support using an orthogonal protecting strategy (Fig. 3). To produce crossed NTH-18, the thiols of Cys 4 and 16 were protected with acid labile trityl groups, while *S*-acetamidomethyl aminothioacetal (Acm) protection was used for Cys 8 and 18. The trityl-groups were removed followed by air oxidation to establish the disulfide bond between Cys 4 and 16. Subsequently, the Acm groups were removed with silver triflate and the second disulfide between Cys 8 and 18 introduced. Similarly, parallel NTH-18 was produced by trityl-protection of Cys 4 and 16 and Acm-protection of Cys 8 and 18. Crossed and parallel NTH-18 displayed different retention times during reversed phase HPLC. Coinjections of the air-oxidised peptides with crossed and parallel NTH-18 indicated that the two products, which had been formed in approximately equal amounts on air oxidation of the fully reduced NTH-18, were indeed the crossed and parallel peptides (Fig. 2). This was in contrast to air oxidation of reduced

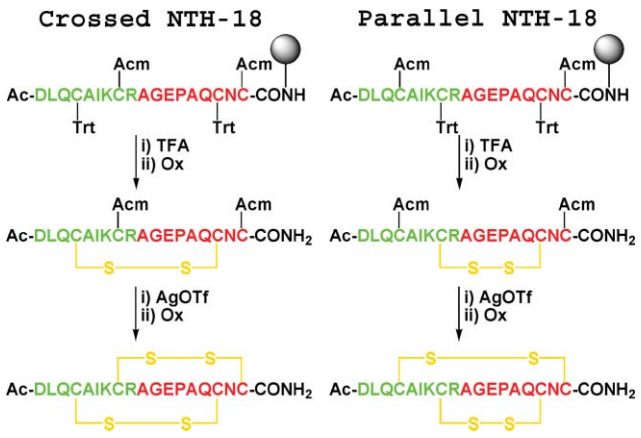

Fig. 3 Strategy to produce "crossed" (left) and "parallel" (right) NTH-18. Both peptides were synthesised on a solid support using standard Fmoc chemistry and cleaved from the resin and partially deprotected. After air oxidation to produce the first disulfide bond, the Acm protecting groups were removed with silver triflate. Air oxidation led to the formation of the second disulfide bond (for details see Experimental).

apamin, which produced fully biologically active peptide with only the crossed disulfide pattern.[50] Air oxidation of endothelin on the other hand, a peptide which naturally occurs with two parallel disulfide bonds, is known to generate a mixture of disulfide patterns on air oxidation.[51]

Characterisation of the secondary structure of crossed and parallel NTH-18 by CD-spectroscopy

The secondary structure of NTH-18 was characterised by circular dichroism spectroscopy. The CD-spectrum (Fig. 4) of HPLC-purified crossed NTH-18 showed a minimum at 206 nm with a mean residue ellipticity of $-20\,500 \pm 400$ deg cm^2 dmol^{-1} residue^{-1} and a shoulder at 222 nm characterised by a $[\theta]_{222,r}$ of $-13\,800 \pm 200$ deg cm^2 dmol^{-1} residue^{-1}. From the mean residue ellipticity at 222 nm the α-helical content was estimated to be 44% suggesting the presence of just over 2 helical turns. The estimated amount of α-helicity in crossed NTH-18 is therefore similar to that calculated for apamin from its NMR structure,[30,52] suggesting that the 2 peptides had similar secondary structures at least with respect to their helices.

The high stability of apamin and other cysteine rich motifs is known to be the result of their disulfide bonds.[53] Similarly, reduction of the disulfide bonds in crossed NTH-18 with

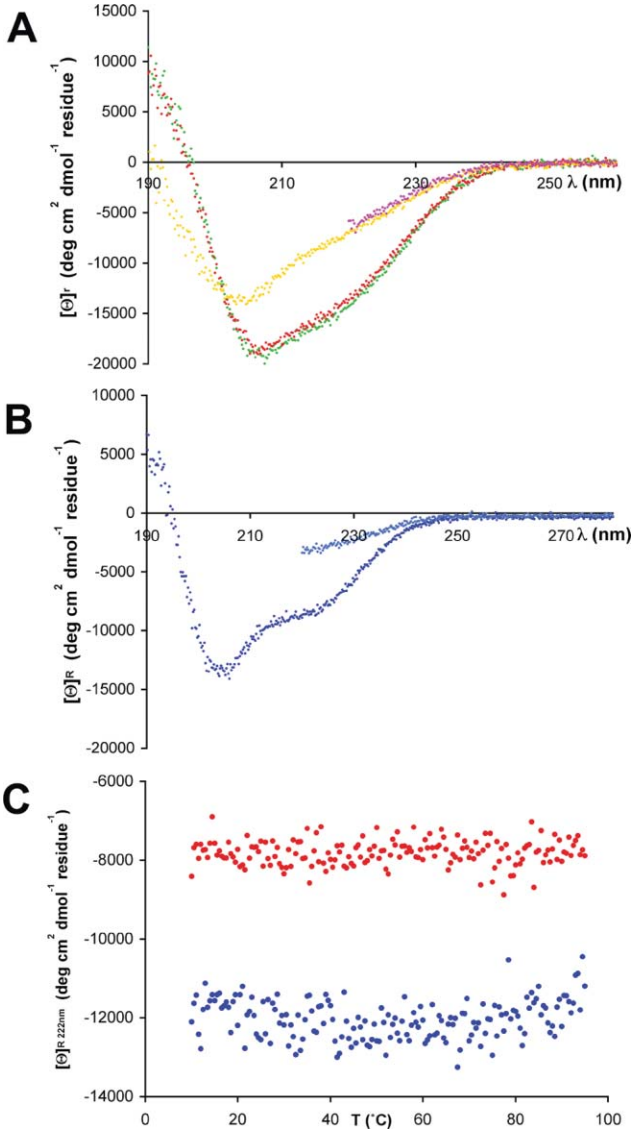

Fig. 4 CD-spectra (20 °C, 10 μM) of (**A**) crossed NTH-18 at pH 7 (red), pH 2 (green), in the presence of 50 mM TCEP (yellow) or 6 M guanidinium hydrochloride (pink), (**B**) parallel NTH-18 in the absence (blue) and presence (light blue) of 6 M G guanidinium chloride and, (**C**) the mean residue ellipticity at 222 nm, $[\theta]_{r,222}$, of crossed (red) and parallel (blue) NTH-18 is given as a function of the temperature.

tris-carboxyethyl phosphine (TCEP) led to a significant change in the CD-spectrum. The mean residue ellipticity at 222 nm was measured as −6800 deg cm^2 dmol^{-1} residue^{-1} but there was no hint of a minimum at that wavelength. The minimum at 206 nm observed for crossed NTH-18 was shifted to 203 nm. The structure of crossed NTH-18 therefore depended on the formation of the disulfide bonds. In their absence the peptide adopted a largely unfolded conformation.

Measuring the CD-spectra as a function of temperature indicated that the secondary structure of crossed NTH-18, like that of apamin, was remarkably stable. Crossed NTH-18 was highly resistant to thermal unfolding and only small changes in the CD-spectrum were observed between 10 and 80 °C (Fig. 4). Above 80 °C, the intensity of the CD-spectrum was reduced slightly without a major change in its shape. This change was gradual and might be the consequence of conformational change of crossed NTH-18 in its entirety rather than a loss of α-helicity. Crossed NTH-18 also showed significant resistance to changes in pH. The CD-spectra of crossed NTH-18 at pH 2 and pH 7 were identical suggesting identical secondary structures under both conditions (Fig. 4). In the presence of 6 M guanidinium hydrochloride the CD-spectrum of crossed NTH-18 resembled that of the peptide with parallel disulfide bonds. The reduction in the secondary structure of crossed NTH-18 in the presence of the denaturant suggested that, in the absence a hydrophobic core, the native form was stabilised by hydrogen bonding.

For parallel NTH-18 the CD-signal at 222 nm was only a shoulder of the minimum at 205 nm (Fig. 4). Such a spectrum has been postulated to be more consistent with a 3_{10}-type helix rather than a regular α-helix.[54] The thermal stability of parallel NTH-18 was similar to that of the peptide with the crossed disulfide pattern in that only minor changes of the CD-spectrum were observed between 5 and 80 °C. The resistance to chemical denaturants was however reduced when compared to that of crossed NTH-18.

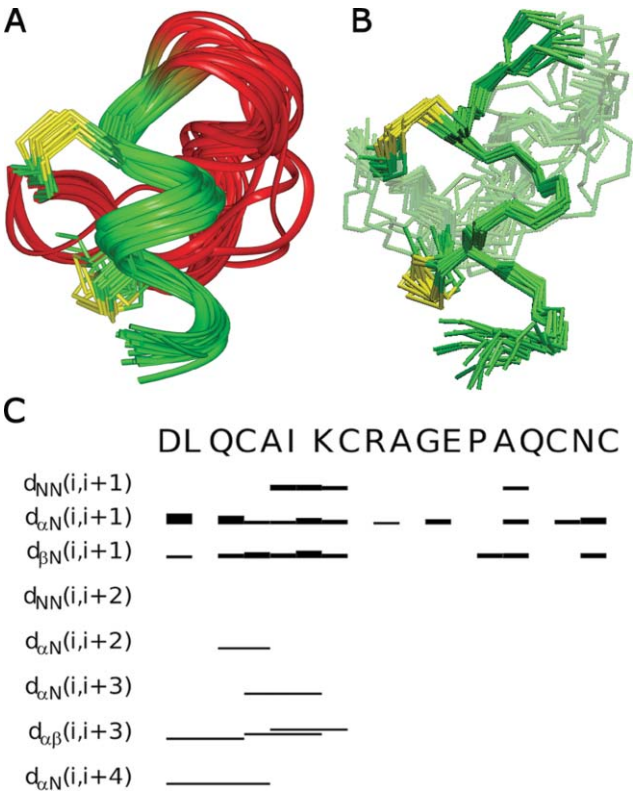

Fig. 5 The 20 lowest energy structures of NTH-18 as calculated in Aria from NOE distance constraints overlaid on all backbone heavy atoms (A) (RMSD 1.33 Å) and for the backbone heavy atoms of residues 3–8 (B) (RMSD 0.33 Å), highlighting the well defined helix within this region. C. Amino acid sequence of NTH-18 and a summary of all the short-range NOEs involving the NH, $C_\alpha H$ and $C_\beta H$. The strength of the NOEs is indicated by the thickness of the lines.

Solution structure of crossed NTH-18

The solution structure of crossed NTH-18 was determined by NMR spectroscopy using two-dimensional NOESY and TOCSY spectra. An almost complete set of resonance assignments for NTH-18 was obtained (Table 1).

The 20 best structures based on 96 uniquely and unambiguously assigned NOESY signals and 12 ambiguously assigned NOESY signals are shown in Fig. 5. The number of NOEs used in this structure determination was almost twice that of the original apamin structure,[30] which is probably a consequence of the higher magnetic field strength (800 *vs.* 500 MHz) combined with improved NMR instrumentation. NOESY derived distance constraints (Fig. 5) defined the α-helix extremely well between residues 3 and 8, where a RMSD of 0.13 Å was achieved for backbone atoms and 0.81 Å for all heavy atoms. The high definition of the α-helix was a consequence of the choice of amino acids and of the stabilisation provided by the disulfides from the C-terminal extension, since the reduced peptides did not show significant structure in the CD-spectrum (Fig. 4). Only slight fraying was observed for the two N-terminal residues of the helix. Fraying of the helix terminus had been observed for the three corresponding C-terminal residues in apamin.[30] The length of the α-helix was similar to that observed in apamin, where the NOE based distance constrains indicated an α-helix between residues 10 and 15.[30] The N-terminal end of the α-helix

Table 1 Assignment of ^{1}H-NMR resonances of NTH-18

Residue	NH	Hα	Hβ	Other
Asp-1	8.183	4.553	2.600, 2.706	
Leu-2	8.375	4.199	1.669	Qγ 1.669, QQδ 0.846, 0.905
Gln-3	8.380	4.194	2.109	Qγ 2.349
Cys-4	8.329	4.504	2.975, 3.061	
Ala-5	8.305	4.082	1.424	
Ile-6	7.540	3.790	1.905	Qγ 2 0.891, Hγ12 1.594, Hγ13 1.171, Qδ1 0.837
Lys-7	7.946	4.068	1.834, 1.987	Qγ 1.424, Hδ2 1.576, Hδ3 1.608
Cys-8	8.738	4.369	3.038, 3.067	
Arg-9	7.701	4.200	1.662, 1.732	Qγ 1.878, Qδ 3.185, Hε 7.169
Ala-10	7.793	4.341	1.423	
Gly-11	7.904	3.830, 4.050		
Glu-12	7.824	4.728	2.013, 1.827	Qγ 2.249
Pro-13		4.189	2.189, 2.193	Qγ 1.922, Hδ2 3.612, Hδ3 3.615
Ala-14	8.381	4.125	1.409	
Gln-15	8.521	4.341	2.213, 2.017	Hγ2 2.332, Hγ3 2.335
Cys-16	8.040	4.638	2.925, 2.909	
Asn-17	8.613	4.747	2.708, 2.901	Hδ21 6.815, Hδ22 7.539
Cys-18	8.578	4.591	2.790, 3.275	

in NTH-18 was however better defined than the C-terminus of the helix in apamin (Fig. 5) making NTH-18 an ideal vehicle for helix stabilisation in chimeric proteins.

A higher level of disorder was observed for all residues downstream of Arg 9 (Fig. 5). However, this region in itself was still relatively well structured yielding an overall RMSD of 1.33 Å for the entire peptide backbone. Whilst this value is comparable to the resolution obtained for the original apamin structure (1.34 Å),[30] the definition of the α-helix of NTH-18 was higher than in apamin. The loop regions in NTH-18 were however more flexible than had been observed for apamin.

The overall pseudoknot topology of the peptide is well defined by 10 long-range distance constraints. Several of those link the side chain of Cys 18 to that of Cys 8. There are also some additional links between the side chains of Cys 4 and 18. These two cysteines are not linked through disulfide bonds and the observed NOEs indicate a good definition of the relative orientation of the two disulfide bonds in NTH-18. The side chain protons of Cys 16 were relatively flexible resulting in only one broad signal for the two H^{β}s, whereas all other diastereotopic cysteine H^{β}s were resolved. This was reflected in a relatively poorly defined disulfide bond between Cys 4 and Cys 16. The side chain of Lys 7 showed NOE contacts to the side chains of Gln 15, Cys 16 and Asn 17 suggesting that this lysine might be an important link between the α-helix and the C-terminal loop region.

Conclusions

In summary, NTH-18, an apamin-like N-terminal helical foldamer, in which the α-helix, was stabilised through two disulfide bonds to a C-terminal extension, has been designed, synthesised and characterised. Its well-defined 3-dimensional fold and its high stability over a wide pH-range and for temperatures up to at least 80 °C were evidence of the structural robustness of these small disulfide stabilised peptides. The high definition of the N-terminal end of NTH-18 makes it an ideal vehicle for the stabilisation of α-helical structures in hybrid proteins thereby providing many opportunities for the examination of the relationship between structure and function of biologically active proteins.

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