The Role of Cystine Knots in Collagen Folding and Stability, Part I.[+] Conformational Properties of (Pro-Hyp-Gly)$_5$ and (Pro-(4*S*)-FPro-Gly)$_5$ Model Trimers with an Artificial Cystine Knot**

Dirk Barth, Hans-Jürgen Musiol, Markus Schütt, Stella Fiori, Alexander G. Milbradt, Christian Renner, and Luis Moroder*[a]

Abstract: In analogy to the cystine knots present in natural collagens, a simplified disulfide cross-link was used to analyse the conformational effects of a C-terminal artificial cystine knot on the folding of collagenous peptides consisting of solely (Pro-Hyp-Gly) repeating units. Assembly of the α chains into a heterotrimer by previously applied regioselective disulfide-bridging strategies failed because of the high tendency of (Pro-Hyp-Gly)$_5$ peptides to self-associate and form homotrimers. Only when side-chain-protected peptides were used, for example in the Hyp(*t*Bu) form, and a new protection scheme was adopted, selective interchain-disulfide cross-linking into the heterotrimer in organic solvents was successful. This unexpected strong effect of the conformational properties on the efficiency of well-established reactions was further supported by replacing the Hyp residues with (4*S*)-fluoroproline, which is known to destabilise triple-helical structures. With the related [Pro-(4*S*)-FPro-Gly]$_5$ peptides, assembly of the heterotrimer in aqueous solution proceeded in a satisfactory manner. Both the intermediates and the final fluorinated heterotrimer are fully unfolded in aqueous solution even at 4 °C. Conversely, the disulfide-crossbridged (Pro-Hyp-Gly)$_5$ heterotrimer forms a very stable triple helix. The observation that thermal unfolding leads to scrambling of the disulfide bridges was unexpected. Although NMR experiments support an extension of the triple helix into the cystine knot, thermolysis is not associated with the unfolding process. In fact, the unstructured fluorinated trimer undergoes an equally facile thermodegradation associated with the intrinsic tendency of unsymmetrical disulfides to disproportionate into symmetrical disulfides under favourable conditions. The experimental results obtained with the model peptides fully support the role of triple-helix nucleation and stabilisation by the artificial cystine knot as previously suggested for the natural cystine knots in collagens.

Keywords: collagen · cystine knot · peptides · protein models · triple helix

Introduction

In their biosynthesis, collagens are generally aligned in the correct register into homo- or heterotrimers by selective recognition/association of C-terminal non-collagenous globular domains. After nucleation of the triple helix, this propagates from the C- to the N-terminus in a zipper-like mode with the *cis/trans* isomerisation of Xaa-Pro imide bonds as the rate limiting step.[1] Oligomerisation and nucleation may also be provided at the N-terminus and folding then proceeds from the N- to the C-terminus.[2] Mature homo- and heterotrimeric collagens are often cross-linked by more or less complex cystine knots, and in the absence of such cystine knots or of the procollagen registration domains, in vitro refolding of collagen is slow and mismatched triple helices are formed.[3] Handling of natural collagens is difficult because of their instability and gelating properties, and related fragments obtained by enzymatic and/or chemical cleavage are thermally rather unstable. Therefore most information about structural and biophysical properties of collagens has been collected with synthetic model peptides.

[a] Prof. Dr. L. Moroder, Dipl. Chem. D. Barth, H.-J. Musiol, Dipl. Chem. M. Schütt, Dr. S. Fiori, Dipl. Biochem. A. G. Milbradt, Dr. C. Renner
Max-Planck-Institut für Biochemie
Am Klopferspitz 18 A, 82152 Martinsried (Germany)
Fax: (49) 89-8578-2847
E-mail: moroder@biochem.mpg.de

[+] For Part II see: D. Barth, O. Kyrieleis, S. Frank, C. Renner, L. Moroder, *Chem. Eur. J.* **2003**, *9*, 3703 – 3714, following paper.

[**] **Abbreviations:** standard abbreviations are used as recommended by the IUPAC-IUB commission on biochemical nomenclature and the ACS Style Guide. All amino acids are of L configuration. Additional abbreviations: CVFF, consistent valence force field; DAST, diethylaminosulfur trifluoride; DBU, 1,8-diazabicyclo[4.4.0]undec-7-ene; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DQF-COSY, double quantum filtered correlation spectroscopy; FPro, 4-fluoroproline; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methyl-methanaminium hexafluorophosphate *N*-oxide; HOBt 1-hydroxybenzotriazole; NEM, N-ethylmaleimide; NMP, *N*-methyl-pyrrolidinone; *o*-Npys, 3-nitro-2-pyridinesulfenyl; *p*-Npys, 5-nitro-2-pyridinesulfenyl; TFE, 2,2,2-trifluoroethanol; TFFH, fluoro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate; TOCSY, total correlation spectroscopy.

DOI: 10.1002/chem.200304917

Since the first studies of Heidemann and co-workers on N- and C-terminal homotrimerisation of collagen model peptides by 1,2,3-propane-tricarboxylic acid and Lys–Lys dipeptide templates, respectively,[4] the natural oligomerisation domains have been mimicked in various modes.[5] Among these, N-terminal homotrimerisations with rigid templates such as Kemp's triacid[6] or cyclotriveratrylene[7] proved to be of high efficacy. However, for correct registration and assembly of heterotrimeric collagen peptides containing functional sequence portions of natural collagens, alternative procedures were required. For this purpose in our preceding studies we made use of a simplified artificial C-terminal cystine knot and exploited regioselective cysteine pairing procedures to align the peptide chains in the desired raster.[8] Modelling and MD simulations served to optimally fit the cystine knot, without steric clashes, into the triple-helical fold.[8a] The synthetic heterotrimers contained native collagen sequences of low propensity for a triple-helix structure adjacent to the cystine knot and were, therefore, N-terminally extended with (Pro-Hyp-Gly)$_n$ triplets to stabilise the collagen fold. The trimers were found to assume the desired triple-helix structure and to fold at rates comparable to those reported for procollagens,[9] pN-collagen type III, collagen type III and related fragments,[1a, 10] thus suggesting that even the artificial cystine knot acts as effective nucleus for triple-helix formation.[8b, 11] In native collagens and related fragments this property has been assigned to the cystine knots.[10, 12] However, a detailed NMR structural analysis of a heterotrimer, which in terms of collagenase substrate specificity perfectly mimicked native collagen type I,[13] revealed rather unexpectedly that only the N-terminal (Pro-Hyp-Gly)$_5$ extensions formed the triple helix. The downstream native collagen sequence portions, disulfide-cross-linked at the C-terminus, were kept in proximity, but in an extended unordered conformation.[14] These structural properties would suggest a contribution of the cystine knot mainly in terms of entropy by converting folding of the trimer into a concentration-independent process and would point to a weak triple-helix nucleation/induction.

To further investigate this aspect, in the present study the two trimers shown in Figure 1 were synthesised and comparatively analysed in their conformational properties. Trimer **I** consists of only (Pro-Hyp-Gly) repeats as the most ideal sequence composition for stabilisation of the collagen triple helix,[15] and based on the results obtained with related homotrimers cross-linked by Kemp's triacid,[6] a chain length of five repeats was selected. In trimer **II** (4*R*)-hydroxyproline (Hyp) was replaced with (4*S*)-FPro, since significant adverse effects on the stability of the triple helix were expected that would allow better estimates of the contribution of the cystine knot. In fact, it has been reported that replacement of Hyp with (4*R*)-FPro in (Pro-Hyp-Gly)$_{10}$ leads to a remarkably enhanced triple-helix stability owing to stereoelectronic effects that favour the required *trans* conformation of the preceding peptide bond.[16] Conversely, substitution with (4*S*)-FPro in (Pro-Hyp-Gly)$_7$ prevents the triple-helical structure.[17] On the basis of a high-resolution X-ray structure analysis of (Gly-Pro-Hyp)$_{10}$ the effects of (4*R*)-FPro were also attributed to its preference for the *exo*-pucker.[18] This agrees with the results of a detailed NMR analysis of the model compounds Ac-(4*R*)-FPro-OMe and Ac-(4*S*)-FPro-OMe which clearly revealed a stabilisation of the *trans* and *cis* conformation, but also of the *exo*- and *endo*-pucker in the two diastereomers, respectively.[17, 19]

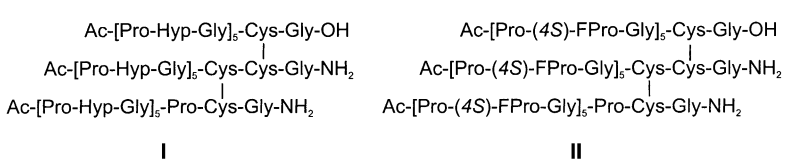


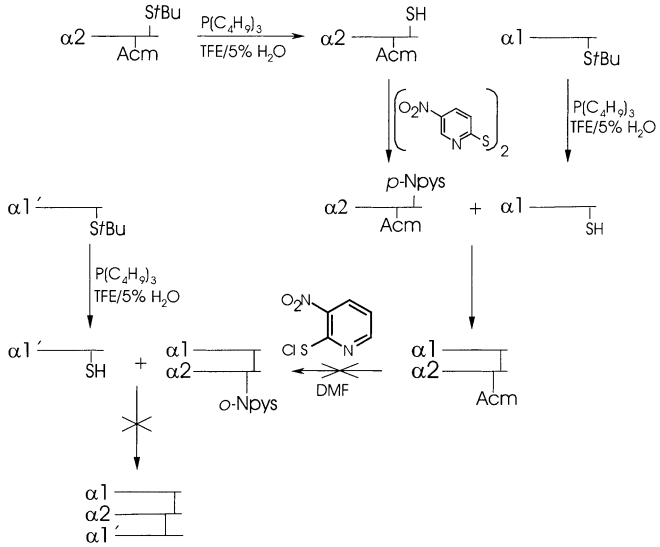
Figure 1. Synthetic heterotrimers containing (Pro-Hyp-Gly)$_5$ (trimer **I**) and (Pro-(4*S*)-FPro-Gly)$_5$ repeats (trimer **II**) and cross-linked C-terminally with a cystine knot designed to fit into a triple-helical collagen-like structure.

Results

Synthesis of the trimers

Synthesis of the *α* chains: In our previous syntheses of the (Pro-Hyp-Gly)$_n$ extensions of collagenous peptides, use was made of both hydroxy-protected and -unprotected (Pro-Hyp-Gly) tripeptide synthons.[8] Since such repetitive sequences result in products difficult to purify, even by HPLC, quantitative acylation steps and maximum suppression of piperazine-2,5-dione (diketopiperazine) formation is required. Thus, in chain elongation steps on resin by the standard HBTU/HOBt/DIEA (1:1:2) procedure, double couplings with a fourfold excess of the tripeptide synthons were employed making these syntheses rather expensive and time-consuming. In the present study the usefulness of TFFH[20] was investigated with an in situ conversion of the synthons into the related fluorides, which were expected to efficiently acylate N-terminal proline residues. In fact, double couplings with an 1.8- and 0.8-fold excess of tripeptides were sufficient for quantitative acylation as monitored by the chloranil test.[21] To suppress the troublesome diketopiperazine formation in the Fmoc cleavage step, a mixture of 2% DBU and 2% piperidine in DMF at 0 °C was used. The reaction time was shortened and the synthesis substantially improved. This deprotection protocol, combined with the fast and efficient fluoride-mediated acylation, led to significant suppression of diketopiperazine-derived deletion sequences.

Moreover, "inverse" capping with Boc_2O was applied after each acylation step in the case of the *N*-acetylated *α* chains used for assembly of the trimers according to Scheme 1 as well as for the synthesis of the *N*-acetylated *α* chains containing (4*S*)-FPro. By this mode of capping, upon TFA-mediated resin-cleavage/deprotection, truncated sequences as side products are unprotected at the N-termini, thus facilitating chromatographic purification of the target products. To prevent side reactions at C-terminal cysteine residues linked directly to the resin,[22] the *α* chains were extended at the C-termini with a glycine residue, and to suppress racemisation, coupling of the side-chain-protected Fmoc-cys-



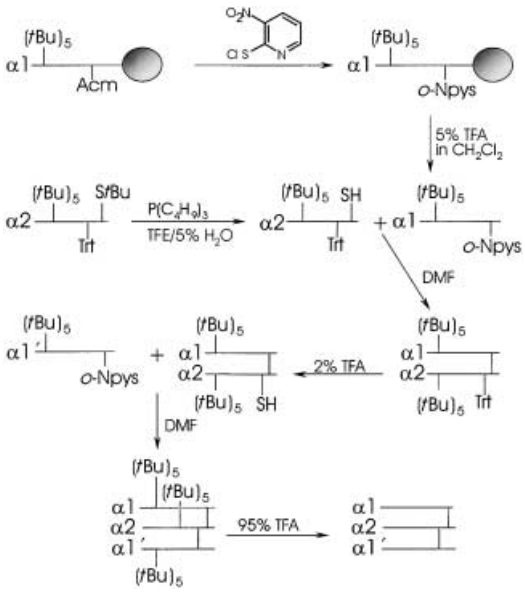
Scheme 1. Attempted assembly of α chains with unprotected Hyp residues into trimer **I** by thiol-disulfide exchange reactions as used in previous studies.[8]

teines was performed with the related pentafluorophenyl esters or, in the case of chlorotrityl resin, with HBTU in the presence of lower amounts of auxiliary base, as previously suggested.[23] It is noteworthy that oxidation of the Cys(S*t*Bu) disulfide to sulfenic acid thiol ester by traces of peroxides was observed even when using methyl *tert*-butyl ether as solvent. These sulfoxides are then reduced in the phosphine-mediated reductive cleavage of the S*t*Bu group yielding the desired cysteine peptides.

Assembly of the heterotrimer I according to Scheme 1: In previous syntheses we have assembled various collagenous heterotrimers by the strategy outlined in Scheme 1. This route allows purification of the α chains protected only at the cysteine residues and foresees activation of selected thiol functions as *o*-Npys or *p*-Npys derivatives with subsequent thiol/disulfide exchange reactions performed in aqueous solutions under slightly acidic conditions.[24] While the assembly of heterotrimers containing the collagenase cleavage site of collagen type I proceeded successfully according to this strategy,[8a–c] in the synthesis of heterotrimers containing the integrin $\alpha1\beta1$ recognition epitope of collagen type IV difficulties were encountered.[8d] These were attributed to the high tendency of the monomeric and dimeric species to self-associate into triple-helical homotrimers in aqueous solution.[25] Only with the proper selection of the α chain to be activated at the thiol function and paying particular attention to the kinetic control of the *o*-Npys derivatisation of the dimers, could the desired trimers be prepared in satisfactory yields and homogeneity.[8d]

The peptide Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ is known to fold into a triple helix in water with a T_m of 18 °C at 0.14 mM concentration.[6b] Consequently, at room temperature the α chains of trimer **I** were expected to be only partly folded (see below). However, reaction of the $\alpha2$ chain, upon reductive cleavage of the S*t*Bu group, with 2,2′-dithiobis(5-nitropyridine)[26] in DMF/AcOH (95:5) to produce, under exclusion of oxygen, the S-activated species, is accompanied by formation of homodimers even when the thiol component is added very slowly to an excess of disulfide reagent. These findings may reasonably be explained by assuming a triple-helical conformation of the $\alpha2$ chain in the reaction medium which leads to a high local concentration of free thiols and thus to reaction with the *S*-*p*-Npys-activated chain to form homodimers at rates competing with the activation reaction. Nonetheless, the desired *S*-*p*-Npys-derivatised $\alpha2$ chain was readily separated from the homodimer species and then reacted according to Scheme 1 with the $\alpha1$ chain to produce the desired heterodimer. However, displacement of the Acm group from the dimer with *o*-Npys-Cl or even with the more reactive *p*-Npys-Cl in slightly acidic aqueous solution to produce the S-activated dimer was found to generate mixtures of products under all the examined conditions. A hindered access of the *S*-Acm thioether has, kinetically, to favour the attack of the sulfenyl chloride on the preformed disulfide which leads to scrambling of the dimer as already observed in our previous studies.[25] Similarly, the subsequent reaction of the purified S-activated dimer with the $\alpha1'$ chain led to mixtures of products which can only be explained in terms of strong self-aggregation phenomena.

Assembly of the heterotrimer I according to Scheme 2: To bypass the difficulties encountered in the synthesis of the trimer **I** following Scheme 1, suppression of self-association of the intermediates into triple helices and/or aggregates was the



Scheme 2. Synthetic route for the assembly of [Pro-Hyp(*t*Bu)-Gly]$_5$- and [Pro-(4*S*)-FPro-Gly]$_5$-containing α chains into trimers **I** and **II**, respectively.

essential premise. To achieve this, the use of *O*-*tert*-butyl-protected hydroxyproline residues in the α chains was examined, since this bulky protecting group was expected to prevent the triple-helical conformation as well as aggregation. For this purpose the single α chains were synthesised on chlorotrityl resin to allow their recovery from the resin in the side-chain-protected form. Attempts to employ the S*t*Bu group for cysteine protection and to cleave this group on resin using phosphines for subsequent conversion of the cysteine residues into the *S*-*p*-Npys derivatives with 2,2′-dithiobis(5-

nitropyridine) failed. Therefore, Cys(Acm) derivatives were used and the $\alpha 1$ and $\alpha 1'$ chains were converted on resin into the $\alpha 1$(*o*-Npys)[I] and $\alpha 1'$(*o*-Npys)[I] compounds by reaction with *o*-Npys-Cl (Scheme 2). Upon mild acid cleavage from the resin, the fully protected peptides were isolated by HPLC. Moreover, as shown by comparing Schemes 1 and 2, even the combination of thiol protecting groups had to be changed to exclude the reaction of *o*-Npys-Cl with the dimer. This crucial intermediate was assembled in DMF, despite the slow thiol/disulfide exchange reaction in aprotic media. Upon acid cleavage of the trityl group from the $\alpha 1\alpha 2$(Trt)[I] dimer, its reaction with the S-activated $\alpha 1'$ chain in DMF under exclusion of oxygen was again found to proceed at low rates. The side-chain protected trimer could, however, be isolated in satisfactory yields by size-exclusion chromatography. The CD spectra of this side-chain-protected trimer **I** derivative in aqueous solution at 4 °C confirmed the absence of triple-helical structure and thus, the strong effect of the *O-tert*-butyl groups on conformational preferences of the related peptides (see below).

Among the various cocktails analysed for the final deprotection step, TFA/Et_3SiH/H_2O (89:10:1) proved to be the most efficient in terms of quantitative cleavage of the *tert*-butyl ether groups without affecting the cystine knot. In fact, the target trimer **I** was obtained directly as an analytically well-defined product.

Synthesis of the heterotrimer II: For the synthesis of the Fmoc-Pro-(4*S*)-FPro-Gly-OH as intermediate, the transformation of (4*R*)-hydroxyproline into (4*S*)-fluoroproline by reaction with DAST[27] was exploited at the level of Z-Pro-Hyp-OH. The aim was to convert simultaneously the C-terminal carboxy group into the fluoride for subsequent coupling with glycine. A combination of thiol protecting groups (as shown in Scheme 2, was selected for the synthesis of trimer **II**. Correspondingly, the $\alpha 1$ and $\alpha 1'$ chains were converted on resin into the related *S-o*-Npys derivatives. Since CD spectra of the single monomeric chains clearly revealed the absence of the triple-helical conformation (data not shown), the thiol/disulfide exchange reactions were carried out according to Scheme 2, but in slightly acidic aqueous solutions. As expected, the reactions were found to proceed at high rates in this medium and generated the desired products without particular difficulties. The absence of self-association of the (4*S*)-FPro-containing peptides and thus, the straightforward assembly of the α chains into the trimers confirmed the strong correlation between propensity for triple helices and synthetic difficulties encountered in the assembly of the trimer **I**.

Conformational characterisation of the collagenous peptides

Circular dichroic properties of the α chains: The CD spectra of the N-acetylated and C-amidated α chains with five (Pro-Hyp-Gly) repeats in water at 4 °C exhibit a positive maximum at 225 nm of significant intensity. The negative maximum at about 200 nm as expected for a triple-helical conformation, and correspondingly the Rpn (see footnote in Table 1 for an explanation) could not be determined because of the high peptide concentration (1 mM) required for self-association of collagenous peptides. However, the related thermal denaturation profiles confirm a triple-helical structure at least to some extent, for example, the T_m of the cysteine-protected $\alpha 2$ chain is 15.6 °C (Table 1), a value which compares well with that previously reported for an analogous (Gly-Pro-Hyp)$_5$ peptide ($T_m = 18$ °C).[6b] Similarly, the absence of ordered structure for the (4*S*)-FPro containing chains at 4 °C was expected from the conformational properties reported for [Pro-(4*S*)-Fpro-Gly]$_7$.[17]

Table 1. Circular dichroic parameters of the collagen peptides as derived from CD spectra recorded at 4 °C after 12 h preequilibration at this temperature under different conditions. The thermal denaturation curves were monitored at 225 nm with a heating rate of 0.2 °C min^{-1}.

Collagen peptides	Solvent	λ_{max-} [Θ_R]	λ_{max+} [Θ_R]	Rpn[a]	T_m [°C]
$\alpha 2$(Acm,S*t*Bu)[I][b]	H_2O	n.d.	224.9 (3113)	n.d.	15.6
$\alpha 1\alpha 2\alpha 1'$(*t*Bu)[I][c]	H_2O	200.4 (−16264)	225.4 (1407)	0.086	n.d.
trimer **I**[c]	H_2O	198.2 (−29120)	225.9 (4137)	0.142	56.7
	pH 7.2[d]	197.5 (−34385)	224.1 (3680)	0.107	56.3
	pH 3.0[e]	198.4 (−24148)	224.7 (3333)	0.138	56.3
trimer **II**[c]	H_2O	199.6 (−14885)	–	–	–

[a] Rpn is defined as the absolute value of the ratio between the CD intensities of the positive band over that of the negative band and is considered as the index of triple helicity.[30] [b] 1×10^{-3} M. [c] 4×10^{-5} M. [d] 20 mM phosphate buffer (pH 7.2) containing 20 mM NaCl, 0.2 mM EDTA and 0.2 mM NEM. [e] 20 mM phosphate buffer (pH 3.0).

Conformational properties of trimer I: As expected from the synthetic accessibility of trimer **I** in the side-chain protected form, its CD spectra in aqueous solution clearly reveals a significantly reduced propensity for triple-helical conformation compared to the unprotected trimer (Table 1). Replacement of Hyp residues by Hyp(*t*Bu) in a structural model of the folded trimer **I** revealed no steric clashes with neighbouring side chains, but shows that the *tert*-butyl group exposes a large hydrophobic area to the solvent. The entropic costs associated with triple-helix formation may, therefore, disfavour the ordered structure. It has been also reported that *O*-acetylation of (Pro-Hyp-Gly)$_{10}$ leads to a decrease of its T_m value from 58 to 25 °C.[28]

Upon removal of the side-chain protecting group, the resulting trimer **I** folds into the collagen-type structure, as determined by its circular dichroic properties. The C-terminal interchain disulfide bridging leads to a significant stabilisation of the triple helix, which is reflected by the high Rpn value of 0.142 and the strong increase of the T_m to ~56 °C (Table 1). Because of the N-terminal acetylation of the three α-chains, significant pH and ion strength effects on triple-helix stability, as reported for non-capped collagen peptides,[29] are not observed (see the CD parameters listed in Table 1).

In molecular dynamics simulations of the heterotrimer **I** a triple-helical conformation is observed not only for the (Pro-Hyp-Gly) repeats, but also for the cystine knot. Only the last glycine of each strand shows marked deviations from the typical dihedral angles of a triple helix. However, two of these C-terminal glycines are also involved in the hydrogen bonds expected for a triple-helical conformation. The third glycine occupies a Y position (Figure 1) and, therefore, is not expected to form a hydrogen bond. The same argument is

valid for the Cys residues that occupy X and Y positions, and consequently, in the computer model, they expose their amide hydrogen atoms to the solvent. Consistent with these findings is the experimental temperature dependence of NMR chemical shifts that show no involvement in hydrogen bonds for the Cys residues. Conversely, for all glycines of the (Pro-Hyp-Gly) repeats and, additionally, one of the C-terminal glycines, hydrogen bonds are indicated by NMR temperature shifts all more positive than $-4.5\ \mathrm{ppb\,K^{-1}}$ (Figure 2).

Conformational properties of trimer II: In contrast to the well-defined structure of trimer **I** no conformational preferences could be derived from the CD properties of trimer **II** containing the [Pro-(4*S*)-FPro-Gly] repeats (Table 1). The CD spectrum with only a negative band centred at 200 nm is fully consistent with a random coil structure. This is further supported by the 1D ^{1}H NMR spectrum of trimer **II** at 10 °C which resembles the NMR spectrum of trimer **I** at high temperature (75 °C) in the fully unfolded state. As shown in Figure 2, for trimer **II** no signal is observed at the chemical shift typical for triple-helical glycines ($\delta \approx 7.7$ ppm). In contrast, most glycine amide protons resonate at $\delta \approx 8$ ppm indicative of random coil. The possible existence of either poly-(Pro)-I or poly-(Pro)-II helices is excluded by the fact that for Gly-Pro and the Pro-(4*S*)-FPro imide bonds both *cis* and *trans* conformations are observed by the NMR spectrum in ratios that are close to those of Pro and (4*S*)-FPro in model compounds.[19a] A broadening of the Cys signals that increases at lower temperatures suggests a slow conformational exchange between different arrangements of the constrained cystine knot. Surprisingly, temperature shifts indicative of protection of the amide from the solvent are observed for two glycine amides and one C-terminal NH_2 proton. A comparison with the spectra of trimer **I** allows a tentative assignment of these glycines as the C-terminal ones. However, H/D exchange experiments did not identify any amide proton of trimer **II** as protected in a stable hydrogen bond. Thus, some conformational preference might be present in the C-terminal part of the cystine knot, without, however, any relevance to the [Pro-(4*S*)-FPro-Gly]$_5$ extensions. A close association of the single chains without formation of a stable structure is likely, but cannot be proven by NMR analysis.

Thermal stability of trimer I

By comparing the thermal denaturation of the self-associated $\alpha 2$ chain and of trimer **I** in water, a large shift of the T_m value from 15.6 to 56.7 °C (Figure 3) is observed. The effect of the cystine knot is lower than that of Kemp's triacid as N-terminal scaffold in KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH_2]$_3$ ($T_m = 70-72$ °C in H_2O[6b] and 62 °C in 10 mM AcOH[7]), but compares well with the triple-helix stabilisation by cyclotriveratrylene in (+)CTV-[Gly-Gly-(Pro-Hyp-Gly)$_5$-NH_2]$_3$ ($T_m = 58$ °C in 10 mM AcOH) and (−)CTV-[Gly-Gly-(Pro-Hyp-Gly)$_5$-NH_2]$_3$ ($T_m = 55$ °C in 10 mM AcOH).[7] In contrast to thermal denaturation of the self-associated $\alpha 2$ chain, the trimer **I** unfolds in a highly cooperative manner as indicated by the sharp two-state transition curve (Figure 3). Rather unexpectedly, however, the thermal refolding trace of trimer **I** in water

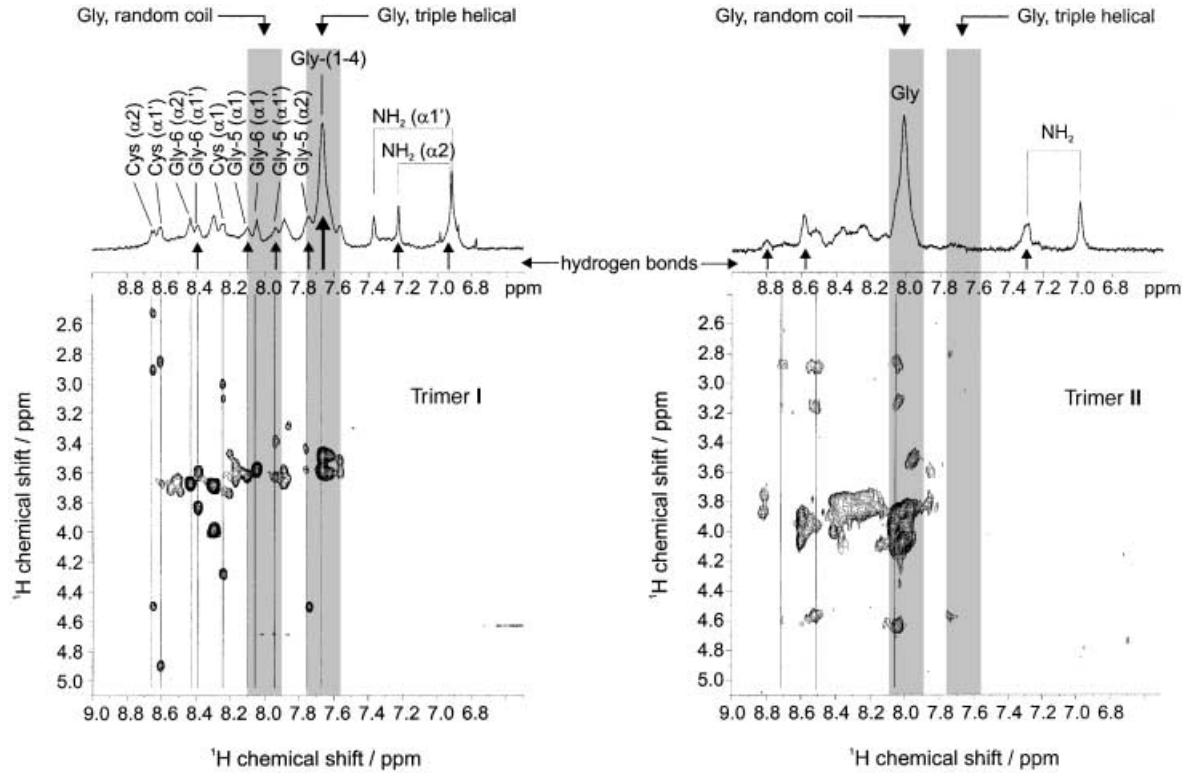


Figure 2. Fingerprint-regions (amide/aliphatic) of the 2D-TOCSY-NMR spectra for trimer **I** (left) and trimer **II** (right) recorded in water at 10 °C. Vertical lines indicate spin systems. On top the corresponding sections of the 1D ^{1}H spectra with partial assignments are given. Vertical arrows below the 1D spectrum mark amide resonances that exhibit a temperature shift indicative of hydrogen bonding ($> -4.5\ \mathrm{ppb\,K^{-1}}$).

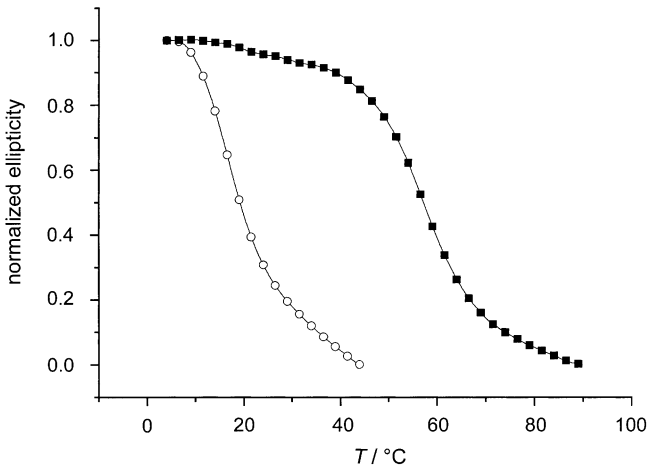
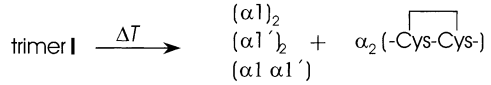


Figure 3. Thermal denaturation of the of the self-associated $\alpha 2$(Acm, S*t*Bu)[I] chain (○) and of the trimer **I** (■) as monitored by CD at 225 nm in water at 1×10^{-3} M and 4×10^{-5} M concentration, respectively.

is reminiscent of the unfolding curve of the $\alpha 2$ chain with a shift of the T_m value to about 20 °C. Mass spectrometric analysis of the sample upon thermal unfolding clearly reveals irreversible denaturation by scrambling of the disulfides into the $\alpha 1\alpha 1$ and $\alpha 1'\alpha 1'$ homodimers, the $\alpha 1\alpha 1'$ heterodimer as well as the intra-chain-disulfide-linked $\alpha 2$ chain which contains two adjacent cysteine residues (Scheme 3). Although



Scheme 3. Thermal decomposition of trimer **I** into the four components which were identified by LC-ESI-MS.

formation of the $\alpha 2$ homodimer cannot be unambiguously excluded from the mass spectral data, all facts support the generation of the rather unusual eight-membered cyclic structure. Intramolecular disulfide-linked adjacent cysteine residues are very rare in peptides and proteins.[31] Typical examples are the bicyclic peptide malformin with disulfide-linked D-Cys – D-Cys[32] and the nicotinic acetylcholine receptors with oxidised L-Cys – L-Cys where this cyclic motif is crucial for ligand binding.[33] X-ray structure analysis of the oxidised L-Cys – L-Cys dipeptide revealed a *cis* conformation of the peptide bond,[34] suggesting a *trans/cis* isomerisation as a requirement for intramolecular oxidation. However, more recent detailed NMR studies of a related model peptide in aqueous solution clearly indicated a preference of the *trans* over the *cis* conformation.[35]

Thermodegradation has recently been reported for the natural cystine knot of collagen type III when H-Gly-Ser-(Gly-Pro-Pro)$_{11}$-Gly-Pro-Cys-Cys-(Gly)$_3$-OH, oxidised to the related trimer, was thermally unfolded.[36] The pathways and mechanisms of irreversible thermal denaturation/inactivation of proteins are still obscure, primarily because of severe conceptual and experimental problems encountered in their investigation. It is, however, well established that in addition to deamidation of Asn and/or Gln residues and scission of labile peptide bonds, the major process is represented by disulfide scrambling as a result of thiol-catalysed intramolecular interchanges of disulfide bonds and by β elimination at cystine residues with formation of dehydroalanine as new reactive species.[37] The thiols required for initiation of the intra- and intermolecular interchange of disulfides are generated in the β elimination at cystine residues.[37, 38]

Although these thiols, if formed, should be trapped with NEM, thus preventing the intermolecular thiol/disulfide exchange processes, thermal decomposition of trimer **I** was not relevantly affected in the presence of 0.2 M NEM in phosphate buffer (pH 7.2). Addition of $CuCl_2$ which was shown to efficiently suppress irreversible denaturation of RNase A,[37b] led, at pH 7.2, to large precipitation of $Cu(OH)_2$. However, operating at pH 3.0 in the presence of 1 mM $CuCl_2$, thermodegradation of the cystine knot was fully prevented. At this acidic pH, even in the absence of $CuCl_2$, scrambling of the disulfides was not observed and the thermal unfolding process was fully reversible as evidenced by superposition of the related transition curves (Figure 4). The thermodynamic

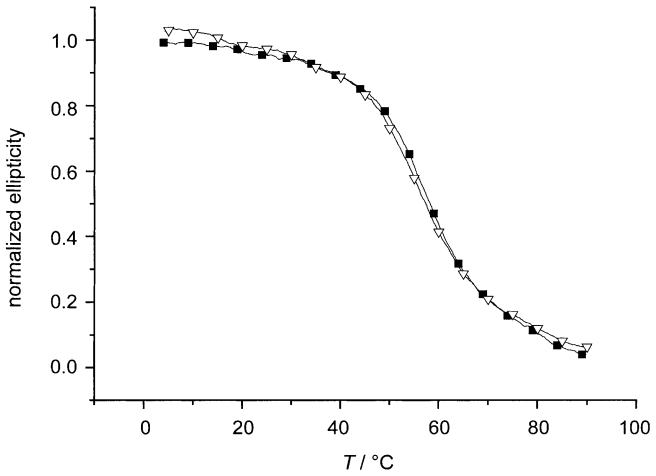


Figure 4. Thermal unfolding (■) and refolding (▽) of the trimer **I** monitored by CD at 225 nm with a heating rate of 0.2 °C min^{-1} at 4×10^{-5} M concentration in 20 mM phosphate buffer (pH 3.0).

parameters extracted from the melting curves are reported in Table 2. The T_m values of the trimer **I** in water and in phosphate buffer at pH 3 are identical within the limits of error of the measurements (Table 1) and similar values were determined by DSC ($T_m = 56.0$ °C in water and 55.7 °C at pH 3.0).

The endotherms of thermal unfolding of the trimer **I** in water (Figure 5) and in phosphate buffer at pH 3.0 (Figure 6) are also very similar suggesting a limited contribution of the disulfide-scrambling reaction to the ΔG° of the unfolding process in water. A comparison of the thermodynamic parameters derived from CD measurements and DSC for

Table 2. Thermodynamic parameters extracted from the thermal denaturation curves monitored by CD at 225 nm for trimer **I** and the self-associated $\alpha 2$(Acm,S*t*Bu)[I] chain in 20 mM phosphate buffer (pH 3.0).

Collagen peptides	T_m [°C]	ΔH_{VH} [kJ mol^{-1}]	ΔS_{VH} [J mol^{-1} K^{-1}]
trimer **I**[a]	56.3	+206	+624
($\alpha 2$(Acm,S*t*Bu)[I])$_3$[b]	14.2	+187	+651

[a] Concentration 4×10^{-5} M. [b] Monomer concentration 1×10^{-3} M.

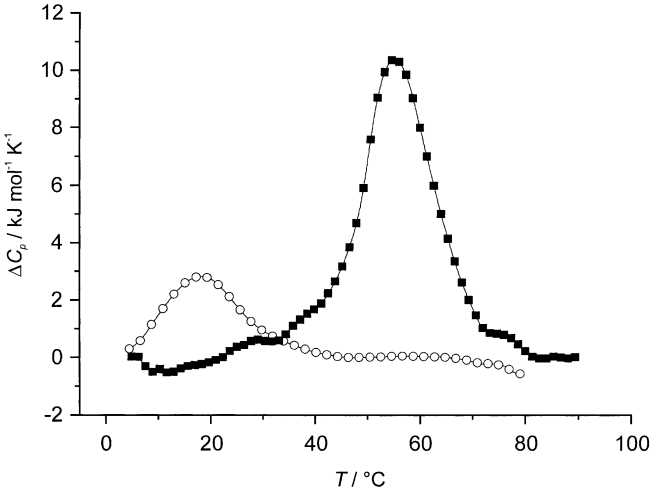


Figure 5. Thermal unfolding of the self-associated homotrimeric α2(Acm,S*t*Bu)[I] (○) and of the trimer **I** (■) in water at 1×10^{-3} M and 4×10^{-5} M concentration, respectively, as monitored by DSC.

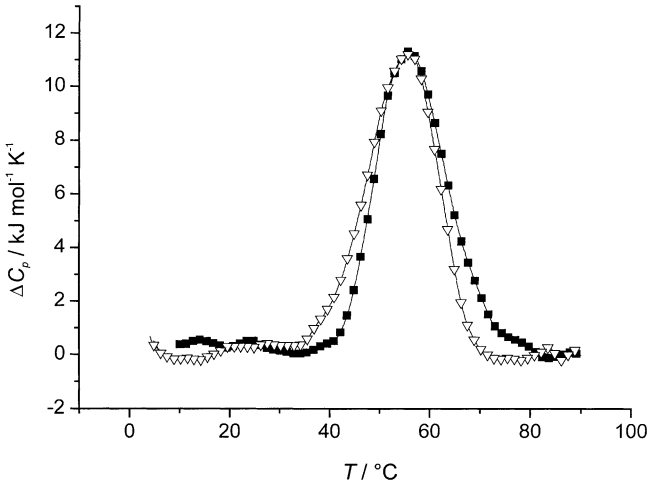


Figure 6. Thermal unfolding (■) and refolding (▽) of trimer **I** at 4×10^{-5} M concentration in 20 mM phosphate buffer (pH 3.0) as monitored by DSC.

unfolding of the self-associated α2 chain with those of the trimer **I** (Tables 2 and 3) further supports the strong effect of the disulfide knot on the stability of the triple helix with a shift of the T_m value by $\sim$40 °C. The favourable entropy term for unfolding of the homotrimeric α2 chain is markedly reduced at the high concentration used (1×10^{-3} M of α2(Acm,S*t*Bu)[I] versus 4×10^{-5} M of trimer **I**) (Table 2).

Table 3. Thermodynamic parameters derived from the thermal denaturation of the collagen peptides as monitored by DSC.

Collagen peptides	Solvent	T_m [°C]	ΔH [kJ mol^{-1}]	ΔS [J mol^{-1} K^{-1}]
trimer **I**[a]	pH 3 (1st melting)	55.7	+191	+583
	pH 3 (1st refolding)	54.5	−204	−623
	pH 3 (2nd melting)	55.7	+202	+613
(α2(Acm,S*t*Bu)[I])$_3$[b]	H_2O	18[c]	–	–

[a] Concentration 4×10^{-5} M in 20 mM phosphate buffer, pH 3.0 (Figure 6). [b] Monomer concentration 1×10^{-3} M in water (Figure 5). [c] The calorimetric curve could not be fitted with the two-state model; T_m for α2(Acm,S*t*Bu)[I] was estimated from the endotherm depicted in Figure 5.

The unfolding and refolding traces of trimer **I** in phosphate buffer (pH 3.0), as monitored by DSC, are reported in Figure 5. Even if slight differences in the peak-tailing can be observed, the thermograms show an identical overall profile as expected from the fully reversible process in this solvent system. The related ΔH and ΔS values (Table 3) compare well with those extracted from the CD measurements.

In the NMR spectra of trimer **I** at temperatures between 4 °C and 75 °C, signal sets corresponding to the triple-helical and unfolded state can clearly be distinguished (data not shown). This, together with the absence of line broadening, indicates folding and unfolding rates slower than 10 s^{-1} at least up to 50 °C. At higher temperatures the signals of the folded state become too weak for analysis of the line shapes.

Discussion

There is a general consensus that disulfide knots in native collagens stabilise the triple-helical structure. Refolding experiments performed on disulfide-cross-linked collagens and collagen fragments were all supportive of a triple-helix nucleation role of such interchain cross-links.[1a, 10] The C-terminal cystine knot of trimer **I** leads to a significant enhancement of the triple-helix stability as evidenced by an increase in the T_m value of $\sim$40 °C. It is not superior to cyclotriveratrylene[7] and particularly to Kemp's triacid[6b] used for N-terminal cross-linking of collagenous peptides of identical Pro-Hyp-Gly triplet composition, unless N- and C-terminal cross-linking produces different effects. In modelling experiments performed for this purpose no difference is observed between N- and C-terminal cystine knots.

While the Cys residues were positioned in trimer **I** in a mode that allows possible extension of the triple helix into the C-terminus, the Kemp's triacid scaffold was spaced from the collagen triplets with a flexible glycine residue to permit correct registration of the chains.[6] NMR conformational analysis clearly revealed that despite this spacing at least one Gly-Pro-Hyp triplet per chain is only partially included in the triple-helix packing.[39] Conversely, both MD simulations and NMR analysis of trimer **I** would support an involvement of all five triplets in the hydrogen bonding network as well as even part of the cystine knot. A possible explanation for the lower stabilisation effect of the disulfides compared to Kemp's triacid could be a slight mismatch of the disulfide geometry with the triple-helical backbone conformation. This mismatch could well account for the observed thermodegradation of the cystine knot. However, by heating the non-structured trimer **II** under identical conditions to trimer **I**, scrambling of the disulfides was found to occur to an identical extent. Thus, solely the high temperature required for complete unfolding of trimer **I** (70–80 °C) is responsible for the observed thermolysis. In fact, scrambling of unsymmetrical disulfides into symmetrical disulfides is known to occur readily under basic and even neutral conditions, particularly in the presence of traces of thiols which may be formed on heating.[37, 38]

Under acidic conditions the nucleophilicity of the thiols required for initiation of the process is significantly reduced, and thermodegradation was not observed.

The NMR-derived structural information for trimer **I** is further supported by the modelling experiments. These would suggest a direct involvement of the simple artificial cystine knot in nucleation of the triple helix and, as expected from the entropy term, in stabilisation of this fold. Nonetheless, replacing the Hyp residues with Hyp(*t*Bu) and particularly with (4*S*)-FPro in the five triplets of all three α chains disfavours the onset of a triple-helical structure. Because of the hydrophobic nature of the bulky *tert*-butyl group the low propensity for the collagen structure in this side-chain-protected trimer **I** is conceivable. The strong unfavourable effect of the (4*S*)-FPro was, however, expected.[17] In model compounds the energy difference between the *cis* and *trans* conformation of Ac-(4*S*)-FPro-OMe is about 2 kJ mol^{-1} smaller than in the related (4*R*)-FPro or Hyp derivatives.[19b] Since the triple helix is a highly cooperative system the substitution of fifteen Hyp residues by (4*S*)-FPro could destabilise the triple-helical state by 30 kJ mol^{-1} or more. Thus, the stabilising effect of the cystine knot is exceeded by the destabilisation due to (4*S*)-FPro.

Experimental Section

Peptide synthesis

Materials and methods: All reagents and solvents were of the highest quality commercially available and were used without further purification, except DMF, which was freshly distilled over ninhydrin. Unless otherwise stated, amino acid derivatives were purchased from Fluka (Taufkirchen, Germany), TFFH and 2-(2-nitropyridyl)sulfenyl chloride and 2,2′-dithiobis(5-nitropyridine) from Aldrich (Taufkirchen, Germany). The Wang- (linker: 4-benzyloxybenzyl alcohol), Rink-MBHA- (linker: 4-[(*R,S*)-α-amino-2′,4′-dimethoxybenzyl]phenoxyacetyl-norleucine-amidobenzhydryl), XAL- (linker: 5-(9-amino-9*H*-xanthen-3-yloxy)valeric acid) and chlorotrityl resin were from Calbiochem-NovaBiochem (Läufelfingen, Switzerland) and TentaGel-S-PHB (linker: *p*-hydroxybenzyl) and TentaGel-S-RAM (linker: 4-[(*R,S*)-α-amino-2′,4′-dimethoxybenzyl]phenoxyacetic acid) were from Rapp Polymere GmbH (Tübingen, Germany). Peptide synthesis was performed manually in a polypropylene syringe fitted with a polyethylene disk. Precoated silica gel 60 TLC plates were obtained from Merck AG (Darmstadt, Germany) and compounds were visualised with chlorine/tolidine or permanganate. Analytical RP-HPLC was performed with Waters equipment (Eschborn, Germany) using reversed-phase Nucleosil C18 columns (0.4 × 25 cm, 10 μm, Macherey & Nagel, Düren, Germany) and linear gradients of MeCN/2 % H_3PO_4 (from 5:95 to 90:10 in 15 min at a flow rate of 1.5 mL min^{-1}) as eluents. UV absorbance was monitored at 210 nm. Preparative RP-HPLC was carried out with Abimed equipment (Langenfeld, Germany) on reversed-phase Nucleosil C18 (2.1 × 25 cm, 5 μm end capped) columns by elution with a linear gradient of MeCN (containing 0.08 % TFA)/0.1 % TFA from 2:8 to 8:2 in 50 min. Elution profiles were monitored by UV absorbance at 210 nm. For preparative size-exclusion column chromatography a 145/1.25 Fraktogel HSK HW-40 S column (145 × 1.25 cm) and isocratic elution with 0.5 % AcOH at a flow rate of 0.4 mL min^{-1} was used. ESI-MS spectra were recorded on a PE Sciex API 165. Amino acid analyses of the acid hydrolysates (6 M HCl containing 2.5 % thioglycolic acid, 110 °C, 72 h) were performed on a LC 6001 Biotronic amino acid analyser.

Fmoc-Pro-Hyp(*t*Bu)-Gly-OH was synthesised as described previously.[8d]

Synthesis of the Hyp-unprotected α chains: The α chains for assembly of trimer **I** according to Scheme 1 were synthesised on TentaGel-S-PHB and TentaGel-S-RAM using Fmoc-Pro-Hyp(*t*Bu)-Gly-OH synthons and purified by preparative HPLC following essentially the protocols described previously.[8d]

Ac-(Pro-Hyp-Gly)$_5$-Cys(S*t*Bu)-Gly-OH, α1(S*t*Bu)[I]: Synthesis on TentaGel-S-PHB (0.224 mM Fmoc-Gly g^{-1}; 1.25 g). Yield: 162 mg (35 %); HPLC: t_R = 7.4 min (98 %); ESI-MS: *m/z*: 823.0 $[M+2H]^{2+}$; M_r = 1644.86 calcd for $C_{71}H_{105}N_{17}O_{24}S_2$.

Ac-(Pro-Hyp-Gly)$_5$-Cys(Acm)-Cys(S*t*Bu)-Gly-NH$_2$, α2(Acm,S*t*Bu)[I]: Synthesis on TentaGel-S-RAM (0.25 mmol g^{-1}; 1 g). Yield: 145 mg (32 %); HPLC: t_R = 7.9 min (98 %); ESI-MS: *m/z*: 910.0 $[M+2H]^{2+}$; M_r = 1818.09 calcd for $C_{77}H_{116}N_{20}O_{25}S_3$; amino acid analysis: Hyp 4.99 (5), Pro 5.01 (5), Gly 6.16 (6), Cys 1.87 (2); peptide content: 91.3 %.

Ac-(Pro-Hyp-Gly)$_5$-Pro-Cys(S*t*Bu)-Gly-NH$_2$, α1′(S*t*Bu)[I]: Synthesis on TentaGel-S-RAM (0.25 mM g^{-1}; 1 g). Yield: 135 mg (46 %); HPLC: t_R = 8.1 min (98 %); ESI-MS: *m/z*: 871.4 $[M+2H]^{2+}$; M_r = 1741.01 calcd for $C_{76}H_{113}N_{19}O_{24}S_2$.

Synthesis of the protected α chains on XAL resin: The Fmoc-protected XAL resin (loading: 0.62 mmol g^{-1}) was treated with 20 % piperidine in DMF (1 × 15 min, 1 × 5 min) and then washed successively with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min) and MeOH (3 × 1 min). Coupling of the first amino acid (Fmoc-Gly-OH) was performed twice with five equivalents of Fmoc-Gly-OH/HBTU/HOBt/DIEA (1:1:1:2) in DMF (2 × 1 h) followed by washing with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min), MeOH (3 × 1 min) and DMF (3 × 1 min). The resin was capped by acetylation with Ac_2O (4 equiv)/DIEA (4 equiv) and DMAP (0.1 equiv) in DMF (2 × 30 min). Loading of the resin with the first amino acid was determined spectroscopically by quantification of the fulvene/piperidine adduct at 301 nm (ε = 7800) upon Fmoc removal under above conditions.

Chain elongation with Fmoc-amino acids was performed by double couplings with four equivalents of Fmoc-amino acid/HBTU/HOBt/DIEA (1:1:1:2) in DMF (2 × 1 h) except for S-protected Fmoc-Cys-OH which was coupled by the HBTU procedure with lower DIEA concentration. The coupling steps were followed by washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min) and MeOH (3 × 1 min). Chain elongation with Fmoc-Pro-Hyp(*t*Bu)-Gly)-OH was carried out by double couplings (2 h) with 1.8 equiv and 0.8 equiv Fmoc-Pro-Hyp(*t*Bu)-Gly-OH/TFFH/DIEA (1:1:2), respectively, in DMF (in the first three coupling steps) and NMP/CH_2Cl_2 (in the last two coupling steps) followed by washings with DMF (3 × 1 min), acylation with Boc_2O (10 equiv) in DMF (20 min) and additional washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min) and MeOH (3 × 1 min). Coupling efficiency was monitored with the Kaiser test[40] except for N-terminal proline residues where the chloranil test[21] was applied.

Cleavage of the Fmoc protecting group from the Fmoc-Pro-peptidyl resin was carried out with 2 % piperidine and 2 % DBU in DMF (0 °C, 1 × 60 s, 1 × 30 s), followed by washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min) and MeOH (3 × 1 min). N-terminal acetylation of the peptides on resin was performed with four equivalents of Ac_2O/DIEA (1:1) in DMF (2 × 30 min) followed by washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min) and MeOH (3 × 1 min).

Synthesis of the protected α chains on chlorotrityl resin: The peptides were synthesised by using Fmoc-Gly-Trt(Cl) resin (loading: 0.57 mmol g^{-1}) essentially as described in the case of the XAL resin.

Synthesis of the heterotrimer I

Ac-[Pro-Hyp(tBu)-Gly]$_5$-Cys(*o*-Npys)-Gly-OH, α1(*t*Bu,*o*-Npys)[I]: Ac-[Pro-Hyp(*t*Bu)-Gly]$_5$-Cys(Acm)-Gly-Trt(Cl) resin was synthesised starting from Fmoc-Gly-Trt(Cl) resin (435 mg, loading: 0.57 mmol g^{-1}) and then treated with *o*-Npys-Cl (2 equiv) in DMF at room temperature for 5 h. The resin was filtered off and washed successively with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min) and Et_2O (3 × 1 min), and then dried under reduced pressure. Cleavage from the resin was performed with 2 % TFA, 2 % Et_3SH and 2 % H_2O in CH_2Cl_2 (1 × 15 min, 2 × 40 min); the resin was washed with CH_2Cl_2. The filtrates were combined and taken to dryness. The crude product was dissolved in MeOH and purified by preparative RP-HPLC. Fractions containing homogeneous product were combined, concentrated and lyophilised. Yield: 55 mg (11 %) of yellowish powder; HPLC: t_R = 11.1 min (98 %); ESI-MS: *m/z*: 996.6 $[M+2H]^{2+}$; M_r = 1991.37 calcd for $C_{92}H_{139}N_{19}O_{26}S_2$; amino acid analysis: Hyp 5.19 (5), Pro 5.02 (5), Gly 6.00 (6), Cys 0.99 (1); peptide content: 76.1 %.

Ac-[Pro-Hyp(*t*Bu)-Gly]$_5$-Cys(Trt)-Cys(S*t*Bu)-Gly-NH$_2$, α2(*t*Bu,Trt,S*t*-Bu)[I]: The synthesis was carried out on XAL resin (400 mg, loading: 0.62 mmol g^{-1}) as described under general procedures. Acidolytic cleavage was performed three times (1 × 5 min, 1 × 40 min, 1 × 90 min) with 2% TFA, 2% Et_3SiH and 2% H_2O in CH_2Cl_2. The resin was washed with CH_2Cl_2 and the filtrates were immediately diluted with DMF, combined and evaporated. The crude product was dissolved in MeOH and purified by preparative RP-HPLC. Fractions containing the desired peptide were pooled, concentrated and lyophilised from *t*BuOH/H_2O (5:1). Yield: 90 mg (16%); HPLC: t_R = 14.3 min (98%); ESI-MS: *m*/*z*: 1135.8 $[M+2H]^{2+}$; M_r = 2269.88 calcd for $C_{113}H_{165}N_{19}O_{24}S_3$.

Ac-[Pro-Hyp(tBu)-Gly]$_5$-Cys(Trt)-Cys-Gly-NH$_2$, α2(*t*Bu,Trt)[I]: Tributylphosphine (10 equiv, 98 μL, 0.4 mmol) was added to Ac-[Pro-Hyp(*t*Bu)-Gly]$_5$-Cys(Trt)-Cys(S*t*Bu)-Gly-NH$_2$, α2(*t*Bu,Trt,S*t*Bu)[I] (90 mg, 39.7 μmol) in TFE/H_2O (95:5, 100 mL) and the solution was stirred at room temperature for 5 h. The bulk of the solvent was removed and the residue lyophilised from *t*BuOH/H_2O (4:1). Yield: 86%; HPLC: t_R = 13.3 min (> 95%); ESI-MS: *m*/*z*: 1091.4 $[M+2H]^{2+}$; M_r = 2181.71 calcd for $C_{109}H_{157}N_{19}O_{24}S_2$; amino acid analysis: Hyp 5.04 (5), Pro 4.90 (5), Gly 6.00 (6), Cys 2.20 (2); peptide content: 73.2%.

Ac-[Pro-Hyp(*t*Bu)-Gly]$_5$-Pro-Cys(*o*-Npys)-Gly-NH$_2$, α1′(*t*Bu,*o*-Npys)[I]: Ac-(Pro-Hyp(*t*Bu)-Gly)$_5$-Pro-Cys(Acm)-Gly-XAL resin was synthesised on XAL resin (400 mg, loading: 0.62 mmol g^{-1}) as described under general procedures. Conversion of the S-Acm derivative with *o*-Npys-Cl into the *S*-*o*-Npys derivative and cleavage from the resin was performed as described for α1(*t*Bu,*o*-Npys)[I]. The crude product was dissolved in MeOH and purified by preparative RP-HPLC. Fractions containing the desired material were pooled, concentrated and lyophilised, Yield: 124 mg (24%) of yellowish powder; HPLC: t_R = 12.05 min (98%); ESI-MS: *m*/*z*: 1044.2 $[M+2H]^{2+}$; M_r = 2087.51 calcd for $C_{97}H_{147}N_{21}O_{26}S_2$; amino acid analysis: Hyp 5.05 (5), Pro 6.03 (6), Gly 6.00 (6), Cys 1.02 (1); peptide content: 82.4%.

Assembly of the heterodimer α1α2(*t*Bu,Trt)[I]: A solution of α2(*t*Bu, Trt)[I] (17.64 mg; 5.92 μmol) and α1(*t*Bu,*o*-Npys) (15.5 mg: 5.92 μmol) in DMF (0.5 mL) was stirred for five days at room temperature under argon. The progress of disulfide formation was monitored by RP-HPLC. The mixture was concentrated to gel and the reaction allowed to proceed for an additional two days under the exclusion of oxygen. The mixture was then diluted with *t*BuOH/H_2O (4:1) and lyophilised. The crude product was purified by size-exclusion chromatography using 0.5 M AcOH/*n*BuOH/2-propanol (7:2:1) as eluent. Fractions containing the desired product (HPLC) were pooled and lyophilised. Yield: 14.7 mg (62%); HPLC: t_R = 12.3 min (98%); ESI-MS: *m*/*z*: 2009.2 $[M+2H]^{2+}$ and 1339.8 $[M+3H]^{3+}$; M_r = 4016.94 calcd for $C_{196}H_{294}N_{36}O_{48}S_3$.

***S*-Deprotection of the heterodimer α1α2(*t*Bu,Trt)[I]**: A solution of the heterodimer α1α2(*t*Bu,Trt)[I] (14.7 mg, 3.67 μmol) in 3% TFA and 3% Et_3SH in CH_2Cl_2 (20 mL) was stirred at room temperature for 15 min, and then diluted with DMF and taken to dryness. The residue was dissolved in *t*BuOH/H_2O (4:1) and lyophilised. Yield: 13.85 mg (100%); HPLC: t_R = 10.3 min (> 98%); ESI-MS: *m*/*z*: 1888.2 $[M+2H]^{2+}$ and 1259.0 $[M+3H]^{3+}$; M_r = 3774.6 calcd for $C_{117}H_{278}N_{36}O_{48}S_3$.

Assembly of the side-chain-protected heterotrimer α1α2α1′(*t*Bu)[I]: The *S*-deprotected heterodimer α1α2(*t*Bu)[I] (13.8 mg; 3.67 μmol) and α1′(*t*Bu,*o*-Npys) (11.2 mg; 4.41 μmol) were dissolved in argon-saturated DMF (0.5 mL) and stirred under argon for five days at room temperature. The solution was then concentrated to gel and allowed to stand for additional two days at room temperature. The mixture was diluted with *t*BuOH/H_2O (4:1) and lyophilised. The crude product was purified by size-exclusion chromatography using 0.5 M AcOH/*n*BuOH/2-propanol (7:2:1) as eluent. Fractions containing the desired product (HPLC) were pooled and lyophilised; yield: 8.5 mg (41%); HPLC: t_R = 12.6 min (> 98%); ESI-MS: *m*/*z*: 1903.0 $[M+3H]^{3+}$, 1427.4 $[M+4H]^{4+}$ and 1142.0 $[M+5H]^{5+}$; M_r = 5704.97 calcd for $C_{269}H_{421}N_{55}O_{72}S_4$.

Heterotrimer I: The side-chain-protected heterotrimer α1α2α1′(*t*Bu)[I] (8.5 mg; 1.49 μmol) was dissolved in ice-cold TFA/Et_3SiH/H_2O (89:10:1, 3 mL) and stirred for 5 min. The solution was diluted with H_2O and lyophilised; yield: 7.2 mg (100%); HPLC: t_R = 7.3 min (> 95%); ESI-MS: *m*/*z*: 1622.4 $[M+3H]^{3+}$ and 1216.8 $[M+4H]^{4+}$; M_r = 4864.34 calcd for $C_{209}H_{305}N_{55}O_{72}S_4$; amino acid analysis: Hyp 14.9 (15), Pro 15.92 (16), Gly 18.17 (18), Cys 3.93 (4); peptide content: 79.0%.

Synthesis of the heterotrimer II

Z-Pro-(4*S*)-FPro-Gly-OBzl: DAST (7.23 mL, 50.4 mmol) was added to an ice-cold suspension of Z-Pro-Hyp-OH[41] (5 g, 12.6 mmol) in CH_2Cl_2 (30 mL) and the mixture was stirred for 4 h at room temperature. Then H-Gly-OBzl · TsOH (5.26 g, 15.1 mmol) was added followed by DIEA (5.2 mL, 30.2 mmol) and the solution was stirred for a further 6 h at room temperature. The solution was poured onto ice and the organic layer extracted with 5% $NaHCO_3$, 5% $KHSO_4$ and H_2O. After drying over $MgSO_4$, the solvent was removed under reduced pressure leaving a dark oily residue. The crude product was chromatographed on silica gel by elution with $CHCl_3$ followed by 0.2% AcOH in 5–15% MeOH in $CHCl_3$. Fractions of the last elute containing homogeneous material (TLC) were pooled and taken to dryness. Yield: 4 g (62%); TLC: R_f = 0.36 (AcOH/MeOH/CH_2Cl_2, 0.5:5:94.5); HPLC: t_R = 12.2 min (> 95%); ESI-MS: *m*/*z*: 512.4 $[M+H]^+$; M_r = 511.56 calcd for $C_{27}H_{30}N_3O_6F$.

H-Pro-(4*S*)-FPro-Gly-OH · TsOH: Z-Pro-(4*S*)-FPro-Gly-OBzl (4 g, 7.8 mmol) was hydrogenated over a 10% Pd/C in MeOH/H_2O (9:1) containing TsOH (1.48 g, 7.8 mmol). After 12 h the catalyst was filtered off and the solution evaporated to dryness. The oily residue was lyophilised from *t*BuOH/H_2O (4:1). Yield: 2.3 g (100%) of colourless oil; TLC: R_f = 0.05 (AcOH/MeOH/CH_2Cl_2, 0.5:5:94.5); HPLC: t_R = 1.2 min (> 98%); ESI-MS: *m*/*z*: 288.2 $[M+H]^+$; M_r = 287.3 calcd for $C_{12}H_{18}N_3O_4F$.

Fmoc-Pro-(4*S*)-FPro-Gly-OH: Fmoc-OSu (3.16 g, 9.36 mmol) was added to H-Pro-(4*S*)-FPro-Gly-OH · TsOH (2.2 g, 7.8 mmol) in H_2O/dioxane (2:1) containing $NaHCO_3$ (1.64 g, 19.5 mmol). The reaction was stirred overnight at room temperature, then the mixture was neutralised and the bulk of the solvent was removed under reduced pressure. The residue was dissolved in 5% $NaHCO_3$ and the excess of Fmoc-OSu extracted with EtOAc. The aqueous solution was acidified with 5% $KHSO_4$ and the product extracted with EtOAc (3 ×). The combined extracts were washed with brine, dried over $MgSO_4$ and taken to dryness. The crude product was chromatographed on silica gel by elution with $CHCl_3$, followed by 0.2% AcOH and 5–15% MeOH in $CHCl_3$. Yield: 2.47 g (62%); TLC: R_f = 0.46 (AcOH/MeOH/CH_2Cl_2, 0.5:5:94.5); HPLC: t_R = 10.9 min (98%); ESI-MS: *m*/*z*: 510.4 $[M+H]^+$; M_r = 509.54 calcd for $C_{27}H_{28}N_3O_6F$.

Ac-[Pro-(4*S*)-FPro-Gly]$_5$-Cys(*o*-Npys)-Gly-OH, α1(*o*-Npys)[II]: Wang resin (1.1 mmol g^{-1}, 280 mg) was loaded with (Fmoc-Gly)$_2$O and DMAP as catalyst at a degree of 0.32 mmol g^{-1} and residual hydroxy groups were capped with a large excess (10 equiv) of Ac_2O/DIEA (1:2) in the presence of DMAP as catalyst according to standard procedures. The peptide was synthesised by coupling Fmoc-Cys(Acm)-OH and Fmoc-Pro-(4*S*)-FPro-Gly-OH (5 ×), intermediate Fmoc cleavage and final acetylation applying the general procedures described above. The *S*-Acm protected peptide was converted on resin to the *S*-*o*-Npys derivative by reaction with *o*-Npys-Cl as described for α1(*t*Bu,*o*-Npys)[I]. The peptide was cleaved from the resin with TFA/Et_3SiH/H_2O (96:2:2) (1 × 5 min, 1 × 30 min, 1 × 60 min). The combined filtrates were evaporated under reduced pressure and the crude product was dissolved in MeOH and purified by preparative RP-HPLC. Fractions containing the desired material were pooled, concentrated and lyophilised. Yield: 35.6 mg (23%) of yellowish powder; HPLC: t_R = 9.0 min (98%); ESI-MS: *m*/*z*: 860.4 $[M+2H]^{2+}$; M_r = 1720.79 calcd for $C_{72}H_{94}N_{19}O_{21}F_5S_2$.

Ac-[Pro-(4*S*)-FPro-Gly]$_5$-Cys(Trt)-Cys(S*t*Bu)-Gly-NH$_2$, α2(Trt,S*t*Bu)[II]: The peptide chain was synthesised on XAL resin (277 mg, 0.62 mM g^{-1}) and worked up as described for α2(*t*Bu,Trt,S*t*Bu)[I]. The crude product was dissolved in MeOH and purified by preparative RP-HPLC. The product was isolated by lyophilisation. Yield: 37.6 mg (11%); HPLC: t_R = 9.2 min (98%); ESI-MS: *m*/*z*: 1000.4 $[M+2H]^{2+}$; M_r = 1999.29 calcd for $C_{93}H_{120}N_{19}O_{19}F_5S_3$.

Ac-[Pro-(4*S*)-FPro-Gly]$_5$-Cys(Trt)-Cys-Gly-NH$_2$, α2(Trt)[II]: Cleavage of the S*t*Bu group was performed as described for α2(*t*Bu,Trt)[I]. Yield: 23.6 mg (84%); HPLC: t_R = 11.3 min (98%); ESI-MS: *m*/*z*: 956.2 $[M+2H]^{2+}$; M_r = 1911.12 calcd for $C_{89}H_{112}N_{19}O_{19}F_5S_2$.

Ac-[Pro-(4*S*)-FPro-Gly]$_5$-Pro-Cys(*o*-Npys)-Gly-NH$_2$, α1′(*o*-Npys)[II]: The title compound was synthesised on Rink-MBHA resin (138.5 mg, loading: 0.65 mmol g^{-1}) as an *S*-Acm protected derivative applying the general procedures described above. Its conversion on resin to the *S*-*o*-Npys derivative was performed with *o*-Npys-Cl as described for α1(*t*Bu,*o*-Npys)[I]. Cleavage from the resin was carried out with TFA/Et_3SiH/H_2O, 96:2:2 (1 × 5 min, 1 × 30 min, 1 × 60 min). The filtrates were combined and

the solvents removed under reduced pressure. The crude product was purified by preparative RP-HPLC. Yield: 47.5 mg (29%) upon lyophilisation; HPLC: $t_R = 10.7$ min (98%); ESI-MS: m/z: 909.6 $[M+2H]^{2+}$; $M_r =$ 1816.92 calcd for $C_{77}H_{102}N_{21}O_{21}F_5S_2$.

Assembly of the heterodimer α1α2(Trt)[II]: α2(Trt)[II] (23.6 mg, 12.3 μmol) and α1(*o*-Npys)[II] chain (21.3 mg, 12.3 μmol) were stirred in degassed and argon-saturated H_2O (0.5 mL) for 6 h at room temperature. The solution was diluted with *t*BuOH/H_2O (4:1) and lyophilised. The crude product was purified by size-exclusion chromatography using 1M AcOH as eluent. Fractions containing the desired product were pooled and lyophilised. Yield: 10.3 mg (24%); HPLC: $t_R = 10.4$ min (> 98%); ESI-MS: m/z: 1159.4 $[M+2H]^{2+}$; $M_r = 3473.79$ calcd for $C_{156}H_{204}N_{36}O_{38}F_{10}S_3$.

***S*-Deprotection of the heterodimer α1α2(Trt)[II]**: The heterodimer α1α2(Trt)[II] (10.3 mg, 2.97 μmol) was stirred in TFA/Et_3SiH/H_2O (94:3:3, 5 mL) for 5 min at room temperature. The mixture was concentrated under reduced pressure to an oil which was then lyophilised from *t*BuOH/H_2O (4:1); yield: 9.6 mg (100%); HPLC: $t_R = 7.9$ min (>98%); ESI-MS: m/z: 1617.8 $[M+2H]^{2+}$, 1078.6 $[M+3H]^{3+}$; $M_r = 3233.44$ calcd for $C_{137}H_{200}N_{36}O_{38}F_{10}S_3$.

Heterotrimer II: A solution of the *S*-unprotected heterodimer α1α2[II] (8.9 mg, 2.75 μmol) and α1′(*o*-Npys)[II] (6.0 mg, 3.3 μMol) in degassed H_2O (0.5 mL) was stirred under argon for 6 h at room temperature. The reaction mixture was then diluted with *t*BuOH/H_2O (4:1) and lyophilised. The crude product was purified by size-exclusion chromatography with 1% AcOH as eluent. The heterotrimer II was isolated by lyophilisation. Yield: 3.9 mg (29%); HPLC: $t_R = 8.2$ min (>98%); ESI-MS: m/z: 1632.0 $[M+3H]^{3+}$ and 1224.0 $[M+4H]^{4+}$; $M_r = 4894.11$ calcd for $C_{209}H_{300}N_{55}O_{57}F_{15}S_4$.

CD measurements: CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a thermostated cell holder and connected to a PC for signal averaging and processing. All spectra were recorded in the range 190–250 nm, employing quartz cuvettes of 0.1 cm optical path length. The average of 10 scans is reported and expressed in terms of ellipticity units per mole of peptide residues ($[\Theta]_R$). The measurements were performed on peptide solutions pre-equilibrated at 4°C for at least 12 h, at 1×10^{-3}M concentration for single α chains and at 4×10^{-5}M concentration for trimers **I** and **II** in water or in 20 mM phosphate buffer at pH 7.2 and 3.0. The concentrations were determined by weight and peptide content as obtained from the quantitative amino acid analysis of the peptides. The thermal denaturation curves were registered on the same peptide solutions following the change in intensity of the CD signal at 225 nm upon increasing the temperature from 4°C to 90°C with a heating rate of 0.2°C min^{-1}. T_m values and thermodynamic parameters were derived from original transition curves using the JASCO software.

DSC measurements: The temperature dependence of the partial heat capacity was determined on a VP-DSC microcalorimeter (MicroCal, Northampton, USA) equipped with a cell feedback network and two fixed-in-place cells with effective volumes of approximately 0.5 mL. The measurements were carried out on peptide solutions at 1×10^{-3}M concentration for the α2(Acm,S*t*Bu)[I] chain and at 4×10^{-5}M concentration for trimer **I** in water or in 20 mM phosphate buffer (pH 3.0) after preequilibration at 4°C for at least 12 h. Thermal denaturations were recorded by monitoring the variation of the heat capacity (C_p) as a function of the temperature in the range 4 to 90°C using a scan rate of 0.2°C min^{-1}. Data analysis was performed with the Origin software modified for microcalorimetric applications (MicroCal, Northampton, USA) using the two-state transition model.[42]

NMR measurements: NMR experiments for conformational analysis of trimers **I** and **II** were carried out at a proton frequency of 500.13 MHz between 10°C and 75°C on a Bruker DRX500 spectrometer. Concentrations were 2 mM for trimer **I** and 1 mM for trimer **II**. For trimer **I** partial resonance assignments could be achieved according to the method of Wüthrich[43] using 2D-DQF-COSY,[44] 2D-TOCSY[45] and 2D-NOESY[46] experiments. Both trimers were measured in a H_2O/D_2O (9:1) mixture using the WATERGATE water suppression scheme.[47] For trimer **II** 2D-DQF-COSY, 2D-TOCSY and 2D-NOESY experiments were also recorded on a 100% D_2O sample. In the D_2O spectra of trimer **II** two spin systems could be identified for each of the Pro and (4*S*)-FPro residues. The major one corresponds in both cases to the *trans* conformation of the peptidyl-proline bond. whereas the minor one belongs to the *cis* conformation. Identification of *trans* and *cis* conformation was based on characteristic NOEs [*trans*: Hα($i-1$) – Hδ(i), *cis*: Hα($i-1$) – Hα(i)]. Temperature shift coefficients for the amide protons were obtained from 1D spectra recorded between 10°C and 20°C. Higher temperatures were not suitable for determination of temperature shifts as the onset of melting of the triple-helical conformation is clearly seen above 20°C. H/D exchange experiments were performed for trimer **II** at 10°C. D_2O was added on ice to a sample lyophilised from H_2O. The first 1D measurement could be performed 10 min after addition of D_2O and already showed a complete lack of amide signals.

Molecular modelling: Molecular modelling was performed on Silicon Graphics O2 R5000 computers (Silicon Graphics Inc., Mountain View, CA) with the INSIGHTII program package (Accelrys, San Diego, CA) using the CVFF force field. Molecular dynamics simulations of trimer **I** were carried out in a water box of size $8 \times 3 \times 3$ nm^3 containing approximately 2100 water molecules. Starting from an ideal triple-helical conformation for the whole backbone, equilibration was performed in two steps. After energy minimising the water molecules in the box with the trimer fixed in place, dynamics simulation was started at 10 K with the trimer backbone tethered to the starting conformation with 40 kJ mol^{-1} Å^{-2}. After 10 ps, tethering was removed and a further 20 ps were simulated at 10 K followed by gradual heating (with time constant of 1 ps) to 100 K during 50 ps. At this point the temperature bath coupling was reduced (time constant of 5 ps) and the temperature was raised to 300 K. After 100 ps, the temperature was set to the final value of 500 K and a further 100 ps were simulated. In all simulations the step size was 1 fs and snapshots were saved each picosecond.

The influence of the *O*-*tert*-butyl groups of the Hyp residues on the triple-helix conformation and stability was investigated using a distance dependent dielectric constant ($\varepsilon = 1.0 \cdot r$ [Å]) as solvent model. In the (Pro-Hyp-Gly) repeats preceding the cystine knot the hydroxy groups of the Hyp were replaced by O-*tert*-butyl. In the first step of minimisation the backbone atoms of the molecule were fixed in the initial triple-helix conformation and amino acid side-chain atoms were tethered to the initial conformation with 40 kJ mol^{-1} Å^{-2}. Only the protecting groups were allowed to move freely. After the first minimisation, overlap of atoms was removed with minimal movement of the side chains (RMSD = 0.06 Å). In a second step extensive minimisation without constraints was performed. In the final structure no steric clashes were observed, with the triple-helix conformation only moderately disturbed. The hydrogen-bonding network of the triple helix remained unaffected. Apparently steric effects do not represent the major destabilising factor in the side-chain-protected trimer **I**. As the *tert*-butyl groups at the outside of the triple helix are oriented towards the solvent, it is more likely that exposure of such large hydrophobic surfaces disfavours triple-helix formation.

Acknowledgement

This study was partly supported by the SFB 533 (grant A 8) of the Ludwig-Maximilians-University of Munich and the SFB 563 (grant C4) of the Technical University of Munich.

[1] a) H. P. Bächinger, P. Bruckner, R. Timpl, D. J. Prockop, J. Engel, *Eur. J. Biochem.* **1980**, *106,* 619–632; b) P. Bruckner, E. F. Eikenberry, D. J. Prockop, *Eur. J. Biochem.* **1981**, *118*, 607–613.

[2] a) A. Snellman, H. Tu, T. Vaisanen, A. P. Kvist, P. Huhtala, T. Pihlajaniemi, *EMBO J.* **2000**, *19*, 5051–5059; b) S. K. Areida, D. P. Reinhardt, P. K. Müller, P. P. Fietzek, J. Kowitz, M. P. Marinkovich, H. Notbohm, *J. Biol. Chem.* **2001**, *276*, 1594–1601.

[3] J. Engel, D. J. Prockop, *Annu. Rev. Biophys. Biophys. Chem.* **1991**, *20*, 137–152.

[4] Y. Greiche, E. Heidemann, *Biopolymers* **1979**, *18,* 2359–2361; b) W. Roth, E. Heidemann, *Biopolymers* **1980**, *19*, 1909–1917.

[5] a) M. Goodman, M. Bhumralkar, E. A. Jefferson, J. Kwak, E. Locardi, *Biopolymers* **1998**, *47*, 127–142; b) L. Moroder, S. Fiori, R. Friedrich, J. C. D. Müller, J. Ottl in *Synthetic Macromolecules with Higher Structural Order* (Ed.: I. M. Khan), ACS Symposium Series *812*, American Chemical Society, Washington (DC), **2002**, pp. 103–116.

[6] a) M. Goodman, Y. Feng, G. Melacini, J. P. Taulane, *J. Am. Chem. Soc.* **1996**, *118*, 5156 – 5157; b) Y. Feng, G. Melacini, J. P. Taulane, M. Goodman, *J. Am. Chem. Soc.* **1996**, *118*, 10351 – 10358.
[7] a) E. T. Rump, D. T. S. Rijkers, P. G. de Groot, R. M. J. Liskamp in *Peptides: The Wave of the Future*, Proceedings of the 17th American Peptide Symposium, San Diego, USA, June 9 – 14, 2001 (Eds.: M. Lebl, R. A. Houghton), American Peptide Society, San Diego, **2001**, pp. 379 – 380; b) E. T. Rump, D. T. S. Rijkers, H. W. Hilbers, P. G. de Groot, R. M. J. Liskamp, *Chem. Eur. J.* **2002**, *8*, 4613 – 4621.
[8] a) J. Ottl, R. Battistuta, M. Pieper, H. Tschesche, W. Bode, K. Kühn, L. Moroder, *FEBS Lett.* **1996**, *398*, 31 – 36; b) J. Ottl, L. Moroder, *J. Am. Chem. Soc.* **1999**, *121*, 653 – 661; c) J. C. D. Müller, J. Ottl, L. Moroder, *Biochemistry* **2000**, *39*, 5111 – 5116; d) B. Saccà, L. Moroder, *J. Pept. Sci.* **2002**, *8*, 192 – 204.
[9] a) P. Bruckner, D. J. Prockop, *Anal. Biochem.* **1981**, *110*, 360 – 368; b) H. P. Bächinger, L. I. Fessler, R. Timpl, J. H. Fessler, *J. Biol. Chem.* **1981**, *256*, 13193 – 13199; c) J. M. Davis, B. A. Boswell, H. P. Bächinger, *J. Biol. Chem.* **1989**, *264*, 8956 – 8962.
[10] P. Bruckner, H. P. Bächinger, R. Timpl, J. Engel, *Eur. J. Biochem.* **1978**, *90*, 595 – 603.
[11] B. Saccà, C. Renner, L. Moroder, *J. Mol. Biol.* **2002**, *324*, 309 – 318.
[12] J. Engel, *Adv. Meat Res.* **1987**, *4*, 145 – 161.
[13] J. Ottl, D. Gabriel, G. Murphy, V. Knäuper, Y. Tominaga, H. Nagase, M. Kröger, H. Tschesche, W. Bode, L. Moroder, *Chem. Biol.* **2000**, *7*, 119 – 132.
[14] S. Fiori, B. Saccà, L. Moroder, *J. Mol. Biol.* **2002**, *319*, 1235 – 1242.
[15] a) S. Sakakibara, K. Inouye, K. Shudo, Y. Kishida, Y. Kobayashi, D. J. Prockop, *Biochim. Biophys. Acta* **1973**, *303*, 198 – 202; b) J. A. M. Ramshaw, N. K. Shah, B. Brodsky, *J. Struct. Biol.* **1998**, 122, 86 – 91; c) R. Dolz, E. Heidemann, *Biopolymers* **1986**, *25*, 1069 – 1080; d) H. P. Bächinger, J. M. Davis, *Int. J. Biol. Macromol.* **1991**, *13*, 152 – 156.
[16] a) S. K. Holmgren, K. M. Taylor, L. E. Bretscher, R. T. Raines, *Nature* **1998**, *392*, 666 – 667; b) S. K. Holmgren, L. E. Bretscher, K. M. Taylor, R. T. Raines, *Chem. Biol.* **1999**, *6*, 63 – 70.
[17] L. E. Bretscher, C. L. Jenkins, K. M. Taylor, M. L. DeRider, R. T. Raines, *J. Am. Chem. Soc.* **2001**, *123*, 777 – 778.
[18] L. Vitagliano, R. Berisio, L. Mazzarella, A. Zagari, *Biopolymers* **2001**, *58*, 459 – 464.
[19] a) E. S. Eberhardt, N. Panasik, Jr, R. T. Raines, *J. Am. Chem. Soc.* **1996**, *118*, 12261 – 12266; b) C. Renner, S. Alefelder, J. H. Bae, N. Budisa, R. Huber, L. Moroder, *Angew. Chem.* **2001**, *113*, 949 – 951; *Angew. Chem. Int. Ed.* **2001**, *40*, 923 – 925; c) M. L. DeRider, S. J. Wilkens, M. J. Waddell, L. E. Bretscher, F. Weinhold, R. T. Raines, J. L. Markley, *J. Am. Chem. Soc.* **2002**, *124*, 2497 – 2505.
[20] L. A. Carpino, A. El-Faham, *J. Am. Chem. Soc.* **1995**, *117*, 5401 – 5402.
[21] T. Christensen in *Peptides, Structure and Biological Functions*, Proceedings of the 6th American Peptide Symposium (Eds.: E. Gross, J. Meienhofer), Pierce Chemical Co.: Rockford, Il., **1979**, pp. 385 – 388.
[22] L. Moroder, H.-J. Musiol, N. Schaschke, L. Chen, B. Hargittai, G. Barany, *Methods of Organic Chemistry,* Houben-Weyl, **2001**, E 22 a, pp. 384 – 423.
[23] a) H.-J. Musiol, F. Siedler, D. Quarzago, L. Moroder, *Biopolymers* **1994**, *34*, 1553 – 1562; b) R. Behrendt, M. Schenk, H.-J. Musiol, L. Moroder, *J. Pept. Sci.* **1999**, *5*, 519 – 529.
[24] J. Ottl, L. Moroder, *Tetrahedron Lett.* **1999**, *40*, 1487 – 1490.
[25] B. Saccà, D. Barth, H.-J. Musiol, L. Moroder, *J. Pept. Sci.* **2002**, *8*, 205 – 210.
[26] F. Rabanal, W. F. DeGrado, P. L. Dutton, *Tetrahedron Lett.* **1996**, *37*, 1347 – 1350.
[27] L. Demange, A. Menez, C. Dugave, *Tetrahedron Lett.* **1998**, *39*, 1169 – 1172.
[28] R. W. Weber, H. Nitschmann, *Helv. Chim. Acta* **1978**, *61*, 701 – 708.
[29] a) R. A. Berg, B. R. Olsen, D. J. Prockop, *J. Biol. Chem.* **1970**, *254*, 5750 – 5763; b) M. G. Venugopal, J. A. M. Ramshaw, E. Braswell, D. Zhu, B. Brodsky, *Biochemistry* **1994**, *33*, 7948 – 7956.
[30] a) D. D. Jenness, C. Sprecher, W. C. Johnson, Jr, *Biopolymers* **1976**, *15*, 513 – 521; b) S. K. Brahmachari, M. Bansal, V. S. Ananthanarayanan, V. Sasisekharan, *Macromolecules* **1979**, *12*, 23 – 28.
[31] J. M. Thornton, *J. Mol. Biol.* **1981**, *151*, 261 – 287.
[32] M. Bodanszky, G. L. Stahl, *Proc. Natl. Acad. Sci. USA* **1974**, *71*, 2791 – 2794.
[33] N. Le Novère, P.-J. Corringer, J.-P. Changeux, *Biophys. J.* **1999**, *76*, 2329 – 2345.
[34] S. Capasso, L. Mazzarella, T. Tancredi, *Biopolymers* **1979**, *18*, 1555 – 1558.
[35] C. J. Creighton, C. H. Reynolds, D. H. S. Lee, G. C. Leo, A. B. Reitz, *J. Am. Chem. Soc.* **2001**, *123*, 12664 – 12669.
[36] S. Boudko, S. Frank, R. A. Kammerer, J. Stetefeld, T. Schulthess, R. Landwehr, A. Lustig, H. P. Bächinger, J. Engel, *J. Mol. Biol.* **2002**, *317*, 459 – 470.
[37] a) T. J. Ahern, A. M. Klibanov, *Science* **1985**, *228*, 1280 – 1284; b) S. A. Zale, A. M. Klibanov, *Biochemistry* **1986**, *25*, 5432 – 5444.
[38] J. R. Whitaker, R. E. Feeney, *CRC Crit. Rev. Food Sci. Nutr.* **1983**, *19*, 173 – 212.
[39] G. Melacini, Y. Feng, M. Goodman, *J. Am. Chem. Soc.* **1996**, *118*, 10359 – 10364.
[40] E. Kaiser, R. L. Colescott, C. D. Bossinger, P. I. Cook, *Anal. Biochem.* **1970**, *34,* 595 – 598.
[41] J. Ottl, H.-J. Musiol, L. Moroder, *J. Peptide Sci.* **1999**, *5*, 103 – 110.
[42] P. L. Privalov, *Adv. Protein Chem.* **1982**, *35*, 1 – 104.
[43] K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, **1986**.
[44] M. Rance, O. W. Sorensen, G. Bodenhausen, G. Wagner, R. R. Ernst, K. Wüthrich, *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479 – 485.
[45] A. Bax, D. G. Davis, *J. Magn. Reson.* **1985**, *65*, 355 – 360.
[46] J. Jeener, B. H. Meier, P. Bachman, R. R. Ernst, *J. Chem. Phys.* **1979**, *71*, 4546 – 4553.
[47] V. Sklenar, M. Piotto, R. Leppik, V. J. Saudek, *J. Magn. Reson. Ser. A* **1993**, *102,* 241 – 245.

Received: March 4, 2003 [F4917]

