Disulfide-Bridged Heterotrimeric Collagen Peptides Containing the Collagenase Cleavage Site of Collagen Type I. Synthesis and Conformational Properties

Johannes Ottl and Luis Moroder*

Contribution from the Max-Planck-Institute of Biochemistry, D-82152 Martinsried, Germany

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Abstract: Collagenous peptides containing the collagenase cleavage site α1(772−784) and α2(772−784) of collagen type I were synthesized and assembled into heterotrimers via regioselective C-terminal interchain-disulfide bridging in a defined α1α2α1′ staggered register of the three peptide strands. Various approaches were attempted to induce and stabilize the collagen-characteristic triple-helical fold even in the sequence portion of the collagenase cleavage site with its weak triple-helix propensity. By N-terminal chain elongation with $(Gly\text{-}Pro\text{-}Hyp)_n$ tripeptide repeats, particularly with $n = 5$, and in an even more pronounced manner, by incorporation of an additional tripeptide repeat adjacent to the cystine knot, a collagenous heterotrimer was obtained which was found to exhibit dichroic properties fully consistent with the triple-helical fold. Thermal denaturation revealed a remarkable stability with a melting temperature of 41 °C. Although the complex cystine knot of natural collagen was reduced in these synthetic heterotrimers to two interchain-disulfide bridges, it showed not only the expected entropic contribution to the refolding process by keeping the three chains assembled, but more importantly a triple-helix nucleation was induced. In fact, temperature jump experiments clearly revealed two-phase refolding kinetics very similar to those of the disulfide-bridged natural collagen fragment of Col 1−3, where refolding without nucleation difficulty was obtained followed by a slower process dominated by the *cis* → *trans* isomerization for triple-helix propagation. These results would indicate that even the simplified artificial cystine knot is capable of aligning the three peptide chains in the defined α1α2α1′ one-residue shift register. Moreover, the synthetic heterotrimers were cleaved by interstitial collagenases in a single cut through all three chains without release of intermediates during the relatively slow enzymatic digestion process. This observation confirms that, with the de novo designed heterotrimers, functional collagen epitopes were mimicked in highly efficient manner; it also strongly suggests that the preselected α1α2α1′ register may indeed represent the correct staggered alignment of the α subunits at least in collagen type I.

Introduction

Collagens are the most abundant structural macromolecules present in the extracellular matrix, and so far, 19 different types are known which are formed by 32 subunit α-chains.[1,2] Each collagen molecule is composed of three identical or of two or three different α-chains of primarily repeating Gly-Xaa-Yaa triplets which induce each single α-chain to adopt a left-handed poly-Pro-II helix and the three chains to intertwine with a one-residue shift into a right-handed triple-helical coiled coil.[3−5] This supercoiled structure is stabilized by the high content of the imino acids proline and hydroxyproline in positions Xaa and Yaa, respectively, and by the presence of glycine as every third residue as well as by an extensive hydrogen bond and hydration network.[6−9] The α-chains are synthesized in vivo in precursor forms that contain globular domains both at the N- and C-terminus. Selection of the α-subunits and their assembly in the correct register is induced by a selective recognition process between the C-terminal propeptides, and the homo- or heterotrimeric structures can be stabilized by C-terminal cystine networks.[10] Upon hydroxylation of proline in Yaa position,[11−15] nucleation of the triple helix occurs at the C-terminus, and its propagation toward the N-terminus is thought to proceed in a zipper-like manner with the relatively slow *cis/trans*-isomerization of the X-Pro imide bonds as the rate-limiting step.[15−18]

* Author for correspondence. Address: Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, D-82152 Martinsried, Germany. Tel.: 49-89-8578-3905. Fax: 49-89-8578-2847. E-mail: moroder@biochem.mpg.de.

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In collagens such as types I and IV the biological activities such as cell adhesion mediated by integrins or proteoglycans, cell activation and signaling, or matrix metalloproteinase binding and catabolism reside in specific loci of the heterotrimers, most probably as structural epitopes.[19]

The aim of our present study is to investigate structure−function correlations of the recognition and cleavage site of collagen type I by the interstitial collagenases. The use of natural collagens for such purpose is difficult because of their insolubility and gelating properties. The alternative is to use considerably shorter fragments. However, production of such fragments by enzymatic and chemical cleavage of natural collagens is also difficult, and generally their triple-helical structure is thermally rather unstable.[20,21] Therefore, synthetic collagen fragments folded into sufficiently stable triple helices should represent the most promising approach.

For the design and synthesis of a fragment containing the collagenase cleavage site of collagen type I, i.e., the sequence portions 772−784 (P_4−P'_9) and 772−785 (P_4−P'_{10}) of the two α1 subunits and 772−784 (P_4−P'_9) of the α2 subunit, two main problems have to be solved: (i) the selective assembly of three peptide chains into a heterotrimer and (ii) the induction of a staggered register of the three α chains in the correct rank order. In the folding process of natural collagens this registration of the chains is induced by the propeptides and stabilized by cystine networks prior or contemporaneously with triple-helix formation. In some collagens complex cystine knots located in triple-helix segments or in their proximity are present even in the processed forms.[22] Extensive kinetic studies on folding and unfolding of procollagen I and III,[23,24] pN-collagen III, collagen III, and a short triple-helical fragment (Col 1−3) derived from the N-terminal propeptide of pN-collagen III[25,26] clearly revealed that chain registration is maintained by the disulfide links between the three subunits and that these disulfide knots located at the C-terminal end of triple-helical regions serve as effective nuclei for triple-helix formation.[25,27] In Col 1−3 the triple-helical segment is flanked by two noncollagenous regions, and the cystine knot of yet unknown connectivity is located at the C-terminus of the triple helix and serves to link three (Gly-Xaa-Yaa)$_n$-Gly-Pro-Cys-Cys chains.[25]

The existing disulfide chemistry is insufficient for an interchain bridging of six Cys residues, located pairwise on three peptide chains, with the correct connectivities. We have, therefore, selected a simplified cystine knot[28] for which procedures were developed that allow the correct assembly of three collagenous synthetic peptides into a heterotrimer of the desired register. Since there are strong indications for a α1α2α1 register at least for type I[29] and type IV[30] collagen, we have chosen this hypothetical rank order for the design of heterotrimeric fragments. A model of the (Gly-Pro-Hyp)$_{10}$ triple helix (Hyp = hydroxyproline) was built up, using the known coordinates,[5,8] and the tripeptide repeats were then replaced in the C-terminal portion of the molecule by the collagenase cleavage sequences P_4−$P'_{9/10}$ of collagen type I in the α1α2α1′ register. Finally the triple helix was cross-linked at the C-terminus with a simplified cystine knot consisting of two interchain disulfide bridges connecting the α1 peptide strand to α2, and the α2 peptide to α1′, respectively; standard disulfide bond length and angles of cystine residues in proteins were used.[31] After energy minimization of this model heterotrimeric collagen peptide a surprisingly good fitting of the two cystines into the triple-helical fold was observed as shown in Figure 1, and no steric clashes were detected.

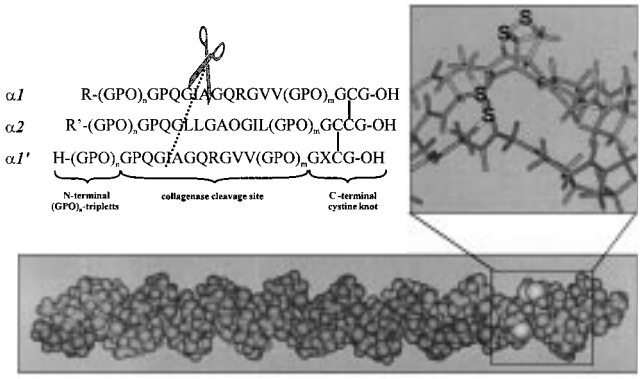

Figure 1. CPK model of the (GPO)$_n$ triple helix with an artificial C-terminal cystine knot (see insert). Alignment of the α1, α2, and α1′ peptide chains containing the collagenase cleavage site 772−784/785 (P_4−$P'_{9/10}$) of collagen type I into α1α2α1′ heterotrimers; n = 3 (heterotrimer A) or 5 (heterotrimers B, C, and D); M = 0 (heterotrimers A, B, and C); R = H (heterotrimers A and B), Ac (heterotrimer C) or Ac-PO (heterotrimer D); R′ = H (heterotrimers A and B); Ac (heterotrimer C) or Ac-O (heterotrimer D); X = L (heterotrimers A, B, and C) or P (heterotrimer D); the one-letter code is used for the amino acid residues and O for hydroxyproline.

From studies on self-associated homotrimeric model collagen peptides it was known that replacement of the imino acids in the consensus Gly-Pro-Hyp repeats by other residues leads to significantly decreased thermal stabilities.[32−35] Since the collagenase cleavage site still contains in every third position of the sequence a Gly residue, it may not represent a nonhelical interruption as present in several other positions of natural collagens.[22,36] Nevertheless, incorporation of "weak helix" triplets into the collagenous heterotrimer was expected to destabilize the triple-helical fold despite the built-in cystine knot.

In the present study we have, therefore, addressed the question of how the functional epitope of the collagenase cleavage site of collagen type I, if arranged in an α1α2α1′ register, can be stabilized in the conformation to possibly mimic its natural fold in the intact collagen type I. For this purpose various heterotrimers were synthesized which are cross-linked at the C-terminus by the cystine knot and differ in the position and number of the "strong helix" tripeptide repeats Gly-Pro-Hyp (Figure 2).

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heterotrimer A

α*1* H_2N-GPOGPOGPOGPQGIAGQRGVVGCGG
α*2* H_2N-GPOGPOGPOGPQGLLGAOGILGCCGG
α*1′* H_2N-GPOGPOGPOGPQGIAGQRGVVGLCGG

heterotrimer B

α*1* H_2N-GPOGPOGPOGPOGPOGPQGIAGQRGVVGCGG
α*2* H_2N-GPOGPOGPOGPOGPOGPQGLLGAOGILGCCGG
α*1′* H_2N-GPOGPOGPOGPOGPOGPQGIAGQRGVVGLCGG

heterotrimer C

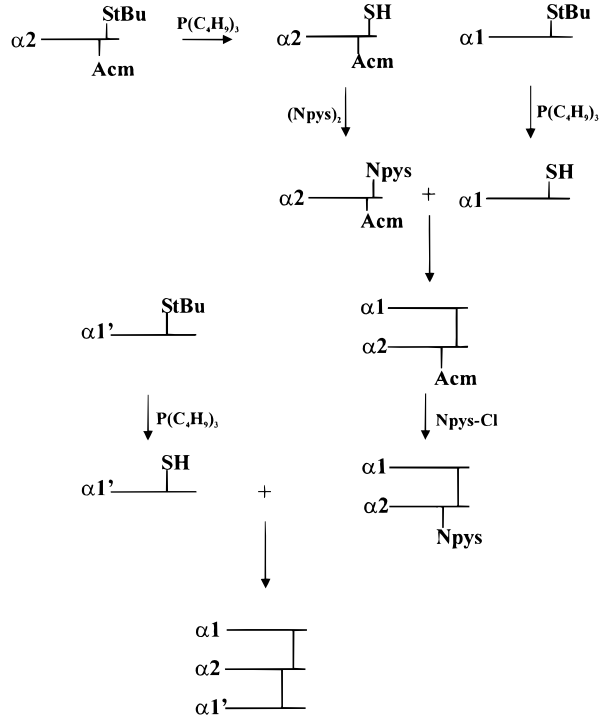
α*1* Ac-GPOGPOGPOGPOGPOGPQGIAGNRGVVGCG
α*2* Ac-GPOGPOGPOGPOGPOGPQGLLGAOGILGCCG
α*1′* H_2N-GPOGPOGPOGPOGPOGPQGIAGQRGVFGLCG

heterotrimer D

α*1* Ac-POGPOGPOGPOGPOGPOGPQGIAGQRGVVGPOGCG
α*2* Ac-OGPOGPOGPOGPOGPOGPQGLLGAOGILGPOGCCG
α*1′* H_2N-GPOGPOGPOGPOGPOGPQGIAGQRGVVGPOGPCG

Figure 2. Amino acid composition of the synthetic heterotrimers A, B, C, and D; the single peptide chains of the trimers are defined by the letter of the trimer, e.g., α1A is the α1 chain of the trimer A.

Scheme 1. Regioselective Assembly of the α1, α2, and α1′ Cysteine Peptides into Heterotrimers with the α1α2α1′ Register.



Results

Synthesis of the Heterotrimeric Collagen Peptides. Purification of collagen[37] and synthetic collagen fragments[38,39] by chromatographic procedures is known to be particularly difficult. Therefore, the synthesis of the single-chain collagen peptides on solid support by the Fmoc/tBu chemistry[40] had to be extensively optimized to allow for their production in good and reproducible yields as homogeneous materials.[41] For this purpose the use of Fmoc-Pro-Hyp-Gly-OH as synthon in analogy to previous syntheses performed by Sakakibara et al.[14,42] with Boc-Pro-Xaa-Gly-OH [Xaa = Pro, Hyp(Bzl)], proved to be the best strategy since cleavage of N-terminal dipeptides via diketopiperazine formation in the chain elongation steps was fully suppressed. Thereby, protection of the Hyp hydroxyl function was not required except when chain elongation was performed with this single residue. In these cases Fmoc-Hyp(tBu)-OH was employed to prevent O-acylation with subsequent O → N acyl shift during base-catalyzed Fmoc-cleavage. Moreover, to avoid side reactions at the level of cysteine residues linked directly to the solid support,[43] these were spaced with a Gly residue or with Gly-Gly.

For assembly of the three α-chains via the cystine-knot into the heterotrimer in a defined α1/α2/α1′ register a new strategy of synthesis had to be elaborated that allows for selective cysteine pairing in the presence of other protected or already disulfide-linked Cys residues. In fact, thiol-induced disproportionation of preformed unsymmetrical disulfides was found to be remarkably favored by the high propensity of collagenous peptides for self-association. For this regioselective disulfide bridging various known procedures were examined, but either they failed or the heterotrimers were obtained only in modest overall yields.[44] Finally, a procedure was developed which in principle should allow the assembly of an unlimited number of cysteine peptides via a cystine ladder into multiple-stranded polypeptides, unless tryptophan is present in the peptide chains. Following the strategy outlined in Scheme 1, the Cys residues of the α1 and α1′ chain as well as one Cys residue of the α2 chain were protected as *S-tert*-butylthio derivatives which were selectively reduced with tributylphosphine to cysteine peptides, whereas the second Cys residue of the α2 chain was protected as an acetamidomethyl derivative to allow for a selective activation of this residue by reaction with 3-nitropyridyl-2-sulfenyl chloride (Npy-Cl).[28] With this orthogonal cysteine protection the thiol functions were activated in a defined order as nitropyridylsulfenyl (Npys) derivatives for subsequent regioselective disulfide bridging under exclusion of air oxygen. Reactions were performed under slightly acidic conditions (pH 4.5−5.0) to prevent undesired thiol/disulfide exchange reactions. By applying this procedure the desired disulfide bridges were formed almost quantitatively, thus allowing for assembly of the three α chains into the heterotrimers A, B, C, and D (Figure 2) in good overall yields. For the analytical characterization of the protein-sized heterotrimers (M_r = 6800−9400) HPLC and mass spectrometry were used, as shown exemplarily in Figure 3 for the trimer B. The heterotrimeric 1:1:1 composition of α1α2α1′ constructs was confirmed by HPLC upon reduction with tributylphosphine.

Oxidative Refolding of Heterotrimeric Collagen Peptides. Previous refolding studies of noncrosslinked natural heterotrimeric collagen fragments revealed a pronounced tendency for

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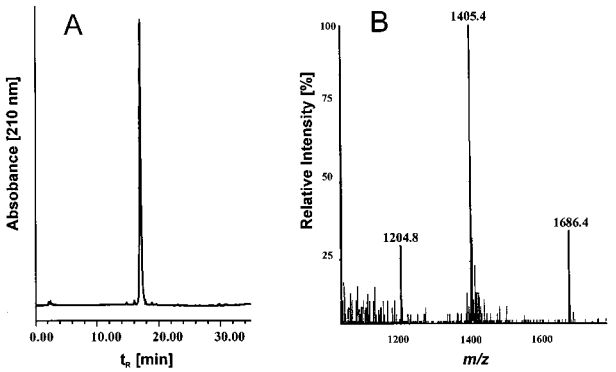

Figure 3. Analytical characterization of the heterotrimer B: (A) HPLC (for experimental conditions see Eperimental Section) and (B) ESI-MS m/z = 8427.6 $[M]^+$; M_r = 8427.2 calcd for $C_{362}H_{559}N_{104}O_{119}S_4$.

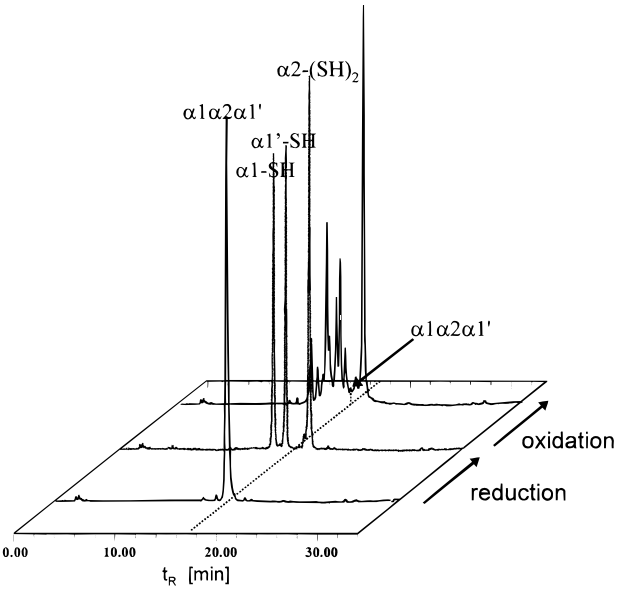

Figure 4. HPLC of heterotrimer B before and after reduction with tributylphosphine to the α1, α2, and α1′ monomeric peptide components, and upon reoxidation by air oxygen for 48 h at room temperature in 50 mM phosphate buffer (pH 7) and at 0.12 mM concentration.

homotrimeric reassembly,[45] which seems to be a general phenomenon encountered in renaturing collagens.[27] Oxidative refolding of the reduced heterotrimer B was performed under air oxygen at room temperature in 50 mM phosphate buffer (pH 7) and at 0.12 mM concentration. HPLC of the reaction solution after 48 h revealed a very complex mixture of products among which the heterotrimer B was formed only in trace amounts (Figure 4). The structural information encoded in the collagenase cleavage site apparently is not at all sufficient to overcome the intrinsic tendency of collagen peptides for self-association into homomeric structures.

Oxidative refolding experiments performed on an equimolar mixture of synthetic α1, α2, and α3 peptides containing the sequence portion related to the cystine knot of collagen type IX extended C-terminally by the noncollagenous NC1 domain, was found to generate the heterotrimer with the correct disulfide network as main product.[46] Conversely, oxidative refolding experiments of the peptide GYCDSSCAG that represents just the sequence of the cystine knot failed. The N-terminal extension

Table 1. Circular Dichroism Data of Monomeric Collagenous Peptides Containing the Collagenase Cleavage Site of Collagen Type I in 50 mM Tris/HCl, pH 7.4 (10 mM $CaCl_2$, 50 mM NaCl) at 4 °C

collagenous peptides	max (nm, Θ_R)	min (nm, Θ_R)	Rpn	T_m (°C)
α1A	222 (75)	197 (−15000)	0.005	
α1B	223 (500)	198 (−22500)	0.022	
$(GPO)_5GPQG$	224 (2600)	198 (−24000)	0.108	18
α1D	223 (550)	198 (−24000)	0.023	

of this COL1−NC1 junction peptide with the triple-helix stabilizing $(Gly\text{-}Pro\text{-}Hyp)_3$ led in reoxidation experiments at −20 °C where triple-helical structure was detected, to about 10% homotrimer containing most probably the native cystine knot.[47] These results would indicate that a concerted action between triple-helical structure and specific recognition of the α subunits in the globular domains is required for the correct reassembly into heterotrimers.

Dichroic Properties of the Monomeric Collagenous Peptides. The triple-helical conformation of collagenous peptides is characterized by a circular dichroism (CD) spectrum with a relatively strong positive maximum (2000−6000 $[\Theta_R]$) centered at 220−223 nm and an intense negative band (−30000 to −55000 $[\Theta_R]$) at 196−200 nm. The poly(Pro)-II helical peptides are known to exhibit a very similar CD spectrum with a slightly red-shifted positive band at 224−226 nm and a negative maximum located at 198−205 nm of weaker intensity. Despite these largely overlapping CD spectra[48] a differentiation between the two types of conformations can be derived from the ratio of the dichroic intensities of the positive band over that of the negative maximum (Rpn). This Rpn parameter has been recently validated by a careful comparative CD and NMR conformational analysis of $(Gly\text{-}Pro\text{-}Hyp)_n$ peptides and their homotrimers cross-linked with Kemp triacid.[49]

The overall pattern of the CD spectra of the α1A (for nomenclature see Figure 2) and α1B peptides with their $(Gly\text{-}Pro\text{-}Hyp)_3$ and $(Gly\text{-}Pro\text{-}Hyp)_5$ N-terminal extensions, as well as that of the α1D peptide where the collagenase cleavage sequence is extended even C-terminally with a Gly-Pro-Hyp repeat and the N terminus acetylated, excludes the presence of a triple-helical structure even at 4 °C (Table 1). Conversely, the N-terminal portion of the α1B peptide, i.e., $(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Gln-Gly, exhibits a CD spectrum that is very similar to the CD spectrum of Ac-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$ for which the triple-helical conformation was well-assessed.[49] Moreover, its thermal denaturation leads to a sigmoidal transition curve with a T_m of 18 °C, again identical to that reported for peptides consisting of 5 Gly-Pro-Hyp repeats.[35,49] Conversely, the α1A, α1B, and α1D peptides show monotonic transitions without the cooperativity that is characteristic for the unfolding of the collagen triple helix.[48,50] These results confirm that the amino acid composition at the collagenase cleavage site of collagen type I is far from the ideal one for a triple helix despite the presence of a Gly residue in every third sequence position.[51] Surprising, however, was the inability of the $(Gly\text{-}Pro\text{-}Hyp)_5$

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Table 2. Circular Dichroism Data of Heterotrimeric Collagenous Peptides Containing the Collagenase Cleavage Site of Collagen Type I in 50 mM Tris/HCl, pH 7.4 (10 mM $CaCl_2$, 50 mM NaCl) at 4 °C

collagenous peptides	max (nm, Θ_R)	min (nm, Θ_R)	Rpn	T_m (°C)
heterotrimer A	222 (2100)	199 (−18200)	0.115	9
heterotrimer B	222 (4160)	196 (−34000)	0.122	33
heterotrimer C	222 (4210)	197 (−33500)	0.126	33
heterotrimer D	223 (4350)	197 (−33700)	0.129	41
collagen type I	222 (4500)	196 (−34600)	0.130	38

extension, which per se folds into the triple helix, to retain this property in the α1B peptide. Even the introduction of an additional Gly-Pro-Hyp repeat at the C-terminus in the α1D peptide does not suffice for inducing self-association of the single chain into a stable triple-helical fold (Table 1). These results agree with previous conformational studies performed on monomeric peptides that contain natural sequence portions of collagens related to cell adhesion or collagenase cleavage sites.[52−54] Both N- and C-terminal $(Gly\text{-}Pro\text{-}Hyp)_n$ tails were required for stabilizing the triple helix.

Dichroic Properties of the Heterotrimeric Collagenous Peptides. The heterotrimers A, B, C, and D are all folded into more or less stable triple-helical structures as evidenced by the Rpn values at 4 °C reported in Table 2. Thus, C-terminal cross-linking of the three α chains, which show no tendency for self-association into triple helices despite their relatively large-size, exerts a dramatic effect on the conformational preferences. This observation strongly suggests that even the simple artificial cystine knot used for construction of the heterotrimers is capable not only of inducing and stabilizing entropically the triple helix but also acts itself for nucleation of supercoiled structures. As shown in Figure 5 by the positive maxima of the CD spectra of the heterotrimers A, B, C, and D, the extension of the N-terminal $(Gly\text{-}Pro\text{-}Hyp)_n$ repetitive sequence from $n = 3$ to $n = 5$ leads to a significant increase in dichroic intensity. Conversely, N-terminal acetylation of two α chains (trimer C) to suppress charge repulsion at the N-terminus of the triple helix or the introduction of an additional tripeptide repeat near the cystine knot, as well as filling the gaps at the N-terminus of the one-residue shifted chains in trimer D to increase the number of hydrogen bonds in this region, results only in minor enhancements of the CD intensities. It leads, however, to Rpn values (Table 2) very close to the value of collagen in water.[49]

Thermal Denaturation of Heterotrimeric Collagenous Peptides. The thermal denaturation curves of the heterotrimers A, B, C, and D, as monitored by the decrease of dichroic intensity at 222 nm as a function of temperature, are reported in Figure 6. For all four heterotrimers a plateau value is observed at lower temperatures that confirms the onset of a stable conformational state consisting mainly of the triple-helical fold according to the Rpn values determined at 4 °C. Upon raising the temperature a highly cooperative transition takes place with the T_m values listed in Table 2. The trimer A with the $(Gly\text{-}Pro\text{-}Hyp)_3$ extension is folded into the less stable triple helix with a T_m of 9 °C. Correspondingly, it is fully unfolded to a gelatin-type state already at room temperature. N-terminal extension by two additional Gly-Pro-Hyp repeats (heterotrimer B) leads to a significant increase of the T_m value to 33 °C. In

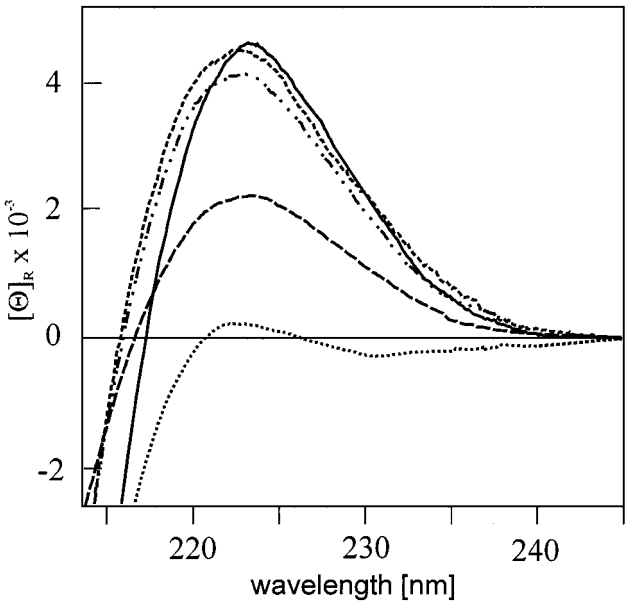

Figure 5. CD spectra in the 215−245 nm range of the monomeric α1A peptide (···), heterotrimer A (− − −), heterotrimer B (−···), heterotrimer C (- - -), and heterotrimer D (—) at 4 °C in 50 mM Tris/HCl, pH 7.4 (10 mM $CaCl_2$, 50 mM NaCl).

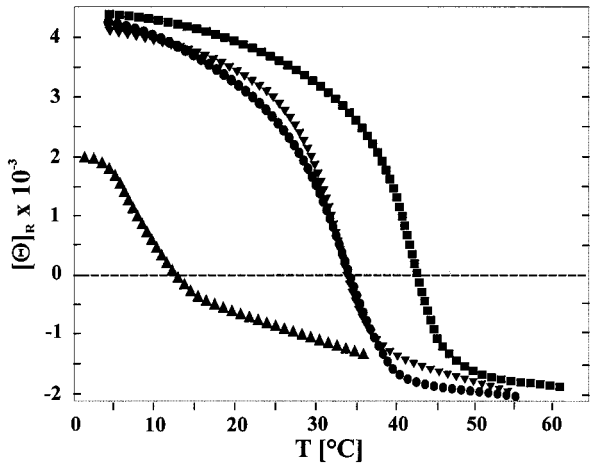

Figure 6. Thermal denaturation of the synthetic heterotrimers in 50 mM Tris/HCl, pH 7.4 (10 mM $CaCl_2$, 50 mM NaCl) as monitored by changes in dichroic intensities at 222 nm: hetrotrimer A (▲), B (▼), C (●), and D (■).

the heterotrimer C where two α chains are acetylated, the lack of charge repulsion at the N-terminus was expected to stabilize the triple-helical fold. In fact, an increase of the Rpn value at 4 °C (Table 2) was observed. Additionally, two mutations were performed in this trimer C at the level of the collagenase cleavage sequence, i.e., $Gln^{23} \rightarrow Asn^{23}$ in the α1 chain and $Val^{27} \rightarrow Phe^{27}$ in the α1′ chain. In the lower temperature range the dichroic intensities decrease faster and in monotonic manner than in the case of trimer B. This fact should reflect a reduced stability of the triple-helical fold of the collagenase cleavage site of the molecule induced by the two mutations. Taking into account the strong effects of solvation on the stability of the collagen structure, mainly the Val → Phe replacement that causes an enhancement of the local surface hydrophobicity, could account for the observed lower T_m value. For the heterotrimer D with its additional Gly-Pro-Hyp repeat adjacent to the cystine knot and with the filled gaps in the N-terminus, an additional stabilization of the 3D structure was obtained with

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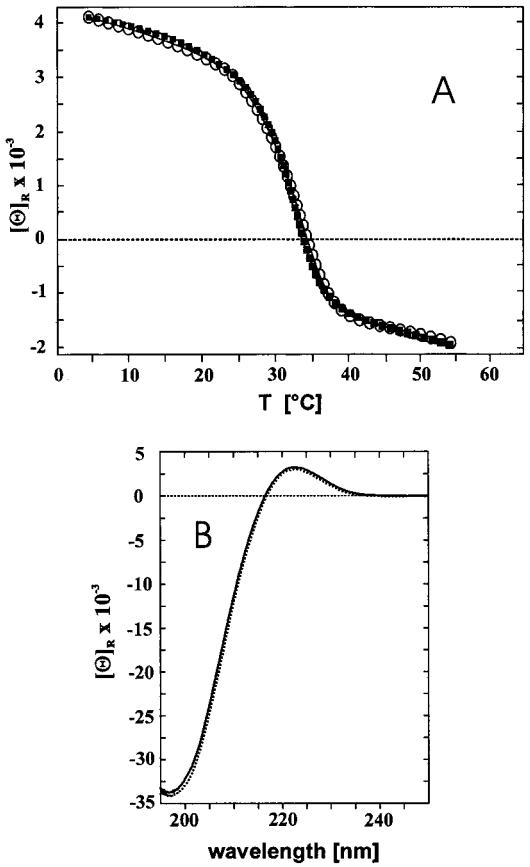

Figure 7. (A) thermal unfolding (■) and refolding (○) of the heterotrimer B in 50 mM Tris/HCl, pH 7.4 (10 mM $CaCl_2$, 50 mM NaCl) as monitored by changes in the dichroic intensity at 222 nm; heating and cooling rates: 0.3 °C/min; (B) CD spectra of the heterotrimer B at 4 °C prior thermal denaturation (—) and after refolding (···).

an almost ideal Rpn value of 0.129 and a T_m of 41 °C that is well above the room temperature.

Refolding of Heterotrimeric Collagenous Peptides. Refolding of thermally denatured collagen fragments and collagenous peptides is known to be a very slow process that requires days and does not occur quantitatively in all cases,[27,55] whereas for the triple helix formation in collagens and procollagens which contain various types of interchain cross-links half times of 30 min to several hours were measured.[26,27] As shown in Figure 8 for the heterotrimer D, after a temperature jump from 53 to 3 °C refolding of the disulfide-bridged heterotrimer at 3 °C is a very fast process consisting of a first kinetically unresolved and a second slower phase with a rate constant of $k = 5.6 \cdot 10^{-4}$ s^{-1}. A similarly fast refolding in two phases has previously been reported for the Col 1−3 fragment derived from the N-terminal propeptide of pN-collagen III[25] and for synthetic collagenous homotrimers cross-linked with the 6-aminohexanoyl-di-lysine template.[56,57]

Thermodynamic Characterization of the Heterotrimeric Collagenous Peptides. Thermal denaturation of natural and synthetic collagenous peptides is generally regarded as a two-state transition.[27] Even for collagenous molecules containing tripeptide repeats of differentiated triple-helical propensity

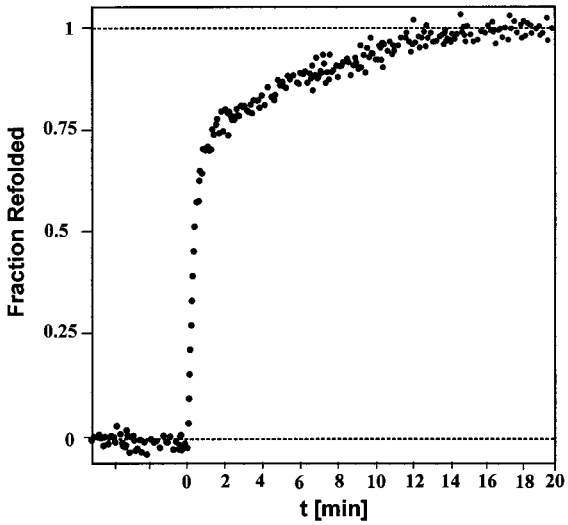

Figure 8. Kinetics of the triple helix refolding of the heterotrimer D in 50 mM Tris/HCl, pH 7.4 (10 mM $CaCl_2$, 50 mM NaCl) after quenching the solution from 53 °C to 3 °C as determined by changes in dichroic intensity at 222 nm. From the semilogarithmic plot of the experimental data against time a first-order rate constant $k_{exp} = 5.6 \cdot 10^{-4}$ s^{-1} was derived.

Table 3. Thermodynamic Data Derived from the Reversible Thermal Folding/Unfolding of Heterotrimeric Collagenous Peptides Containing the Collagenase Cleavage Site of Collagen Type I in 50 mM Tris/HCl, pH 7.4 (10 mM $CaCl_2$, 50 mM NaCl)

collagenous peptides	$\Delta H°$ [kJ mol^{-1}]	$\Delta S°$ [J mol^{-1} K]	$\Delta G°$ (T_m) [kJ mol^{-1}]	T_m [K]
heterotrimer A	−353	−1084	−47	282
heterotrimer B	−416	−1190	−51	306
heterotrimer C	−367	−1031	−51	306
heterotrimer D	−438	−1226	−53	314

multiphasic transitions were not observed unless well-differentiated structural domains are present in the whole molecule as in the case of collagen type IV in which the main triple helix is frequently interrupted by noncollagenous segments.[58] For the heterotrimeric collagenous peptides cross-linked at the C-terminus, unfolding of the triple helix has to start at the opposite end which at the same time represents the triple-helix stabilizing portion of the molecule. According to the Rpn values even the collagenase cleavage portion of the molecule with its weak triple-helix propensity is folded into this supercoiled structure. However, enhanced local conformational flexibility in this part of the molecule is suggested by the initial monotonic denaturation profiles and is further supported by the effects observed in the lower temperature range upon mutation of single residues. Nevertheless, the process of unfolding of the less stable triple-helical portion is not independent and overlaps with the total unfolding process. Correspondingly, the curves reflect the cooperative thermal unfolding of the whole molecules. Thermal denaturation of the four heterotrimers was found to be a fully reversible process as was well-evidenced by the superimposable unfolding and refolding curves obtained at identical heating and cooling rates (Figure 7). Correspondingly, thermodynamic data were derived from this process at equilibrium which are reported in Table 3.

Discussion

To study the process of self-association of collagenous peptides into collagen-like triple helices and to uncover factors

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that stabilize this triple-helical fold, synthetic peptides consisting of Gly-Xaa-Yaa repeats were used.[35,59,60] To overcome the unfavorable entropy of self-association of these peptides and to facilitate nucleation of the triple-helix, their N- and/or C-terminal cross-linking with polyfunctional templates such as Lys-Lys-Gly,[39,56,61−63] Glu-Glu,[64] or 1,2,3-propanetricarboxylic acid[56] to homotrimers has been extensively investigated whereby spacers differing in length and flexibility were applied to allow a staggered alignment of the three chains in the triple-helical fold. More recently the conformationally constrained Kemp triacid,[65] i.e., *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid, with its functionalities aligned in parallel orientation was found to represent a very efficient template in terms of triple-helix nucleation and stabilization.[49,66,67] However, cross-linking of different peptide chains on such templates to produce heterotrimers that mimic functional epitopes of collagens would require a highly selective and sophisticated chemistry, and thus efficient syntheses are difficult to achieve.[68] In this context our approach based on a mimicry of the natural cystine knots in collagens proved to be an efficient and synthetically more feasible procedure.

Although the cystine knot used to assemble the heterotrimers is largely simplified in the cystine connectivities if compared to that of natural collagens, e.g., that of the Col 1−3 fragment,[25] refolding kinetics revealed a surprisingly homologous effect in terms of triple-helix nucleation. In the structural model of our heterotrimers the disulfide bonds do not disturb the triple helix, and all possible hydrogen bonds between glycine and the residue in position 2 in the adjacent chain can be formed without distortions or CDM penetration of the van der Waals radii as reported for the structural model of the Col 1−3 fragment.[25] Nucleation of the triple helix consists of finding the first three tripeptide units in the correct alignment and interaction via the proper hydrogen bonds.[25] This was recently confirmed with the homotrimer $(Hyp\text{-}Pro\text{-}Gly)_3$ linked to Kemp triacid, where CD and NMR conformational analysis indicated the onset of an incipient triple helix.[67] This first step of triple-helix formation is thought to be less probable than the subsequent helix propagation steps. The disulfide bridges impart constraints on the adjacent residues and apparently keep these in an arrangement that closely resembles the nucleus of the triple helix such that this first event is no longer the slowest process. In fact, as shown in Figure 8 for the refolding of heterotrimer D, a time-unresolved very fast first phase is observed even at 3 °C which is followed by a second slower process. The rate constant of $5.6 \cdot 10^{-4}$ s^{-1} determined for the slow phase in the case of the trimer D at 3 °C is slower than the rate constant of $8 \cdot 10^{-3}$ s^{-1} determined for the slow phase of the refolding process of the Col 1−3 fragment at 20 °C.[25] This second slow-phase process was attributed to the rate of the zipper-mode propagation of the triple helix which is dominated by the *cis* → *trans* isomerization of the first imino acid encountered in a *cis*-configuration.[27] The difference in the refolding kinetics observed for the trimer D and the natural fragment may reasonably be explained by the lower temperature applied in the case of the trimer D (3 vs 20 °C) which significantly affects the rate of *cis* → *trans* isomerization.

In conclusion, with the artificial C-terminal cystine knot and the N-terminal stabilization of the triple helix with (Gly-Pro-Hyp) tripeptide repeats, the collagenase cleavage sequence of collagen type I was forced into an overall triple-helical fold. Conversely, the α1(I)CB-7 fragment, a 36-membered peptide that contains the cleavage site,[19] and the shorter 15-membered synthetic peptide[69] were unable to self-associate into structured homotrimers.

It is a general tenet of collagen research that the cystine knots are formed after registration of the three chains by specific interactions between the propeptide domains and that it is not the sequence-encoded information in and around the multiple-cysteine sequence portion that dictates the correct assembly of the chains.[10,27] The role of the cystine knots is, therefore, to stabilize the triple helix in the staggered register of the three α subunits. Correspondingly, even in the design of the heterotrimers we had to select a priori a defined register for the collagenase cleavage sequences α1(772−784) and α2(772−784) of collagen type I on the basis of the proposed α1α2α1 subunit alignment.[29] The interstitial collagenases MMP-1 and, in an even more efficient manner, MMP-8 are known to process collagen type I at this locus with a single scission across all three α-chains into a $^3/_4$ and $^1/_4$ collagen fragment. Because of the high specificity of this cleavage site, it is reasonable to assume that this substrate epitope exhibits very peculiar structural features which may strongly depend on the register of the α-chains. The heterotrimer B, as reported elsewhere,[28] and the heterotrimer D in the α1/α2/α1′ register, are cleaved in a single cut into the two expected fragments without detectable intermediates in the time course of the relatively slow enzymatic processing by MMP-1 and MMP-8. These results strongly suggest that the selected α1α2α1′ alignment represents indeed the register of native collagen type I.

Experimental Section

Materials and Methods. All reagents and solvents used in the synthesis were of the highest quality commercially available. Amino acid derivatives were purchased from Alexis (Grünberg, Germany) or were prepared according to standard protocols. TentagelGel-S-PHB-resin was from Rapp (Tübingen, Germany). Analytical HPLC was carried out on Nucleosil 300/C8 (Machery & Nagel, Düren) using a linear gradient of acetonitrile/2% H_3PO_4 from 5:95 to 80:20 in 30 min and preparative HPLC on Nucleosil 5 C18 PPN with a linear gradient of acetonitrile (0.08% trifluoroacetic acid (TFA))/0.1% TFA from 15:85 to 60:40 in 70 min. Gel chromatography was carried out on Fraktogel HSK 40 with 0.5% AcOH as eluent. Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectra were recorded on a Bruker Reflex II instrument, fast atom bombardment mass spectrometry (FAB-MS) on Finnigan MAT 900 and electron spray ionization mass spectrometry (ESI-MS) on PE Sciex API 165 (Perkin-Elmer). Amino acid analyses of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid, 110 °C; 72 h) were performed on a Biotronic analyzer (LC 6001).

Peptide Synthesis. The single α-chains were synthesized on TentagelGel-S-PHB-resin using optimized procedures reported elsewhere.[41]

α1(StBu) Chains. H-(Gly-Pro-Hyp)$_3$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Cys(StBu)-Gly-Gly-OH, α1(StBu)A:

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16% yield; HPLC t_R 15.62 min; MALDI-TOF-MS $m/z = 2302.6$ $[M]^+$; $M_r = 2302.1$ calcd for $C_{97}H_{157}N_{30}O_{31}S_2$; amino acid analysis Glu 2.00, Pro 3.77 (4), Gly 9.81 (10), Ala 1.04 (1), Cys 0.89 (1), Val 1.85 (2), Ile 0.99 (1), Leu 0.94 (1), Arg 1.00 (1), Hyp 2.88 (3); peptide content 74%.

H-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Cys(StBu)-Gly-Gly-OH, α1(StBu)B: for analytical characterization see ref 41.

Ac-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Asn-Arg-Gly-Val-Val-Gly-Cys(StBu)-Gly-OH, α1(StBu)C: 12% yield; HPLC t_R 15.12 min; MALDI-TOF-MS $m/z = 2807.6$ $[M]^+$; $M_r = 2807.3$ calcd for $C_{120}H_{188}N_{35}O_{79}S_2$; amino acid analysis Asp 0.99 (1); Glu 1.00, Pro 5.58 (6), Gly 10.80 (11), Ala 1.02 (1), Cys 0.93 (1), Val 1.78 (2), Ile 0.94 (1), Arg 1.04 (1), Hyp 4.78 (5); peptide content 70%.

Ac-Pro-Hyp-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Pro-Hyp-Gly-Cys(StBu)-Gly-OH, α1-StBu D: for analytical characterization see ref 41.

α2(Acm,Npys) Chains.

The crude α2(Acm,StBu) peptides obtained upon cleavage from the resin were dissolved at 10 mM concentration in 95% aqueous trifluoroethanol (TFE) and reacted with 5 equiv of tributylphosphine. The reaction mixtures were evaporated and the residues reprecipitated from TFE with methyl *tert*-butyl ether. The 4 mM solutions of the crude α2(Acm,SH) peptides in degassed argon-saturated dimethylformamide (DMF)/AcOH (95:5) were added dropwise to a 100 mM solution of 5 equiv of di-(5-nitro-2-pyridine)-disulfide in DMF/AcOH (95:5) under exclusion of air oxygen. The reaction was monitored spectroscopically at 430 nm, and after completion, (1 to 2 h) the solvent was evaporated; the resulting residues were dissolved in water, and excess reagent was filtered off. The water was evaporated, and the residues were reprecipitated from TFE with methyl *tert*-butyl ether and purified by preparative HPLC.

H-(Gly-Pro-Hyp)$_3$-Gly-Pro-Gln-Gly-Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Cys(Acm)-Cys(Npys)-Gly-Gly-OH, α2(Acm,Npys)A: 17% yield; HPLC t_R 16.81 min; MALDI-TOF-MS $m/z = 2512.2$ $[M]^+$; $M_r = 2511.1$ calcd for $C_{106}H_{162}N_{30}O_{35}S_3$; amino acid analysis Glu 1.00, Pro 3.92 (4), Gly 9.85 (10), Ala 1.00 (1), Cys 1.94 (2), Ile 0.96 (1), Leu 3.02 (3), Hyp 3.66 (4); peptide content 68%.

H-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Cys(Acm)-Cys(Npys)-Gly-Gly-OH, α2(Acm,Npys)B: 13% yield; HPLC t_R 17.68 min; MALDI-TOF-MS $m/z = 3046.8$ $[M]^+$; $M_r = 3045.4$ calcd for $C_{130}H_{196}N_{36}O_{43}S_3$; amino acid analysis Glu 1.00, Pro 5.66 (6), Gly 11.61 (12), Ala 1.02 (1), Cys 1.96 (2), Ile 0.97 (1), Hyp 5.88 (6); peptide content 77%.

Ac-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Cys(Acm)-Cys(Npys)-Gly-OH, α2(Acm,Npys)C: 11% yield; HPLC t_R 16.08 min; MALDI-TOF-MS $m/z = 3031.9$ $[M]^+$; $M_r = 3030.3$ calcd for $C_{130}H_{195}N_{35}O_{43}S_3$; amino acid analysis Glu 1.00, Pro 5.72 (6), Gly 10.45 (11), Ala 1.10 (1), Cys 1.93 (2), Ile 0.99 (1), Leu 3.06 (3), Hyp 5.48 (6); peptide content 71%.

Ac-Hyp-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Pro-Hyp-Gly-Cys(Npys)-Cys(StBu)-Gly-OH, α2-(Acm,Npys)D: 10% yield; HPLC t_R 15.13 min; ESI-MS $m/z = 3409.6$ $[M]^+$; $M_r = 3410.5$ calcd for $C_{147}H_{219}N_{39}O_{49}S_3$; amino acid analysis Glu 1.00, Pro 6.84 (7), Gly 11.66 (12), Ala 1.03 (1), Cys 1.74 (2), Ile 0.94 (1), Leu 2.99 (3), Hyp 7.90 (8); peptide content 65%.

α1′(StBu) Chains. H-(Gly-Pro-Hyp)$_3$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Cys(StBu)-Gly-Gly-OH, α1′(StBu)A: 16% yield; HPLC t_R 16.56 min; MALDI-TOF-MS $m/z = 2416.4$ $[M]^+$; $M_r = 2415.2$ calcd for $C_{103}H_{168}N_{31}O_{32}S_2$; amino acid analysis Glu 2.00, Pro 3.80 (4), Gly 9.80 (10), Ala 1.01 (1), Cys 0.90 (1), Val 1.95 (2), Ile 0.97 (1), Arg 1.02 (1), Hyp 2.86 (3); peptide content 79%.

H-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Cys(StBu)-Gly-Gly-OH, α1′(StBu)B: 14% yield; HPLC t_R 17.82 min; MALDI-TOF-MS $m/z = 2949.2$ $[M]^+$; $M_r = 2949.4$ calcd for $C_{127}H_{202}N_{37}O_{40}S_2$; amino acid analysis Glu 2.00, Pro 5.56 (6), Gly 11.82 (12), Ala 1.06 (1), Cys 1.99 (2), Val 0.96 (1), Leu 0.93 (1), Ile 0.97 (1), Arg 1.03 (1), Hyp 4.78 (5); peptide content 76%.

H-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Phe-Val-Gly-Leu-Cys(StBu)-Gly-OH, α1′(StBu)C: 11.5% yield; HPLC t_R 16.11 min; MALDI-TOF-MS $m/z = 2940.2$ $[M]^+$; $M_r = 2940.1$ calcd for $C_{129}H_{199}N_{36}O_{39}S_2$; amino acid analysis Glu 2.00, Pro 5.66 (6), Gly 10.72 (11), Ala 1.00 (1), Cys 0.93 (1), Val 0.95 (1), Ile 0.97 (1), Phe 0.98 (1), Leu 0.96 (1), Arg 1.00 (1), Hyp 4.80 (5), peptide content 76%.

H-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Pro-Hyp-Gly-Pro-Cys(StBu)-Gly-OH, α1′(StBu)D: 18% yield; HPLC t_R 15.51 min; ESI-MS $m/z = 3143.5$ $[M]^+$; $M_r = 3142.5$ calcd for $C_{136}H_{212}N_{39}O_{43}S_2$; amino acid analysis Glu 2.00, Pro 7.84 (8), Gly 11.49 (12), Ala 1.09 (1), Cys 0.98 (1), Val 1.88 (2), Ile 0.97 (1), Arg 1.02 (1), Hyp 5.66 (6); peptide content 79%.

α1α2(Acm) Heterodimers. To 10 mM solutions of α1-chains in 95% aqueous TFE 5 equiv of tributylphosphine were added. The reaction mixtures were evaporated and the residues reprecipitated from TFE with methyl *tert*-butyl ether. The precipitates were lyophilized from 50 mM AcOH. The α1(SH) peptides were then dissolved at 4 mM concentration in degassed and argon-saturated 50 mM NH_4OAc (pH 5.5), and the solutions were added dropwise to 10 mM solutions of 1 equiv of α2(Acm,Npys) peptides in 50 mM NH_4OAc (pH 5.5) under exclusion of air oxygen. The reactions were monitored spectroscopically at 430 nm. After 2−3 h the reaction mixtures were evaporated and the heterodimers were isolated by gel chromatography or HPLC.

α1α2(Acm)A: 82% yield; HPLC t_R 15.42 min; MALDI-TOF-MS $m/z = 4569.6$ $[M]^+$; $M_r = 4567.2$ calcd for $C_{194}H_{305}N_{58}O_{64}S_3$.

α1α2(Acm)B: 68% yield; HPLC t_R 16.45 min; MALDI-TOF-MS $m/z = 5638.4$ $[M]^+$; $M_r = 5637.7$ calcd for $C_{242}H_{373}N_{70}O_{80}S_3$.

α1α2(Acm)C: 70% yield; HPLC t_R 14.23 min; MALDI-TOF-MS $m/z = 5593.5$ $[M]^+$; $M_r = 5591.6$ calcd for $C_{241}H_{369}N_{68}O_{80}S_3$.

α1α2(Acm)D: 55% yield; HPLC t_R 14.22 min; ES-MS $m/z = 6461.8[M]^+$; $M_r = 6463.0$ calcd for $C_{281}H_{426}N_{77}O_{93}S_3$.

Activated α1α2(Npys) Heterodimers. To 50 mM solutions of the heterodimers α1α2(Acm) in TFA/AcOH (1:2) 1.1 equiv of freshly prepared 3-nitro-2-pyridine-sulfenyl chloride[70] was added. After 30 min the bulk of the solvent was evaporated, and the residues were purified by gel chromatography.

α1α2(Npys)A: 77% yield; HPLC t_R 17.22 min; MALDI-TOF-MS $m/z = 4651.8$ $[M]^+$; $M_r = 4651.3$ calcd for $C_{196}H_{302}N_{59}O_{64}S_4$.

α1α2(Npys)B: 71% yield); HPLC t_R 18.32 min; MALDI-TOF-MS $m/z = 5720.5$ $[M]^+$; $M_r = 5719.8$ calcd for $C_{244}H_{370}N_{69}O_{80}S_4$.

α1α2(Npys)C: 74% yield; HPLC t_R 16.42 min; MALDI-TOF-MS $m/z = 5676.8$ $[M]^+$; $M_r = 5675.7$ calcd for $C_{243}H_{366}N_{69}O_{80}S_4$.

α1α2(Npys)D: 70% yield; HPLC t_R 15.42 min; ESI-MS $m/z = 6546.0$ $[M]^+$; $M_r = 6547.1$ calcd for $C_{283}H_{422}N_{77}O_{94}S_4$.

α1α2α1′ Heterotrimers. The α1′ peptides were dissolved in 95% aqueous TFE (10 mM) and reduced with 5 equiv of tributylphosphine. The bulk of the solvent was evaporated, and the residues were reprecipitated from TFE with methyl *tert*-butyl ether and then lyophilized from 50 mM AcOH. Solutions of the α1′(SH) peptides in degassed argon-saturated 50 mM NH_4OAc (pH 5.5) at 4 mM concentration were added dropwise to 10 mM solutions of 1 equiv of the activated heterodimers α1α2(Npys) in 50 mM NH_4OAc (pH 5.5) under exclusion of air oxygen. The reaction was monitored spectroscopically at 430 nm and by HPLC. After 2−3 h the bulk of the solvent was removed and the heterotrimers were isolated by preparative HPLC (heterotrimers A, B, and C) or gel chromatography (heterotrimer D).

α1α2α1′A: 76% yield; HPLC t_R 14.56 min; MALDI-TOF-MS $m/z = 6824.9$ $[M]^+$; $M_r = 6821.3$ calcd for $C_{290}H_{457}N_{88}O_{95}S_4$; amino acid analysis Glu 5.00, Pro 17.10 (18), Gly 28.79 (30), Ala 3.05 (3), Cys 4.23 (4), Val 3.28 (4), Ile 2.95 (3), Leu 4.08 (4), Arg 2.09 (2), Hyp 9.99 (10); peptide content 76%.

α1α2α1′B: 35% yield; HPLC t_R 16.42 min; ESI-MS $m/z = 8427.6$ $[M]^+$; $M_r = 8427.2$ calcd for $C_{362}H_{559}N_{104}O_{119}S_4$; amino acid analysis: Glu 5.00, Pro 17.10 (18), Gly 35.25 (36), Ala 3.02 (3), Cys 4.30 (4),-Val 3.25 (4), Ile 2.97 (3), Leu 4.07 (4), Arg 2.11 (2), Hyp 15.78 (16); peptide content 78%.

α1α2α1′C: 55% yield; HPLC t_R 14.95 min; MALDI-TOF-MS $m/z = 8372.6$ $[M]^+$; $M_r = 8371.0$ calcd for $C_{363}H_{552}N_{103}O_{118}S_4$; amino acid analysis Glu 4.00, Asp 0.99 (1), Pro 17.21 (18), Gly 32.09

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(33), Ala 3.02 (3), Cys 4.30 (4), Val 2.65 (3), Phe 1.02 (1), Ile 2.97 (3), Leu 4.06 (4), Arg 2.21 (2), Hyp 15.56 (16); peptide content 78%.

α1α2α1′D: 73% yield; HPLC t_R 14.78 min; MALDI-TOF-MS m/z = 9443.1 $[M]^+$, M_r = 9443.4 calcd for $C_{410}H_{621}N_{116}O_{136}S_4$; amino acid analysis Glu 5.13 (5), Pro 21.36 (21), Gly 30.96 (31), Ala 3.01 (3), Cys 3.64 (4), Val 3.10 (4), Ile 3.02 (3), Leu 2.75 (3), Arg 2.00, Hyp 19.83 (20); peptide content 79.5%.

N-Terminal Peptide of the α1B Chain. H-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-OH was synthesized in the same manner as the α-chains by the optimized procedures reported elsewhere[41] and purified by preparative HPLC; 10% yield; HPLC t_R 9.84 min; MALDI-TOF-MS m/z = 1693.9 $[M]^+$; M_r = 1692.8 calcd for $C_{74}H_{108}N_{20}O_{26}$; amino acid analysis Glu 1.00, Pro 5.35 (6), Gly 7.67 (7), Hyp 4.45 (5); peptide content 66%.

CD Measurements. The CD spectra were recorded on a Jobin-Yvon dichrograph Mark IV equipped with a thermostated cell holder and connected to a data station for signal averaging and processing. All spectra are averages of 10 scans, and the spectra were recorded, employing quartz cells of 0.1-cm optical path length. The spectra are reported in terms of ellipticity units per mole of peptide residues (Θ_R). The concentrations were determined by weight and peptide content as it was determined by quantitative amino acid analysis of the peptides. Solutions of the peptides (0.2 mg/mL) were prepared in the collagenase assay buffer (10 mM $CaCl_2$, 50 mM NaCl, 50 mM Tris/HCl, pH 7.4) and pre-equilibrated for 12 h in an ice-bath. Melting curves were determined by monitoring the changes in dichroic intensity at 222 nm every 3 s using a heating rate of 0.3 °C/min with a Lauda RKS thermostat. The temperature jump refolding experiments were performed by equilibrating the system at 53 °C for 15 min. The solution was then cooled to 3 °C in 2 s. The refolding kinetics were monitored at 222 nm every 2 s for 30 min.

The van't Hoff enthalpy (ΔH°) was calculated from the triple-helical transition curves using eq 1

$$\Delta H^\circ = 8\, RT_m^{\,2}\, (dF/dT)_{F=0.5} \quad (1)$$

where F refers to the fraction of triple helicity.[51] The standard entropy (ΔS°) was calculated using eq 2

$$\Delta S^\circ = \Delta H^\circ/T_m - R \ln(0.75c^2) \quad (2)$$

where c is the concentration of the collagenous peptides and R is the gas constant.

Molecular Modeling. Molecular modeling was performed on a Silicon Graphics Workstation (INDIGO). The collagen coordinates were obtained from the Brookhaven National Laboratory Protein Databank (accession code 1CLG). The complex was submitted to 1000 steps of energy minimization with the "steepest descent" algorithm and "conjugated gradient" algorithm using the InsightII molecular modeling and energy minimization programs.[71]

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