# Heterotrimeric Collagen Peptides as Fluorogenic Collagenase Substrates: Synthesis, Conformational Properties, and Enzymatic Digestion†

J. Constanze D. Müller, Johannes Ottl,‡ and Luis Moroder*

*Max Planck Institute of Biochemistry, D-82152 Martinsried, Germany*

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ABSTRACT: 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-chains and the sequence portion 772−784 ($P_4$−$P_9'$) of the α2-chain, were assembled in an α1α2α1′ register by C-terminal cross-linking of these peptides with an artificial cystine knot. The triple-helical conformation of the construct was stabilized by N-terminal extensions with $(Gly\text{-}Pro\text{-}Hyp)_5$ repeats. The gaps in the sequence alignment were filled up, and the α1-chain was dansylated and the α1′-chain was acylated with a tryptophan residue to place in spatial proximity the two chromophores for an efficient fluorescence resonance energy transfer. Although the incorporation of the two N-terminal chromophores leads to partial destabilization of the overall triple-helical fold, the heterotrimer behaved as a collagen-like substrate of the matrix metalloproteinases MMP-1 and MMP-13. Cleavage of the fluorogenic heterotrimer leads to a 6-fold increase in fluorescence intensity, thus making it a useful fluorogenic substrate for interstitial collagenases. With this folded heterotrimeric collagen molecule it was shown that fluorescence resonance energy transfer, as applied so far only for the design of linear fluorogenic enzyme substrates, can also be exploited in conformation dependency.

Collagen is the most abundant tissue protein that serves as structural material, but also as anchorage, for all stationary cells and as a track for cell migration. Each collagen molecule is composed of three identical or of two or three different α-chains of primarily repeating Gly-Xaa-Yaa triplets with Xaa being mainly proline and Yaa hydroxyproline. This sequence composition induces 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 supercoil. The high content of proline and hydroxyproline in positions Xaa and Yaa, respectively, and the presence of glycine at every third sequence position stabilizes the triple-helical structure by an extensive hydrogen-bond and hydration network (*1*−*4*). In the turnover of the extracellular matrix the degradation of collagen by matrix metalloproteinases (MMPs)[1] plays a decisive role not only in processes of organogenesis and differentiation but also in pathophysiological processes such as tumor metastasis and rheumatoid diseases. Thereby, fibrillar type I, II and III collagens are cleaved by vertebrate interstitial collagenases in a highly specific manner by a single scission across all three α-chains, generating characteristic $^3/_4$ and $^1/_4$ fragments (*5*−*8*).

To study these processes of collagen catabolism, the use of natural collagens is troublesome due to the solubility problems and gelating properties. Nonetheless, radiolabeled collagen is commonly applied, which is produced by reaction of natural collagens with [$^{14}$C]acetic anhydride or even biosynthetically in animals by supplying $^{14}$C-labeled glycine and proline (*9*).

We have recently developed synthetic procedures for the correct assembly of collagenous peptides into heterotrimers using an artificial cystine knot for the regioselective cross-linking of the three chains in a defined order (*10*). With this methodology, heterotrimers 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-chains and the sequence portion 772−784 ($P_4$−$P_9'$) of the α2-chain, were assembled in the α1α2α1′ register (*11*, *12*). The C-terminally positioned cystine knot was found to facilitate nucleation of the triple-helical fold, which was further stabilized N-terminally by chain elongations with $(Gly\text{-}Pro\text{-}Hyp)_n$ repeats. Collagenous heterotrimers of different conformational stability were obtained by this approach, which at room temperature mimic optimally natural collagen in both its folded and denatured (gelatin) states (*12*) and which allow HPLC monitoring of their enzymatic degradation by collagenases and gelatinases (*11*, *13*). The main drawback of this synthetic substrate is the relatively high concentrations required for the UV-based HPLC monitoring and thus the need of correspondingly high concentrations of the precious enzymes, since an optimal substrate/enzyme ratio has to be maintained due to the known very slow degradation of natural collagen preparations and related synthetic triple-helical molecules by interstitial collagenases.

Such problems are generally bypassed by the use of intramolecularly quenched fluorogenic substrates (*14*). These consist of single peptide chains that match the specificity of

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* Corresponding author: 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.

‡ Present address: Novartis, Pharma AG, WKl-136.3.62, 4002 Basel-CH, Switzerland.

[1] Abbreviations: Acm, acetamidomethyl; AcOH, acetic acid; CD, circular dichroism; Dns, dansyl; ESI-MS, electrospray ionization mass spectrometry; FRET, fluorescence resonance energy transfer; GdmCl, guanidinium chloride; HPLC, high-performance liquid chromatography; Hyp, 4-hydroxyproline; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MMP, matrix metalloproteinase; Npys, nitropyridinesulfenyl; Rpn, ratio of positive peak intensity over negative peak intensity in CD spectra of collagenous peptides; StBu, *tert*-butylthio; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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the particular enzyme and bear suitable chromophores at both sides of the scissile bond. One of the chromophores is a fluorophore, and the other acts as quencher that causes significant reduction of fluorescence intensity, either by direct intramolecular collision or by radiationless fluorescence resonance energy transfer (FRET). Upon enzymatic substrate cleavage, the quenching effect is suppressed and the enhanced fluorescence is exploited spectroscopically to monitor enzyme kinetics.

This principle has been efficiently applied for the design of monomeric substrates of MMPs by Stack and Gray (*15*), and in the present study it was used for the design of a fluorogenic triple-helical collagen-like substrate for collagenases.

## 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. TentaGel-S-PHB resin (Rapp Polymere GmbH, Tübingen, Germany) was loaded with Fmoc-Gly-OH by the symmetric anhydride/DMAP procedure in dimethylformamide (DMF). Cleavage of the Fmoc group with 20% piperidine in DMF was used to determine spectroscopically the loading (0.21 mmol/g). The syntheses on resin were performed manually or with the synthesizer ABI 431A (Applied Biosystems) by the standard FastMOC protocol. Analytical HPLC was carried out on Nucleosil 100−5/C8 (Machery & Nagel, Düren, Germany) with a linear gradient of acetonitrile/2% $H_3PO_4$ from 5:95 to 80:20 in 15 min and preparative HPLC on Nucleosil 100−5 C18 PPN with a linear gradient of 0.08% TFA in acetonitrile/0.1% aqueous TFA from 18:82 to 45:55 in 60 min. Gel chromatography was carried out on Sephacryl S-200 HR with 1% aqueous AcOH as eluent. Electrospray ionization mass spectra (ESI-MS) were recorded 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). The CD spectra were recorded on a Jasco J715 spectropolarimeter with the power supply PS-150J and temperature controller PFD-350S. The program J700 for Windows was used as software. Quartz cells of 0.1 cm optical path length and concentrations of $10^{-5}$ M peptide in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$ (pH 7.4) were used. Thermal denaturation was determined by monitoring the changes in dichroic intensity at 222 nm as a function of the temperature. Fluorescence spectra were measured with a Perkin-Elmer luminescence spectrometer (LS50B) equipped with digital software FL-WinLab (2.01) in quartz cells of 0.4 × 1 cm.

(*p*-Aminophenyl)mercuric acetate (APMA) was purchased from Sigma. Pro-MMP-1 and pro-MMP-13 were a gift from Gillian Murphy (School of Biological Sciences, University of East Anglia, Norwich, Norfolk, England) and MMP-3 was a gift of H. Nagase (The Kennedy Institute of Rheumatology, Hammersmith, London).

### *Peptide Synthesis*

*Synthesis of the* α*-Chains.* The single α-chains were synthesized on TentaGel-S-PHB resin as described previously (*12*, *16*). The dansyl group at the N-terminus of the α1-chain was introduced by reaction of the resin-bound peptide with a solution of 2 equiv of dansyl chloride and 2 equiv of DIEA in DMF for 1 h at room temperature. Deprotection and cleavage from the resin was performed with TFA/triethylsilane/water (95:3:2) in 1.5 h, and purification of the peptides was by preparative HPLC or gel chromatography.

*Dns-(Pro-Hyp-Gly)$_6$-Pro-Gln-Gly-Ile-Ala-Gly-Asn-Arg-Gly-Val-Val-Gly-Cys(StBu)-Gly-OH [Dns-*α*1(StBu)-OH].* Yield 19% after HPLC purification. ESI-MS: $m/z = 3209.6$ $[M]^+$; $M_r = 3209.7$ calcd for $C_{140}H_{210}N_{38}O_{43}S_3$. Amino acid analysis: Gln 1.00 (1), Pro 5.87 (7), Gly 9.86 (11), Ala 1.08 (1), Cys 1.23 (1), Val 1.84 (2), Ile 0.99 (1), Arg 1.04 (1), Asn 1.06 (1), Hyp 4.78 (6). Peptide content: 78%.

*Dns-(Pro-Hyp-Gly)$_6$-Pro-Gln-Gly-Ile-Ala-Gly-Asn-Arg-Gly-Val-Val-Gly-Cys-Gly-OH, [Dns-*α*1(SH)-OH].* To a 10 mM solution of Dns-α1(StBu)-OH in 95% aqueous TFE was added 5 equiv of tributylphosphine. After 12 h, the reaction mixture was evaporated and the residue was reprecipitated from TFE with methyl *tert*-butyl ether. The precipitate was lyophilized from 50 mM AcOH; yield 91%. ESI-MS: $m/z = 3121.2$ $[M]^+$; $M_r = 3121.5$ calcd for $C_{136}H_{202}N_{38}O_{43}S_2$. Amino acid analysis: Gln 0.93 (1), Pro 5.84 (7), Gly 10.86 (11), Ala 1.04 (1), Cys 1.00 (1), Val 1.93 (2), Ile 0.92 (1), Arg 1.00 (1), Asn 1.05 (1), Hyp 5.41 (6). Peptide content: 85%.

*Dns-(Pro-Hyp-Gly)$_6$-Pro-Gln-Gly-Ile-Ala-Gly-Asn-Arg-Gly-Val-Val-Gly-Cys(Npys)-Gly-OH [Dns-*α*1(Npys)-OH].* A 50 mM solution of Dns-α1(SH)-OH in degassed argon-saturated DMF/AcOH (95:5) was added dropwise to a 50 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 by analytical HPLC and after completion (1−2 h), the solvent was evaporated; the resulting residue was dissolved in water, and excess reagent was filtered off. The aqueous solution was washed twice with ethyl acetate and lyophilized; yield 27% after gel chromatography; ESI-MS: $m/z = 3276.0$ $[M]^+$; $M_r = 3275.6$ calcd for $C_{141}H_{204}N_{40}O_{45}S_3$. Amino acid analysis: Gln 0.97 (1), Pro 5.46 (7), Gly 8.80 (11), Ala 1.06 (1), Cys 0.77 (1), Val 1.77 (2), Ile 0.92 (1), Arg 1.00 (1), Asn 1.02 (1), Hyp 4.77 (6). Peptide content: 96%.

*H-Hyp-Gly-(Pro-Hyp-Gly)$_5$-Pro-Gln-Gly-Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Cys(Acm)-Cys-Gly-OH [H-*α*2(Acm,StBu)-OH].* Yield 10% after HPLC. MALDI-TOF-MS: $m/z = 3035.22$ $[M]^+$; $M_r = 3035.42$ calcd for $C_{132}H_{206}N_{34}O_{42}S_3$.

*H-Hyp-Gly-(Pro-Hyp-Gly)$_5$-Pro-Gln-Gly-Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Cys(Acm)-Cys-Gly-OH [H-*α*2(Acm,SH)-OH].* The α2(Acm,StBu) peptide was dissolved at 10 mM concentration in 95% aqueous TFE and reacted with 5 equiv of tributylphosphine. The reaction mixture was evaporated and the residue was reprecipitated from TFE with methyl *tert*-butyl ether; yield 86%. ESI-MS: $m/z = 2949.2$ $[M]^+$; $M_r = 2949.3$ calcd for $C_{128}H_{198}N_{34}O_{42}S_2$. Amino acid analysis: Gln 1.00 (1), Pro 5.51 (6), Gly 9.93 (10), Ala 1.04 (1), Cys 1.36 (2), Ile 1.03 (1), Leu 2.95 (3), Hyp 6.62 (7). Peptide content: 84%.

*H-Trp-Gly-(Pro-Hyp-Gly)$_5$-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Cys(StBu)-Gly-OH [H-Trp-*α*1′(StBu)-OH].* Yield 31% after HPLC purification; ESI-MS: *m/z*

= 3079.0 $[M]^+$; $M_r$ = 3079.5 calcd for $C_{136}H_{208}N_{38}O_{40}S_2$. Amino acid analysis: Gln 1.95 (2), Pro 5.91 (6), Gly 10.20 (11), Ala 1.00 (1), Cys 0.96 (1), Val 1.42 (2), Ile 0.96 (1), Leu 1.00 (1), Trp 1.05 (1), Arg 0.96 (1), Hyp 4.69 (5). Peptide content: 77.5%.

*H-Trp-Gly-(Pro-Hyp-Gly)$_5$-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Cys-Gly-OH [H-Trp-α1′(SH)-OH].* To a 10 mM solution of H-Trp-α1′(StBu)-OH in 95% aqueous TFE was added 5 equiv of tributylphosphine . The reaction mixture was evaporated and the residue was reprecipitated from TFE with methyl *tert*-butyl ether; yield 93%. ESI-MS: $m/z$ = 2990.8 $[M]^+$; $M_r$ = 2991.4 calcd for $C_{132}H_{200}N_{38}O_{40}S_1$. Amino acid analysis: Gln 2.06 (2), Pro 5.89 (6), Gly 10.23 (11), Ala 1.16 (1), Cys 0.49 (1), Val 2.00 (2), Ile 0.96 (1), Leu 1.02 (1), Trp 0.83 (1), Arg 1.00 (1), Hyp 5.00 (5). Peptide content: 79.95%.

*Assembly of the Heterotrimer **2***

*Dns-α1-OH/H-α2(Acm)-OH.* H-α2(Acm,SH)-OH was dissolved at 50 mM concentration in degassed argon-saturated 50 mM $NH_4OAc$ (pH 5.5), and the solution was added dropwise to a 50 mM solution of 1 equiv of Dns-α1(Npys)-OH in 50 mM $NH_4OAc$ (pH 5.5) under exclusion of air−oxygen. The reaction was monitored by analytical HPLC. After 4 h, the aqueous solution was washed with ethyl acetate and lyophilized; yield 53% after HPLC purification. MALDI-TOF-MS: $m/z$ = 6069.44 $[M]^+$; $M_r$ = 6068.79 calcd for $C_{264}H_{398}N_{72}O_{85}S_4$. Amino acid analysis: Gln 2.00 (2), Pro 11.95 (13), Gly 18.96 (22), Ala 2.13 (2), Cys 2.70 (3), Val 1.75 (2), Ile 1.96 (2), Leu 2.92 (3), Arg 1.03 (1), Asn 1.05 (1), Hyp 12.04 (13). Peptide content: 84%.

*Dns-α1-OH/H-α2(Npys)-OH.* To a 50 mM solution of Dns-α1-OH/H-α2(Acm)-OH in TFA/AcOH (1:2) was added 1.2 equiv of freshly prepared 3-nitropyridine-2-sulfenyl chloride (*17*). The reaction was monitored by analytical HPLC. After 3 h, the mixture was diluted with 50 mM HOAc and lyophilized; yield 51% after HPLC purification. Amino acid analysis: Gln 2.02 (2), Pro 11.73 (13), Gly 18.70 (22), Ala 2.17 (2), Cys 2.39 (3), Val 1.73 (2), Ile 2.01 (2), Leu 3.10 (3), Arg 1.01 (1), Asn 1.00 (1), Hyp 12.00 (13). Peptide content: 79%.

*Hetrotrimer **2** (Dns-α1-OH/H-α2-OH/H-Trp-α1′-OH).* A 50 mM solution of H-Trp-α1′(SH)-OH in degassed argon-saturated 50 mM $NH_4OAc$ (pH 5.5) was added dropwise to a 50 mM solution of 1 equiv of Dns-α1-OH/H-α2(Npys)-OH in 50 mM $NH_4OAc$ (pH 5.5) under exclusion of air-oxygen. The reaction was monitored by HPLC. After 5 h, the aqueous solution was lyophilized; yield 50% after gel chromatography. Amino acid analysis: Gln 4.74 (4), Pro 18.94 (19), Gly 32.46 (33), Ala 3.58 (3), Cys 4.11 (4), Val 4.43 (4), Ile 3.47 (3), Leu 5.40 (4), Trp 1.02 (1), Arg 2.42 (2), Asn 1.00 (1), Hyp 19.94 (18); peptide content: 81%. UV shows a 1:1 ratio of the α1 and α1′-chains containing the chromophores.

*Enzyme Assays*

Pro-MMP-1 was converted to MMP-1 essentially as described by Suzuki et al. (*18*). The latent enzyme was incubated in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$ (pH 7.4) with 0.02 equiv of the catalytic domain of MMP-3 and 0.2 mM APMA at 25 °C for 15 h. Pro-MMP-13 was activated by incubation in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$ (pH 7.4) with 1 mM APMA at 37 °C for 3.5 h. The enzyme activation was controlled by SDS−PAGE under reducing conditions according to Laemmli (*19*) with 10% polyacrylamide gels.

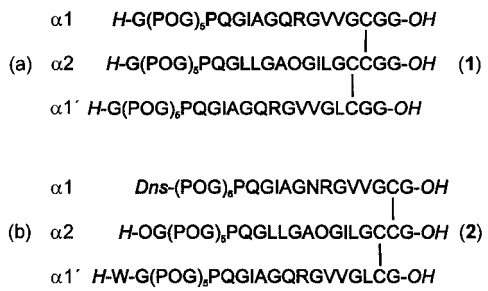


FIGURE 1: Sequences of (a) the minimum-size triple-helical collagen peptide **1** that acts as substrate for human neutrophil collagenase (MMP-8) and (b) the related fluorogenic heterotrimer **2**; O = Hyp, *trans*-L-hydroxyproline.

To a 50 μM solution of the collagen peptide in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$ (pH 7.4) at 23 °C were added aliquots of the stock solutions of the MMPs to reach a concentration of 0.5 μM. At time intervals 10 μL aliquots of the assay solution were taken and quenched with 2 μL of 0.1 M HCl. The quenched probes were analyzed by analytical HPLC. At the same time intervals 5 μL aliquots of the assay solution were taken and diluted with 600 μL of buffer (50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$, pH 7.4) and stored at 0 °C for 15 h. After warming to 5 °C, fluorescence spectra were recorded in the wavelength range of 300−460 nm upon excitation at 280 nm (slits 5 nm).

## RESULTS

*Design and Synthesis of a Fluorogenic Heterotrimeric Collagen Peptide.* By cross-linking C-terminally via a cystine knot the collagen type I cleavage sites 772−784 ($P_4$−$P_9$') and 772−785 ($P_4$−$P_{10}$') of the two α1-chains and 772−784 ($P_4$−$P_9$') of the α2-chain and stabilizing the triple helix with N-terminal $(Gly\text{-}Pro\text{-}Hyp)_5$ extensions, the heterotrimer **1** (Figure 1a) was obtained in previous studies that at room temperature is folded into the triple helix and thus is recognized and degraded by collagenases with a single cut through the three chains (*11*). With this heterotrimer the correctness of the register proposed for the three α-chains in collagen type I (*20*) was confirmed, and since it represents the minimum size of this type of collagen-like trimer in terms of triple-helix stability (*12*), it was used for the design of a fluorogenic heterotrimeric substrate for collagenases.

Fluorescence resonance energy transfer (FRET) requires a spectral overlap between the emission spectrum of the donor and the absorbance spectrum of the acceptor. This is fulfilled by the emission spectrum of tryptophan ($\lambda_{max}$ = 350 nm) and the absorption spectrum of the dansyl group ($\lambda_{max}$ = 330 nm). The efficiency of energy transfer between the two groups depends on their critical transfer distance $R_0$, for which resonance energy transfer is 50%. For the Trp/Dns pair this critical transfer distance $R_0$ is 21.3 Å (*21*, *22*). Since the energy transfer efficiency decreases with the sixth power of the distance between the chromophores, we have estimated on a structural model derived from X-ray crystallographic data (*1*, *23*) the distance between amino acids located N-terminally on a vertical cut through the collagen triple helix. With values in the range of 4.7−4.9 Å, a good

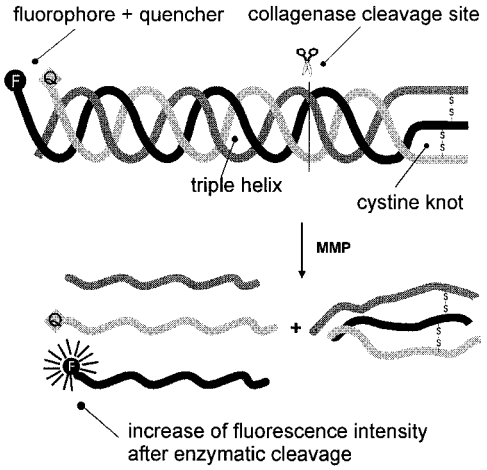


FIGURE 2: Schematic representation of the fluorescence resonance energy transfer (FRET) principle applied for the design of the fluorogenic triple-helical collagen peptide **2** as substrate for collagenases.

quenching efficiency was expected for this pair of chromophores. These were therefore incorporated at the N-termini of two α-chains as shown in Figure 1b to possibly exploit FRET induced by their proximity in the triple-helical structure. Thereby, to align the chromophores at least theoretically in close proximity, the gaps resulting from the α1α2α1′ register were filled up by extending the α1-chain with the dansyl-Pro-Hyp-Gly tripeptide, the α2-chain with the Hyp-Gly dipeptide, and the α1′-chain with the Trp-Gly dipeptide. Digestion of the heterotrimer by a single cut through the three chains was expected to unfold the triple helix with release of the fluorescence as schematically outlined in Figure 2.

Following previously optimized synthetic methods (*16*), the single peptide chains were synthesized on a solid support by Fmoc/tBu chemistry (*24*). Thereby Fmoc-Pro-Hyp-Gly-OH was used as synthon to avoid diketopiperazine formation in the $N^{\alpha}$-deprotection steps. When required, chain elongation with single Hyp residues was performed by the use of Fmoc-Hyp(tBu)-OH to prevent O-acylation with subsequent O → N acyl shift during the piperidine-promoted Fmoc cleavage. The N-terminal Trp residue was incorporated into the α1′-chain by standard coupling procedures, while $N^{\alpha}$-dansylation of the α1-chain was performed on resin by reaction with dansyl chloride.

For the selective cross-linking of the three α-chains into the heterotrimer **2** according to the α1α2α1′ register, the cystine knot was built up following essentially the strategy elaborated previously (*10, 12*). This foresees activation of the heterodimer α1α2(Acm) with 3-nitropyridine-2-sulfenyl chloride (Npys-Cl) for subsequent reaction with the free thiol group of the α1′-chain. Therefore the Trp residue was placed into the α1′-chain to prevent its contact with this reagent. In fact, it is well-known that the indole group reacts rapidly with sulfenyl chlorides under acidic conditions to form the related 2-thioether derivatives (*25*). All disulfide-bridging reactions were performed under exclusion of air−oxygen and in slightly acidic medium (pH 4.5−5.0) to prevent homodimerizations and undesired thiol/disulfide exchange reactions, respectively. The final fluorogenic heterotrimeric peptide **2** was characterized in its homogeneity by HPLC and amino acid analysis as well as by UV and fluorescence

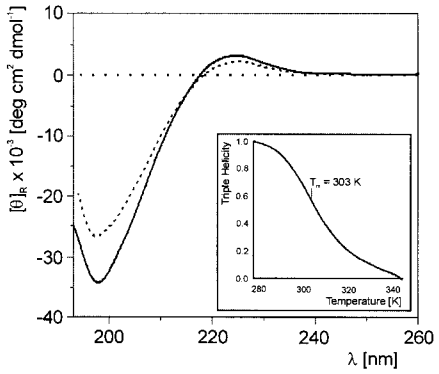


FIGURE 3: CD spectra of the heterotrimeric collagen peptide **2** at 4 °C (—) and 20 °C (---) in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$ (pH 7.4). (Inset) Thermal denaturation of the heterotrimer **2** in the identical buffer.

spectroscopy. In view of the mass spectra obtained for the heterotrimer **1** (Figure 1) (*12*), it was surprising that mass-spectrometric analysis by both ESI and MALDI-TOF failed in the case of the heterotrimer **2** even with the matrix 2,5-dihydrydroxybenzoic acid/2-hydroxy-5-methoxybenzoic acid (9:1) as proposed recently in the case of collagen peptides assembled on a dilysine core (*26*). However, upon enzymatic digestion and thus disruption of this supercoiled structure, mass spectrometry of the single fragments confirmed the correct composition and the high degree of homogeneity of the parent heterotrimer.

*Conformational Properties of the Heterotrimeric Collagen Peptide.* The triple-helical conformation of collagenous peptides is characterized by a typical circular dichroism (CD) spectrum with a relatively strong positive maximum centered at 220−223 nm and an intense negative band at 196−200 nm (*27*). 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 minimum located at 198−205 nm of weaker intensity (*27*). A differentiation between these two types of conformations can be derived from the ratio of the positive peak intensity over the negative peak intensity (Rpn value), which represents an index of the degree of triple helicity (*28, 29*). Since the dichroic contributions of the Trp side chain and the Dns group are negligible in the far UV, the Rpn values are not affected by these chromophores. As shown in Figure 3, the CD spectrum of the fluorogenic heterotrimer **2** at 4 °C is supportive of a triple-helical conformation, although the Rpn value is significantly lower than that of the parent heterotrimer **1** without the chromophores (Table 1). The contribution of the additional hydrogen bond resulting from filling the gaps between the N-termini of the chains in the heterotrimer **2** has previously been shown to contribute only marginally to the stability of the triple helix (*12*). The thermal denaturation of the heterotrimer **2**, as monitored by the decrease of dichroic intensity at 222 nm as a function of temperature (Figure 3), revealed a melting temperature ($T_m$) of 30 °C vs 33 °C measured for the heterotrimer **1** (*12*). Nonetheless, the cooperative thermal denaturation (Figure 3) and the comparison of the CD spectra prior to and after enzymatic cleavage of the fluorogenic substrate by interstitial collagenases (Figure 4) clearly support a triple-helical conformation, although of lower stability than that of the heterotrimer **1** that lacks the two N-terminal chromophores and the filled gaps.

Table 1: Circular Dichroism Data of Heterotrimeric Collagenous Peptides Containing the Collagenase Cleavage Site of Collagen Type I[a]

| heterotrimeric molecules | $\lambda_{min}$ (nm) | $[\theta] \times 10^3$ (deg cm$^2$ dmol$^{-1}$) | $\lambda_{max}$ (nm) | $[\theta] \times 10^3$ (deg cm$^2$ dmol$^{-1}$) | Rpn | $T_m$ (°C) |
|---|---|---|---|---|---|---|
| heterotrimer **1** | 196 | −34 | 222 | 4.16 | 0.122 | 33 |
| heterotrimer **2** | 197.8 | −33.6 | 224.5 | 2.92 | 0.087 | 30 |
| heterotrimer **2** upon enzymatic cleavage | 199.6 | −21.3 | 226.1 | 1.42 | 0.066 | |
| collagen type I | 196.5 | −34.6 | 220.0 | 4.50 | 0.13 | 38 |

[a] Measurements were performed in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$, pH 7.4 at 4 °C.

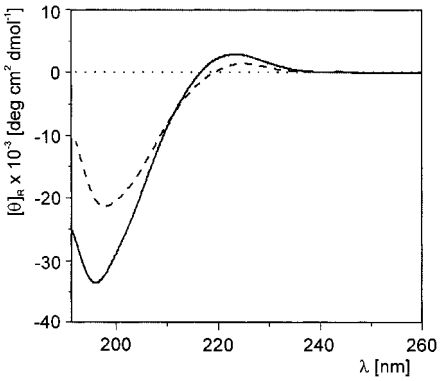


FIGURE 4: CD spectra of the fluorogenic collagen peptide **2** at 4 °C in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$ (pH 7.4) before (—) and after (---) enzymatic cleavage by the collagenases MMP-1 and MMP-13.

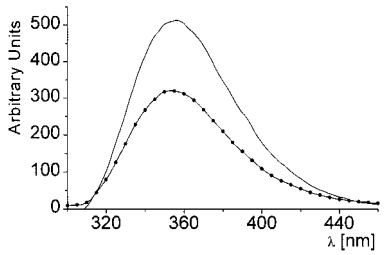


FIGURE 5: Trp fluorescence spectra of the heterotrimer **2** (excitation at 280 nm) in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$, pH 7.4 (●) and in 8 M GdmCl (—); 2 $\mu$M peptide concentration.

Incorporation of the two hydrophobic residues at the N-terminus of the heterotrimer **2** was expected to additionally stabilize the triple helix via a hydrophobic collapse of the indole side chain and the dansyl group. Apparently, the opposite effect is induced with at least N-terminal destabilization of the triple helix. This is further supported by the observation that renaturation of the triple-helical structure is significantly slower for this collagenous peptide than in the case of the trimer **1**. In fact, only upon storage for 10 h at 4 °C were we able to record the identical CD spectrum as prior to thermal denaturation.

*Fluorescent Properties of the Heterotrimeric Collagen Peptide.* A comparison of the Trp fluorescence spectra in buffer and in 8 M guanidinium chloride (GdmCl) (Figure 5) reveals a 2-fold increase of the fluorescence intensity in GdmCl, i.e., under conditions known to denature the collagen triple helix (*30*). As expected, in the unfolded state of the heterotrimer **2** FRET is weakened.

An increase in temperature generally leads to a decrease of the fluorescence intensity due to thermal quenching. This phenomenon could be observed in the spectra of the Trp containing single α1′-chain, but the opposite result was obtained with the heterotrimer **2** as shown in Figure 6. Thus conformational changes have to be responsible for the

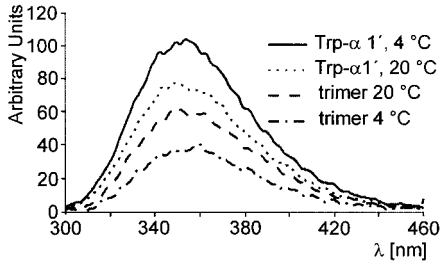


FIGURE 6: Fluorescence spectra of the Trp-containing α1′-chain and of the heterotrimer **2** (excitation at 280 nm) at 4 and 20 °C in 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$ (pH 7.4) at 2 $\mu$M concentration.

increased Trp fluorescence. An inspection of the CD spectra of the heterotrimer **2** (Figure 3) clearly reveals partial denaturation already at 20 °C compared to 4 °C. These experimental data indicate that a higher N-terminal mobility at increased temperatures leads to a lower efficiency of FRET, possibly due to increased distances between donor and acceptor chromophore. The conformational effect is apparently stronger than the thermal quenching, leading to an overall increased fluorescence intensity at 20 °C compared to that at 4 °C. Correspondingly, for monitoring enzyme kinetics with the fluorogenic heterotrimer **2**, the fluorescence spectra have to be recorded at low temperatures.

*Enzyme Assays.* Enzymatic cleavage of the heterotrimer **2** with the activated full-length MMP-1 and MMP-13 was comparatively monitored by HPLC analysis and fluorescence spectroscopy. At time intervals, samples of the incubation medium were taken for the analysis. For HPLC monitoring, the reaction was quenched by addition of HCl and the product distribution was analyzed. At 1:100 enzyme/substrate ratios, both MMP-1 and MMP-13 were found to cleave the substrate at comparable rates. An inspection of the product distribution, as monitored by HPLC in the time course of enzymatic digestion of the heterotrimeric substrate, would suggest that the α2-chain is the first to be cleaved. LCMS analysis of the final digestion medium confirmed that cleavage occurs selectively at the Gly−Ile bond of the α1- and α1′-chain and at the Gly−Leu bond of the α2-chain.

Parallel to the HPLC experiments, samples for fluorescence spectroscopy were taken, diluted at 1:100 with the assay buffer, and kept in the cold overnight prior to the fluorescence measurements to exploit the stronger FRET at low temperature. The Trp fluorescence spectra were recorded at 5 °C and a plot of the fluorescence intensity as a function of time was compared with a similar plot obtained by the HPLC analysis. As shown in Figure 7, the resulting curves are practically identical, thus confirming the usefulness of the new fluorogenic heterotrimeric collagenase substrate.

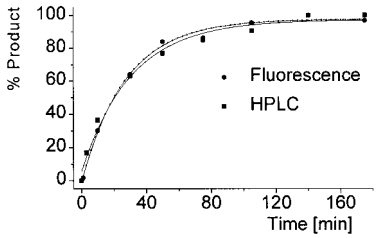


FIGURE 7: Monitoring of the enzymatic cleavage of the fluorogenic triple-helical collagen peptide **2** by HPLC and fluorescence spectroscopy.

## DISCUSSION

Previous studies on the conformational properties of heterotrimeric collagen peptides containing the large 772−784/785 ($P_4$−$P_9'$/$P_{10}'$) collagenase cleavage site of collagen type I clearly confirmed the low propensity of these sequence portions for a triple-helical fold (*12*). In fact, besides the C-terminal cystine knot, an N-terminal extension with at least five (Gly-Pro-Hyp) repeats, as present in the heterotrimer **1** (Figure 1), was required to stabilize the triple helix at room temperature. Incorporation of two hydrophobic groups at the N-termini of two α-chains was expected to further stabilize the triple helix. However, the opposite effect was observed, which may derive from steric clashes that could destabilize the triple helix N-terminally but more probably has to be attributed to an enhancement of the local hydrophobicity that may interfere with the known strong effects of solvation on the stability of the collagen structure. Nonetheless, even at 20 °C where enzyme assays were performed, i.e., 10 °C below the $T_m$, a sufficiently large portion of the molecule is folded into the triple helix to be recognized and digested by the collagenases MMP-1 and MMP-13 at rates comparable to those observed for the heterotrimer **1**.

At low temperature (4−5 °C) the triple helix is stabilized, and correspondingly, even FRET is enhanced. Although the fluorescence of the cleaved substrate is enhanced only by a factor of 6 over the intact molecule, it would suffice to monitor directly the digestion of the fluorogenic substrate **2** by collagenases. However, digestion of collagen and related triple-helical substrates by collagenases is known to be one of the slowest enzymatic processes, and thus at 4−5 °C this proteolysis is almost totally quenched. Therefore, the fluorogenic properties of the substrate **2** could only be exploited indirectly by cooling aliquots taken at time intervals for fluorescence measurements.

Although the advantages of the collagen-like heterotrimer **2** as a fluorogenic substrate for collagenases are limited if compared to the HPLC monitoring, the results obtained clearly show that FRET, so far applied only in the design of linear fluorogenic enzyme substrates, can also be exploited in conformation dependency. For further improvements of the efficiency of such types of substrates, either the position of the pair of chromophores has to be changed or a more efficient donor/acceptor system has to be applied.

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