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Synthesis of Heterotrimeric Collagen Peptides Containing the $\alpha 1\beta 1$ Integrin Recognition Site of Collagen Type IV

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Abstract: Collagen type IV provides a biomechanically stable scaffold into which the other constituents of basement membranes are incorporated, but it also plays an important role in cell adhesion. This occurs with collagen type IV mainly via the $\alpha 1\beta 1$ integrin, and the proposed epitope involved in this type of collagen/integrin interaction corresponds to a non-sequential R/Xaa/D motif, where the arginine and aspartate residues are provided by the $\alpha 2$ and $\alpha 1$ chains of the collagen molecule, respectively. Since the stagger of the three α chains in native collagen type IV is still unknown and different alignments of the chains lead to different spatial epitopes, two heterotrimeric collagen peptides containing the natural 457–469 sequences of the cell adhesion site were synthesized in which the single chains were assembled via disulfide bonds into the two most plausible $\alpha 1\alpha 2\alpha 1'$ and $\alpha 2\alpha 1\alpha 1'$ registers. The differentiated triple-helical stabilities of the two heterotrimers suggest a significant structural role of the chain register in collagen, although the binding to $\alpha 1\beta 1$ integrin is apparently less affected as indicated by preliminary experiments.

Keywords: collagen; synthesis; cystine knot; conformation; cell adhesion

INTRODUCTION

Cells interact with the extracellular matrix (ECM) in processes such as cell differentiation and migration, wound repairs and maintenance of tissue integrity by cell adhesion to the basement membrane [1]. The main structural component of the basement membrane is collagen type IV [2] whose major and ubiquitous form consists of one $\alpha 2$ and two $\alpha 1$ chains [3,4]. With its mesh-like structure, it forms a network that determines the biochemical stability and macromolecular organization of the basement membrane and provides a scaffold into which other constituents of the tissue are incorporated [5].

Cell adhesion to collagen type IV [6] is specifically mediated by cell-surface receptors, i.e. the integrins which are transmembrane heterodimeric glycoproteins composed of non-covalently associated α and β subunits. Among the various known integrins, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were identified as specific collagen receptors by a combination of different approaches [7–11].

While other integrin ligands such as fibronectin or fibrinogen are recognized via contiguous adhesion motifs consisting of an aspartic/glutamic acid and often of an additional arginine residue (RGD motif) [12], collagen binding to integrin is known to depend critically upon its native triple-helical fold. In fact, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins exhibit higher affinity for native than denatured collagen. As a consequence, attempts to identify the integrin binding sites of collagen were difficult since its chemical and enzymatic fragmentation leads to destabilization of the collagen triple helix.

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In addition, such fragmentation itself generally requires collagen denaturation. Nevertheless Vanderberg *et al.* [3] succeeded in isolating and identifying a disulfide-stabilized triple-helical fragment CB3(IV) as the major cell-binding site within collagen type IV. Subsequently, a detailed study by Eble *et al.* [13] allowed the restriction of the adhesion epitope to the *N*-terminal portion of the F4 subfragment of CB3(IV), and more precisely to Arg-461 of the α2(IV) chain and Asp-461 of the α1(IV) chain. Such an adhesion motif that is provided by a defined display of the residues on different chains is expected to depend not only on the triple-helical fold, but even more decisively on the stagger of the single chains within the triple helix.

Although there is some evidence for an $\alpha2\alpha1\alpha1'$ register of chains in native collagen type IV [14], the aim of the present study was to confirm this proposed chain assembly via the more or less selective recognition of synthetic collagen heterotrimers, containing the adhesion motif, by the $\alpha1\beta1$ integrin. For this purpose heterotrimeric constructs had to be designed containing the sequence portions 457–469 of the α1 and α2 chains of collagen type IV in the most plausible $\alpha2\alpha1\alpha1'$ and $\alpha1\alpha2\alpha1'$ registers, but at the same time being folded into a sufficiently stable triple helix under the conditions of integrin binding experiments. In the related syntheses we made use of our previous experience gained during the preparation of collagen heterotrimers capable of mimicking the collagenase cleavage site of collagen type I [15,16].

MATERIALS AND METHODS

All reagents and solvents used were of the highest quality commercially available. Amino acid derivatives were from Alexis (Grünberg, Germany) or were prepared according to standard protocols. TentaGel-S-PHB resin (loading 0.25 mmol/g) was purchased from Rapp Polymere GmbH (Tübingen, Germany). Precoated silica gel 60 TLC plates were from Merck AG (Darmstadt, Germany) and compounds were visualized with the chlorine/tolidine or permanganate reagent. Analytical RP-HPLC was carried out on 125/4 Nucleosil 100-5 C_8 (Macherey & Nagel, Düren, Germany) or on 150/3.9 X-Terra MS 300-5 C_8 (Waters) columns using a linear gradient of ACN/2% H_3PO_4 from 5 : 95 to 90 : 10 in 15 min at a flow rate of 1.5 ml/min and monitoring at 210 nm. Preparative RP-HPLC was performed on 250/21 Nucleosil 100-5 C_{18} PPN (Macherey & Nagel) and on 100/19 X-Terra MS 300-5 C_{18} (Waters) columns using a linear gradient of ACN (0.08% TFA)/0.1% TFA from 15 : 85 to 40 : 60 in 40 min, 50 min or 80 min for the monomers, dimers and trimers, respectively. The flow rate was 10 ml/min and the elution was monitored at 210 nm. The peak fractions were collected manually and lyophilized. Gel chromatography was carried out on 145/1.25 Fraktogel HSK HW-40 S and 100/1.0 Sephadex G-50 Superfine using an isocratic elution of 0.5% AcOH at a flow rate of 0.2 ml/min. Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectra were recorded on a Bruker Reflex II instrument and electro spray ionization mass spectrometry (ESI-MS) on a PE Sciex API 165 from 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 LC 6001 Biotronic amino acid analyser.

Synthesis of Reagents and Intermediates

(3-Nitro-2-pyridine)sulphenyl chloride. Di-(3-nitro-2-pyridine)disulfide was freshly prepared following essentially the method described by Grassetti *et al.* [17] and recrystallized from DMF; TLC: R_f 0.5 (AcOEt/hexane, 2 : 1), R_f 0.8 ($CHCl_3$/MeOH, 9 : 1); RP-HPLC: t_R 11.73 min; mp 246°–247 °C (249°–250 °C) [18]; ESI-MS: $m/z = 311.0$ $[M + H^+]$; $M_r = 310.3$ calcd for $C_{10}H_6N_4O_4S_2$.

The disulfide was converted to the sulphenyl chloride (Npys-Cl) according to known procedures [18] by reaction with sulphuryl chloride; TLC: R_f 0.3 (CH_2Cl_2), R_f 0.5 (hexane/methyl *tert*-butyl ether/AcOH, 95 : 5 : 5); mp 100°–110 °C (96°–98 °C) [18–20]. The reagent has a limited stability of 3–4 weeks at 4 °C.

Z-Pro-Hyp(tBu)-Gly-OBzl. The tripeptide derivative was synthesized by standard procedures in solution via acylation of H-Hyp(tBu)-OH with Z-Pro-OSu followed by condensation of Z-Pro-Hyp(tBu)-OH with H-Gly-OBzl or by direct *tert*-butylation of Z-Pro-Hyp-Gly-OBzl [21] and recrystallization from AcOEt/ether; TLC: R_f 0.4 ($CHCl_3$/MeOH/AcOH, 9 : 1 : 0.5); RP-HPLC: t_R 11.44 min; ESI-MS: $m/z =$ 566.4 $[M + H^+]$; $M_r = 565.7$ calcd for $C_{31}H_{39}N_3O_7$.

H-Pro-Hyp(tBu)-Gly-OH·Tos-OH. Z-Pro-Hyp(tBu)-Gly-OBzl (26.6 g; 47.1 mmol) was hydrogenated over Pd/C in 500 ml water/MeOH (9 : 1) containing *p*-toluenesulphonic acid monohydrate (8.9 g; 47.1 mmol). The catalyst was removed by filtration

and the solution evaporated to dryness. The residue was precipitated with ether; yield: 23.2 g (96%); TLC: R_f 0.3 (ACN/H_2O/AcOH, 4 : 1 : 0.5), R_f 0.2 ($CHCl_3$/MeOH/AcOH, 4 : 1 : 0.5); RP-HPLC: t_R 3.41 min; ESI-MS: $m/z = 342.2$ [M+H^+ − Tos-OH]; $M_r = 341.4$ calcd for $C_{16}H_{27}N_3O_5$.

Fmoc-Pro-Hyp(tBu)-Gly-OH. To a solution of H-Pro-Hyp(tBu)-Gly-OH·Tos-OH (10.5 g; 20.4 mmol) in 300 ml water/dioxane (2 : 1) containing $NaHCO_3$ (4.3 g; 51.2 mmol) was added slowly a solution of Fmoc-OSu (8.3 g; 24.6 mmol) in 100 ml dioxane. After 12 h, the solution was neutralized with 2 M HCl and the bulk of the solvent evaporated. The residue was distributed between 2% $NaHCO_3$ and ether, the aqueous layer was acidified to pH 3.5 with 2 M HCl and the product was extracted with AcOEt. The organic layer was dried (Na_2SO_4) and concentrated to a small volume. Upon addition of ether, the solid was collected by filtration and purified by flash-chromatography (eluent: CH_2Cl_2/MeOH/AcOEt, 3 : 2 : 1); yield: 8.2 g (71%); TLC: R_f 0.7 (ACN/H_2O/AcOH, 4 : 1 : 0.5), R_f 0.6 (CH_2Cl_2/MeOH/AcOEt, 3 : 2 : 1); RP-HPLC: t_R 10.39 min; ESI-MS: $m/z = 564.4$ [M + H^+]; M_r = 563.6 calcd for $C_{31}H_{37}N_3O_7$.

Synthesis of the α Chains

TentaGel-S-PHB resin (10 g; 2.5 mmol; substitution grade: 0.25 mmol/g) was loaded with Fmoc-Gly-OH (7.4 g; 25 mmol) by reaction with HOBt (3.4 g; 25 mmol)/DIC (3.9 ml; 25 mmol) in the presence of DMAP (30.5 mg; 0.25 mmol) followed by capping with acetic anhydride (473 µl; 5 mmol) and DIEA (873 µl; 5 mmol); loading: 72%. The peptide chains were assembled in sequence order using Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Hyp(tBu)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH and Fmoc-Phe-OH and the active esters Fmoc-Cys(StBu)-OPfp and Fmoc-Cys(Acm)-OPfp. Double couplings were performed with four-fold excesses of the active esters (in the absence of base), of the Fmoc-Xaa-OH/HBTU/HOBt/DIEA (1 : 1: 1: 2) and of the Fmoc-Pro-Hyp(tBu)-Gly-OH/HBTU/HOBt/DIEA (1 : 1: 1: 2) in NMP/CH_2Cl_2 (4 : 1). Coupling reactions were allowed to proceed for 90 min for the single amino acid derivatives and for 3 h for the tripeptide fragment, and the coupling efficiency was monitored by the Kaiser [22] or chloranil test [23]. A capping step with 20% acetic anhydride/2% DIEA in CH_2Cl_2 for 20 min was included after each coupling to minimize the complexity of failure sequences. Fmoc deprotection was carried out twice with 20% piperidine in NMP (1 × 2 min; 1 × 20 min). After each step, the resin was washed alternatively with CH_2Cl_2 and NMP/CH_2Cl_2 (4 : 1). Peptides were deprotected and cleaved from the resin with TFA/triethylsilane/H_2O (95 : 3 : 2) for 3 h at room temperature. The crude products were precipitated from ice-cold methyl *tert*-butyl ether/hexane (2 : 1), recovered by centrifugation and lyophilized twice from 5% acetic acid. Purification was carried out by preparative HPLC.

Ac-Gly-(Pro-Hyp-Gly)$_4$-Asp-Gln-Gly-Pro-Hyp-Gly-Ile-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(StBu)-Gly-Gly-OH, α1(StBu) (A). Yield: 26.8%; RP-HPLC: t_R 7.67 min; MALDI-TOF-MS: $m/z = 2876.9$ [M^+]; M_r = 2877.1 calcd for $C_{123}H_{182}N_{32}O_{44}S_2$; amino acid analysis: Asp 0.98 (1), Cys 0.87 (1), Glu 1.00 (1), Gly 11.56 (12), Hyp 8.08 (8), Ile 0.98 (1), Pro 7.21 (7); peptide content: 91.2%.

Ac-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Acm)-Cys(StBu)-Gly-Gly-OH, α2(Acm,StBu) (A). Yield: 8.8%; RP-HPLC: t_R 7.61 min; MALDI-TOF-MS: $m/z = 3073.8$ [M^+]; $M_r = 3074.5$ calcd for $C_{132}H_{201}N_{37}O_{42}S_3$; amino acid analysis: Ala 1.97 (2), Arg 0.98 (1), Cys 2.04 (2), Gly 11.69 (12), Hyp 7.15 (7), Leu 0.97 (1), Lys 0.93 (1), Phe 1.00 (1), Pro 5.08 (5); peptide content: 87.9%.

Ac-Gly-(Pro-Hyp-Gly)$_4$-Asp-Gln-Gly-Pro-Hyp-Gly-Ile-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Acm)-Cys(StBu)-Gly-Gly-OH, α1(Acm,StBu) (B). Yield: 11.5%; RP-HPLC: t_R 7.58 min; MALDI-TOF-MS: m/z = 3051.5 [M^+]; $M_r = 3051.4$ calcd for $C_{129}H_{192}N_{34}O_{46}S_3$; amino acid analysis: Asp 1.00 (1), Cys 1.78 (2), Glu 1.03 (1), Gly 10.27 (12), Hyp 7.37 (8), Ile 0.96 (1), Pro 5.89 (7); peptide content: 87.8%.

Ac-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(StBu)-Gly-Gly-OH, α2(StBu) (B). Yield: 40.8%; RP-HPLC: t_R 7.64 min; MALDI-TOF-MS: $m/z = 2900.0$ [M^+]; $M_r = 2900.3$ calcd for $C_{126}H_{191}N_{35}O_{40}S_2$; amino acid analysis: Ala 2.13 (2), Arg 0.97 (1), Cys 0.87 (1), Gly 10.87 (12), Hyp 7.05 (7), Leu 1.00 (1), Lys 1.00 (1), Phe 1.02 (1), Pro 4.46 (5); peptide content: 83.1%.

H-Gly-(Pro-Hyp-Gly)$_4$-Asp-Gln-Gly-Pro-Hyp-Gly-Ile-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(StBu)-Gly-Gly-Gly-OH, α1(StBu) (C). Yield: 16%; RP-HPLC: t_R 7.33 min; MALDI-TOF-MS: $m/z = 2891.2$ [M^+];

$M_r = 2892.1$ calcd for $C_{123}H_{183}N_{33}O_{44}S_2$; amino acid analysis: Asp 1.00 (1), Cys 0.68 (1), Glu 1.10 (1), Gly 11.84 (13), Hyp 7.80 (8), Ile 0.97 (1), Pro 6.04 (7); peptide content: 70.1%.

H-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Acm)-Cys(StBu)-Gly-Gly-OH, α2(Acm,StBu) (C). Yield: 10%; RP-HPLC: t_R 7.95 min; MALDI-TOF-MS: $m/z = 3030.0$ [M$^+$]; $M_r = 3032.4$ calcd for $C_{130}H_{199}N_{37}O_{41}S_3$; amino acid analysis: Ala 2.03 (2), Arg 0.96 (1), Cys 1.27 (2), Gly 10.23 (12), Hyp 6.03 (7), Leu 1.02 (1), Lys 1.00 (1), Phe 1.04 (1), Pro 4.51 (5); peptide content: 76.1%.

H-Gly-(Pro-Hyp-Gly)$_4$-Asp-Gln-Gly-Pro-Hyp-Gly-Ile-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Pro-Cys(StBu)-Gly-Gly-OH, α1′(StBu) (A/B/C). Yield: 6.4%; RP-HPLC: t_R 7.86 min; MALDI-TOF-MS: $m/z = 2931.6$ [M$^+$]; $M_r = 2932.2$ calcd for $C_{126}H_{187}N_{33}O_{44}S_2$; amino acid analysis: Asp 1.00 (1), Cys 1.01 (1), Glu 1.00 (1), Gly 10.36 (12), Hyp 7.29 (8), Ile 0.98 (1), Pro 7.76 (8); peptide content: 91.8%.

Reduction of the Cys(StBu) Protected α Chains

To a 1 mM solution of (StBu)-peptide in 95% aqueous TFE 10 equiv tributylphosphine was added. After stirring for 2 h at room temperature, the solution was concentrated and the residue diluted with 50 mM AcOH. The excess phosphine was removed by extraction with methyl *tert*-butyl ether, and the aqueous layer was then lyophilized twice from 50 mM AcOH. The (SH)-peptides were obtained in quantitative yields.

Ac-Gly-(Pro-Hyp-Gly)$_4$-Asp-Gln-Gly-Pro-Hyp-Gly-Ile-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys-Gly-Gly-OH, α1(SH) (A). Yield: quantitative; RP-HPLC: t_R 6.54 min; MALDI-TOF-MS: $m/z = 2788.0$ [M$^+$]; $M_r = 2789.0$ calcd for $C_{119}H_{174}N_{32}O_{44}S_1$.

Ac-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Acm)-Cys-Gly-Gly-OH, α2(Acm,SH) (A). Yield: quantitative; RP-HPLC: t_R 6.86 min; MALDI-TOF-MS: $m/z = 2986.4$ [M$^+$]; $M_r = 2986.3$ calcd for $C_{128}H_{193}N_{37}O_{42}S_2$.

Ac-Gly-(Pro-Hyp-Gly)$_4$-Asp-Gln-Gly-Pro-Hyp-Gly-Ile-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Acm)-Cys-Gly-Gly-OH, α1(Acm,SH) (B). Yield: quantitative; RP-HPLC: t_R 6.74 min; MALDI-TOF-MS: $m/z = 2963.0$ [M$^+$]; $M_r = 2963.2$ calcd for $C_{125}H_{184}N_{34}O_{46}S_2$.

Ac-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys-Gly-Gly-OH, α2(SH) (B). Yield: quantitative; RP-HPLC: t_R 6.84 min; MALDI-TOF-MS: $m/z = 2811.6$ [M$^+$]; $M_r = 2812.1$ calcd for $C_{122}H_{183}N_{35}O_{40}S_1$.

H-Gly-(Pro-Hyp-Gly)$_4$-Asp-Gln-Gly-Pro-Hyp-Gly-Ile-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys-Gly-Gly-Gly-OH, α1(SH) (C). Yield: quantitative; RP-HPLC: t_R 5.01 min; MALDI-TOF-MS: $m/z = 2803.5$ [M$^+$]; $M_r = 2803.9$ calcd for $C_{119}H_{175}N_{33}O_{44}S_1$.

H-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Acm)-Cys-Gly-Gly-OH, α2(Acm,SH) (C). Yield: quantitative; RP-HPLC: t_R 5.09 min; MALDI-TOF-MS: $m/z = 2944.0$ [M$^+$]; $M_r = 2944.3$ calcd for $C_{126}H_{191}N_{37}O_{41}S_2$.

H-Gly-(Pro-Hyp-Gly)$_4$-Asp-Gln-Gly-Pro-Hyp-Gly-Ile-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Pro-Cys-Gly-Gly-OH, α1′(SH) (A/B/C). Yield: quantitative; RP-HPLC: t_R 6.77 min; MALDI-TOF-MS: $m/z = 2844.5$ [M$^+$]; $M_r = 2844.0$ calcd for $C_{122}H_{179}N_{33}O_{44}S_1$.

Synthesis of the Cys(Npys) Activated α Chains

A 1 mM solution of the (SH)-chains in degassed and argon-saturated DMF/AcOH 95 : 5 was added dropwise to a 10 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 (1–2 h), the solvent was removed and the residue dissolved in 50 mM AcOH. The excess reagent was removed by extraction with AcOEt, the aqueous layer lyophilized and the product purified by preparative RP-HPLC.

Ac-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Acm)-Cys(Npys)-Gly-Gly-OH, α2(Acm,Npys) (A). Yield: 59%; RP-HPLC: t_R 7.43 min; MALDI-TOF-MS: $m/z = 3140.2$ [M$^+$]; $M_r = 3140.5$ calcd for $C_{133}H_{195}N_{39}O_{44}S_3$.

Ac-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Npys)-Gly-Gly-OH, α2(Npys) (B). Yield: quantitative; RP-HPLC: t_R 7.45 min; MALDI-TOF-MS: $m/z = 2966.4$ [M$^+$]; $M_r = 2966.2$ calcd for $C_{127}H_{185}N_{37}O_{42}S_2$.

H-Gly-(Pro-Hyp-Gly)$_3$-Ala-Lys-Gly-Arg-Ala-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-(Pro-Hyp-Gly)$_2$-Cys(Acm)-Cys(Npys)-Gly-Gly-OH, α2(Acm,Npys) (C). Yield: 78%; RP-HPLC: t_R 5.34 min; MALDI-TOF-MS: $m/z = 3097.6$ [M$^+$]; $M_r = 3098.4$ calcd for $C_{131}H_{193}N_{39}O_{43}S_3$.

Synthesis of the Cys(Acm) Protected Heterodimers

The (SH)-peptide was dissolved at 1 mM concentration in degassed and argon-saturated 50 mM NH_4OAc (pH 5.5), and the solution was added dropwise to a 5 mM solution of 1.1 equiv of the (Npys)-peptide in 50 mM NH_4OAc (pH 5.5) under exclusion of air oxygen. The reaction was monitored spectroscopically at 430 nm, and after 4–5 h stirring at room temperature, the mixture was washed with AcOEt to remove the byproduct 5-nitro-2-thiopyridine. The aqueous layer was lyophilized and the product was isolated by preparative RP-HPLC.

α1α2(Acm) (A). The product was prepared from α1(SH) (A) (34.0 mg, 11.1 μmol) and α2(Acm,Npys) (A) (40.1 mg; 11.2 μmol) in 47% yield; RP-HPLC: t_R 6.82 min; MALDI-TOF-MS: $m/z = 5773.2$ [M$^+$]; $M_r = 5773.3$ calcd for $C_{247}H_{365}N_{69}O_{86}S_3$.

α2α1(Acm) (B). The title compound was prepared from α1(Acm,SH) (B) (50.0 mg; 14.8 μmol) and α2(Npys) (B) (53.3 mg; 14.9 μmol) in 71% yield; RP-HPLC: t_R 6.94 min; MALDI-TOF-MS: $m/z = 5772.4$ [M$^+$]; $M_r = 5773.3$ calcd for $C_{247}H_{365}N_{69}O_{86}S_3$.

α1α2(Acm) (C). The product was prepared from α1(SH) (C) (16.5 mg, 4.1 μmol) and α2(Acm,Npys) (C) (18.5 mg; 4.5 μmol) in 44% yield; RP-HPLC: t_R 5.08 min; MALDI-TOF-MS: $m/z = 5747.2$ [M$^+$]; $M_r = 5746.2$ calcd for $C_{245}H_{364}N_{70}O_{85}S_3$.

Synthesis of the Cys(Npys) Activated Heterodimers

To a 2 mM solution of the S-Acm protected heterodimer in AcOH a 20 mM solution of 5 equiv of freshly prepared 3-nitro-2-pyridinesulphenyl chloride in TFA was added in one portion. The reaction was monitored spectroscopically at 430 nm and after 10 min, the solution was diluted in 50 mM AcOH, and the excess reagent was extracted with AcOEt. The aqueous layer was lyophilized and the activated dimer was isolated by preparative RP-HPLC.

α1α2(Npys) (A). The title compound was obtained by reaction of α1α2(Acm) (A) (20 mg; 3.5 μmol) with Npys-Cl (3.3 mg; 17.5 μmol) in 32% yield; RP-HPLC: t_R 7.71 min; MALDI-TOF-MS: $m/z = 5856.7$ [M$^+$]; $M_r = 5856.3$ calcd for $C_{249}H_{362}N_{70}O_{87}S_4$.

α2α1(Npys) (B). α2α1(Acm) (B) (46 mg; 7.9 μmol) was reacted with Npys-Cl (7.4 mg; 39.5 μmol) to produce the title compound in 31% yield; RP-HPLC: t_R 7.39 min; MALDI-TOF-MS: $m/z = 5856.3$ [M$^+$]; $M_r = 5856.3$ calcd for $C_{249}H_{362}N_{70}O_{87}S_4$.

α1α2(Npys) (C). The title compound was obtained by reaction of α1α2(Acm) (C) (13 mg; 2.3 μmol) with Npys-Cl (2.2 mg; 11.5 μmol) in 30% yield; RP-HPLC: t_R 5.33 min; MALDI-TOF-MS: $m/z = 5829.5$ [M$^+$]; $M_r = 5829.3$ calcd for $C_{247}H_{361}N_{71}O_{86}S_4$.

Synthesis of the Heterotrimers A, B and C

A 1 mM solution of α1′(SH) (A/B/C) in degassed and argon-saturated 50 mM NH_4OAc (pH 5.5) was added dropwise to a 5 mM solution of 1.1 equiv of the S-Npys activated heterodimer in 50 mM NH_4OAc (pH 5.5) under exclusion of air oxygen. The reaction was monitored spectroscopically at 430 nm and after 6–7 h stirring at room temperature, the mixture was diluted with 50 mM AcOH and washed with AcOEt to remove the byproduct 3-nitro-2-thiopyridine. The aqueous layer was lyophilized and the product was isolated by preparative RP-HPLC.

α1α2α1′ Heterotrimer A. Reaction of α1′(SH) (A/B/C) (8.4 mg; 2.9 μmol) with α1α2(Npys) (A) (19.0 mg; 3.2 μmol) produced the heterotrimer A in 29% yield; RP-HPLC: t_R 7.20 min; amino acid analysis: Ala 2.28 (2), Arg 1.00 (1), Asp 1.94 (2), Cys 3.61 (4), Glu 2.02 (2), Gly 33.61 (36), Hyp 21.19 (23), Ile 1.71 (2), Leu 1.01 (1), Lys 1.02 (1), Phe 1.03 (1), Pro 18.01 (20); peptide content: 90.7%.

α2α1α1′ Heterotrimer B. From α1′(SH) (A/B/C) (17.0 mg; 5.5 μmol) and α2α1(Npys) (B) (32.5 mg; 5.6 μmol) the heterotrimer B was obtained in 37% yield; RP-HPLC: t_R 7.24 min; amino acid analysis: Ala 2.87 (2), Arg 1.25 (1), Asp 1.89 (2), Cys 4.18 (4), Glu 2.00 (2), Gly 37.81 (36), Hyp 22.11 (23), Ile 1.84 (2), Leu 1.32 (1), Lys 1.24 (1), Phe 1.33 (1), Pro 17.49 (20); peptide content: 72.1%.

α1α2α1′ Heterotrimer C. Reaction of α1′(SH) (A/B/C) (5.1 mg; 1.7 μmol) with α1α2(Npys) (C) (15.6 mg; 1.9 μmol) produced the heterotrimer C as an oil; RP-HPLC: t_R 6.58 min; amino acid analysis:

Ala 2.48 (2), Arg 1.00 (1), Asp 2.83 (2), Cys 4.14 (4), Glu 3.38 (2), Gly 40.25 (37), Hyp 25.88 (23), Ile 2.35 (2), Leu 1.02 (1), Lys 0.99 (1), Phe 0.94 (1), Pro 23.10 (20); peptide content: 39.8%.

Circular Dichroism

The CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a thermostatted cell holder and connected to a data station for signal averaging and processing. All spectra were recorded in the range 190–250 nm, employing quartz glass 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 a concentration of 3×10^{-5} M in Tris buffer (50 mM Tris·HCl, pH 7.4, 50 mM NaCl, 10 mM $CaCl_2 \cdot 2H_2O$), unless stated differently. 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 222 nm versus the temperature, in the range 4°–80 °C with a heating rate of 0.2 °C/min.

RESULTS AND DISCUSSION

Design of the Heterotrimeric Collagen Peptides in the $\alpha 1\alpha 2\alpha 1'$ and $\alpha 2\alpha 1\alpha 1'$ Register

In analogy to our previous syntheses of heterotrimeric collagen peptides in defined registers [15,16], an artificial *C*-terminal cystine knot was selected to assemble regioselectively the three synthetic chains in the desired order (Figure 1). With the assumption that a stable triple helix represents an essential prerequisite for recognition of the adhesion epitope by the integrin $\alpha 1\beta 1$, the sequence portions 457–468 of the $\alpha 1$ and $\alpha 2$ chains of collagen type IV were extended *C*- and *N*-terminally with two and three (Gly-Pro-Hyp) repeats, respectively, which are known to represent the most ideal sequence composition for induction and stabilization of the collagen fold [24–26]. A selective protection scheme was foreseen for the cysteine thiol groups to exploit regioselective cysteine chemistry for the assembly of the two constructs in the $\alpha 1\alpha 2\alpha 1'$ (heterotrimer A) and $\alpha 2\alpha 1\alpha 1'$ (heterotrimer B) registers (Figure 1). Thereby two of the three α chains were *N*-acetylated leaving the third amino group accessible for further postsynthetic manipulations. Moreover, to analyse the triple-helix destabilizing effect of electrostatic repulsion of the *N*-termini, the heterotrimer C was synthesized with all three *N*-termini non-acetylated.

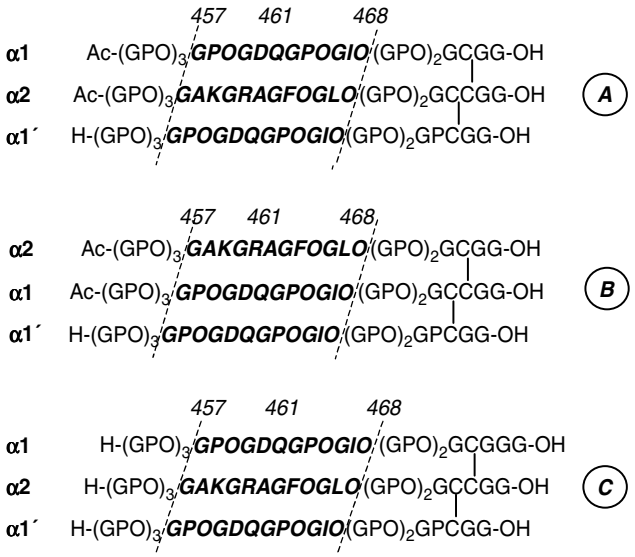


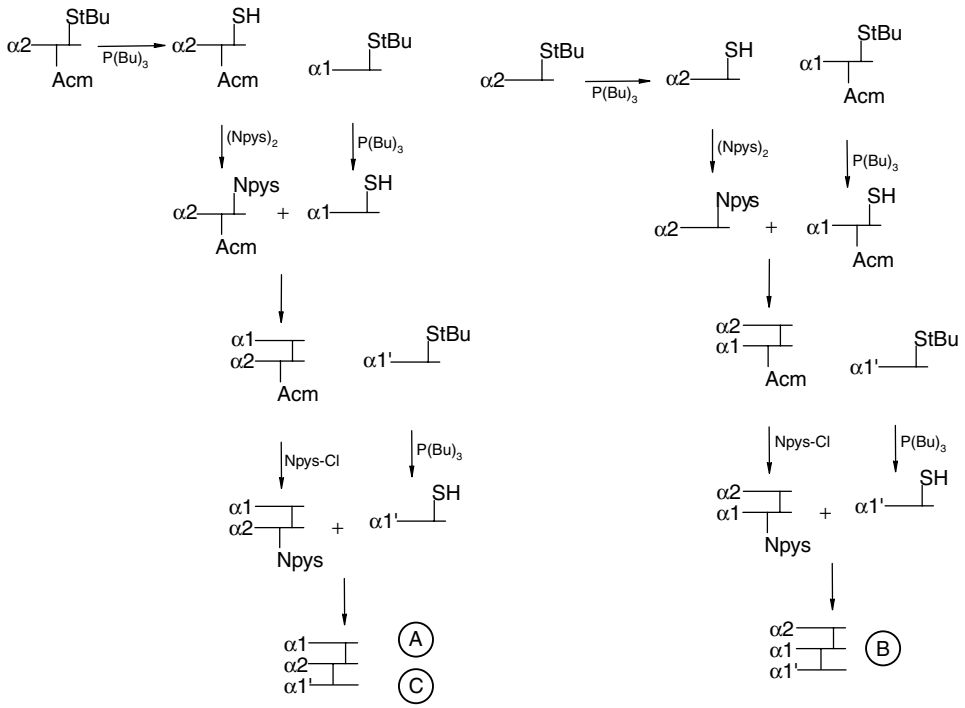
Figure 1 Synthetic heterotrimeric collagen peptides A, B and C containing the adhesion epitope 457–469 of collagen type IV for $\alpha 1\beta 1$ integrin recognition. The three α chains were assembled into the two most plausible registers; i.e. $\alpha 1\alpha 2\alpha 1'$ and $\alpha 2\alpha 1\alpha 1'$ for the heterotrimers A and B, respectively. The heterotrimer C has the same chain-composition and alignment of the heterotrimer A, but lacks the *N*-terminal acetyl group at the $\alpha 1$ and $\alpha 2$ chains.

Synthesis of the Single-Chain Collagenous Peptides

Taking into account the high tendency of collagenous peptides to self-aggregate into homotrimers and thus, the resulting difficult purification of such highly compact structures by means of chromatographic procedures [27], great care was taken to optimize the synthesis of the single chains [28,29]. The Fmoc/tBu chemistry [30] was selected for assembly of the chains, and the Fmoc-Pro-Hyp(tBu)-Gly-OH synthon was used whenever possible to minimize undesired cleavage of *N*-terminal dipeptides via diketopiperazine formation in the base-catalysed Fmoc-cleavage steps as reported previously for the synthesis of peptides containing the collagenase cleavage site [21]. However, in contrast to the latter peptides, the target peptide chains exhibit a sequence composition which according to the triple-helical propensity rules [31–33] would predict a significantly enhanced tendency to self-aggregation. In order to prevent such a phenomenon occurring during chain assembly on the resin, the Hyp hydroxy group was protected as *tert* -butyl ether not only when Hyp was incorporated as a single residue, but also as a tripeptide synthon. This strategy bears the advantage of excluding O-acylation and the possible O→N acyl shift during the Fmoc-cleavage step at *N*-terminal Hyp residues. Finally, a *C*-terminal Gly-Gly spacer was adopted to avoid side reactions at the level of cysteine residues directly attached to the solid support [34]. With this strategy the single peptide chains suitably protected at the cysteine residues became accessible as highly homogeneous materials in good and reproducible yields.

Regioselective Assembly of the Heterotrimers

For the regioselective cysteine crossbridging of the three α chains into the heterotrimers in the desired $\alpha 1 \alpha 2 \alpha 1'$ (heterotrimer A) and $\alpha 2 \alpha 1 \alpha 1'$ (heterotrimer B) registers a cysteine chemistry was applied as outlined in Scheme 1. Among the various strategies examined in the past [16], this approach to selective interchain-disulfide formation was found to be the most efficient [15]. It is based on the use of two orthogonal thiol-protecting groups, i.e. the *S*-*tert*-butylthio and the *S*-acetamidomethyl derivatives. Accordingly, the α chains with a single cysteine residue, i.e. $\alpha 1$ and $\alpha 1'$ of trimer A, and $\alpha 2$ and $\alpha 1'$ of trimer B as well as one of the two cysteine residues of the $\alpha 2$ chain of trimer A and of the $\alpha 1$ chain of trimer B were protected as *S*-*tert*-butylthio



Scheme 1 Regioselective assembly of the $\alpha 1$, $\alpha 2$ and $\alpha 1'$ cysteine peptides into the $\alpha 1 \alpha 2 \alpha 1'$ (heterotrimer A and C) and $\alpha 2 \alpha 1 \alpha 1'$ (heterotrimer B) registers.

derivatives to allow for their selective deprotection by reduction of the unsymmetric disulfide with tributylphosphine. Activation of the thiol-deprotected α2(Acm,SH) (A) and α1(Acm,SH) (B) chains as *S*-nitropyridylsulphenyl derivatives by reaction with di-(3-nitro-2-pyridine)disulfide was found to proceed straightforward in the case of the α2(Acm,SH) (A) chain, but is accompanied by formation of homodimer in the case of the α1(Acm,SH) (B) chain despite precautions taken to avoid air oxidation. This different behaviour of the two peptides can rationally be attributed to their differentiated propensities for self-aggregation into homotrimers which may facilitate this side reaction (*vide infra*). For this reason, in the synthetic approach adopted for the heterotrimer B the single-chain activation as *S*-nitropyridylsulphenyl derivative was performed on the reduced α2(SH) (B) chain, obtaining the product in good yields. Subsequent reaction of these activated α2(Acm,Npys) (A) and α2(Npys) (B) chains with the free thiol group of the *S*-deprotected α1(SH) (A) and α1(Acm,SH) (B) chains allowed for the selective disulfide bridging into the desired α1α2(Acm) (A) and α2α1(Acm) (B) heterodimers in satisfactory yields. The reactions were performed in degassed and argon-saturated aqueous solution under slightly acidic conditions (pH 5.0–5.5) in order to prevent undesired thiol/disulfide exchanges.

The subsequent step of assembly of the heterotrimers required activation of the second *S*-acetamidomethyl protected cysteine residue of the heterodimers without affecting the preformed disulfide bond. In view of our previous positive experiences gained in the synthesis of collagenous heterotrimers with 3-nitropyridyl-2-sulphenyl chloride (Npys-Cl) for direct conversion of *S*-Acm protected dimers into the activated *S*-Npys derivatives [16], rather unexpected were the difficulties encountered with this reagent in the case of the present target molecules. Therefore various alternative procedures were examined such as the reaction of the *S*-Acm derivatives of the heterodimers with 5-nitropyridyl-2-sulphenyl chloride [35] or analogously with 2-nitrophenylsulphenyl chloride [36] or the more reactive 2,4-dinitrophenysulphenyl chloride as well as with silver trifluoromethanesulphonate [37]. However, the use of Npys-Cl still proved to be the most efficient procedure although not at all devoid of side reactions. These will be discussed in more detail in the accompanying article [38]. Based on the observation that the side reactions can be at least partially controlled by kinetic means, the activated *S*-Npys derivatives of the heterodimers A and B, i.e. α1α2(Npys) (A) and α2α1(Npys) (B), could finally be obtained in satisfactory yields by using a maximum of 5 equiv of freshly prepared Npys-Cl which was added in a single portion, and by quenching the reaction after 10 min via extraction of the excess Npys-Cl reagent.

The subsequent regioselective disulfide bridging of the *S*-Npys activated heterodimers A and B with the reduced α1′ chain was performed under exclusion of air oxygen as described above for the synthesis of the heterodimers. By this procedure both the heterotrimers A and B became accessible in good yields and with a high degree of homogeneity as assessed by RP-HPLC (Figure 2). For further analytical characterization of the trimers quantitative amino acid analysis allowed us to establish the correct ratios of the three α chains. Conversely, both ESI-MS and MALDI-TOF failed as reported previously for other collagenous heterotrimers [39]. Finally, the heterotrimer C was prepared by following the synthetic route of the trimer A.

Dichroic Properties of the Monomeric Collagenous Peptides

The triple-helical conformation of collagenous-peptides is characterized by a CD spectrum with

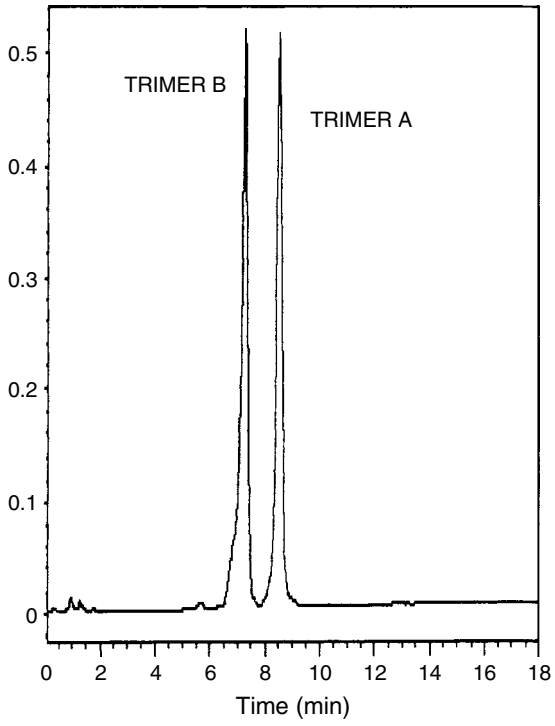


Figure 2 Chromatographic RP-HPLC profiles of the purified heterotrimers A and B.

a relatively strong positive maximum (2000–6000 $[\theta]_R$) centred at 220–223 nm and an intense negative band (−30000 to −55000 $[\theta]_R$) located at 196–200 nm. A very similar CD spectrum is typical for the poly(Pro)-II helix, which differs however from that of the triple helix by the slightly red-shifted positive maximum at 224–226 nm and the less intense negative maximum located at 198–205 nm [40]. Despite these largely overlapping CD spectra, the two types of conformations can be well differentiated by the Rpn value which is defined as the absolute value of the ratio between the dichroic intensities of the positive maximum over that of the negative maximum [41,42]. (Rpn = 0.06 for poly(Pro)-II-like structures in aqueous solutions and Rpn = 0.13 for collagen type I in water [43]). This Rpn parameter has been validated by a careful comparative CD and NMR conformational study of $(Gly\text{-}Pro\text{-}Hyp)_n$ peptides and their homotrimers cross-linked with Kemp's triacid [44,45].

The conformational properties of the single α chains containing the cell-adhesion epitope of collagen type IV were analysed by CD and the most relevant data are summarized in Table 1. Buffered solutions (pH 7.4) of the peptides were prepared at a concentration (3×10^{-5} M) largely below that generally required for self-association into homotrimers of analogous collagenous peptides containing the same total number of (Gly-Pro-Hyp) repeats [44]. The solutions were equilibrated for 12 h at 4 °C prior to recording the CD spectra. The pattern of the CD spectrum of the α2(Acm,StBu) (A) chain at 4 °C and the related Rpn value of 0.072 would agree with a poly(Pro)-II conformation as further supported by the monotonic thermal transition reported in Figure 3a. Such a non-cooperative thermal denaturation is typical for poly(Pro)-II helices [46]. Conversely, the α1(StBu) (A) chain exhibits a CD spectrum similar to those reported for collagenous peptides [44] with a Rpn value of 0.116 and a T_m of 40 °C (Figure 3b). The α1(StBu) (A) and α2(Acm,StBu) (A) peptides contain, in addition to the natural sequences 457–468 of the related α1 and α2 chains of collagen type IV, the identical number of (Gly-Pro-Hyp) repeats at the *C*- and *N*-terminus, with $n = 2$ and 3, respectively (Figure 1). However, the α1(StBu) (A) chain contains two additional, although non-consecutive repeats which markedly enhance the tendency to self-association into a relatively stable triple helix.

The strong triple-helical stabilizing effect of the *N*-terminal acetylation, i.e. of suppression of electrostatic repulsions, is well evidenced by comparing

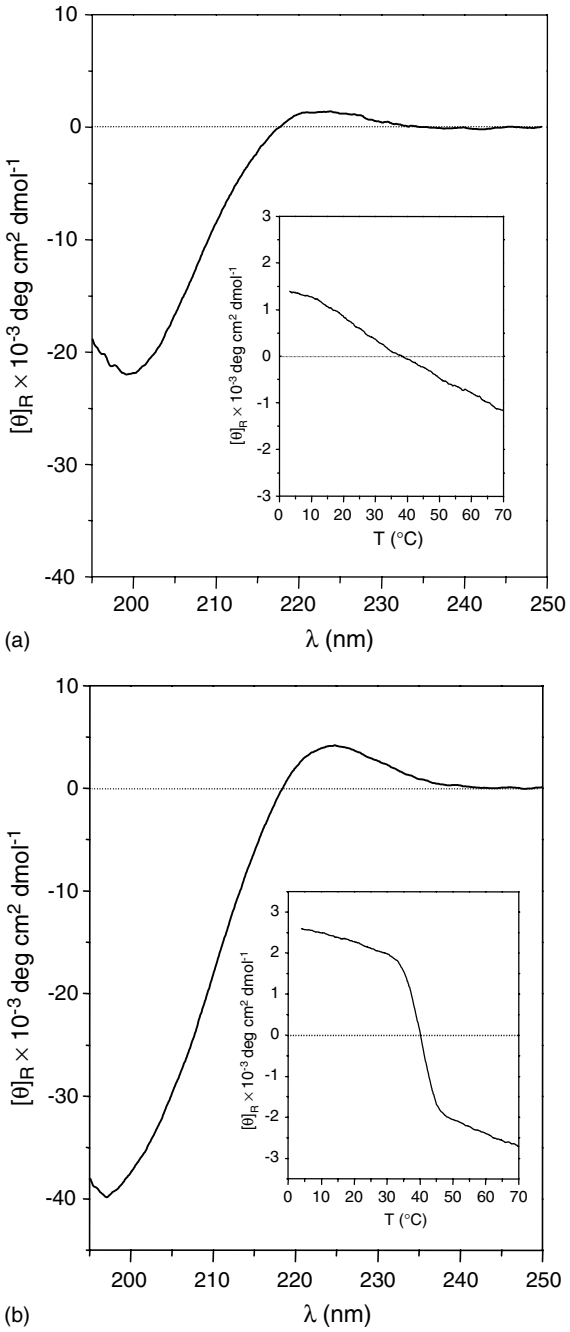


Figure 3 CD spectrum and thermal denaturation curve (inserted panel) of (a) monomeric α2(Acm,StBu) (A) chain (b) monomeric α1(StBu) (A) chain.

the dichroic properties of the *N*-acetylated α1(StBu) (A) chain with those of the corresponding non-acetylated α1′(StBu) (A/B/C) which shares the identical amino acid sequence. Despite the low Rpn value determined for the non-acetylated peptide (0.055), its sigmoidal thermal transition with a T_m value

of 35 °C still suggests a triple-helical conformation, but to a lower extent and of weaker stability. Very similar results were obtained with the α1(Acm,StBu) and α2(StBu) chains of the trimer B, since these differ from those of the trimer A only in their *C*-termini in terms of cysteine residues (data not shown).

By comparing the CD spectra of these single α chains consisting of nine tripeptide repeats and containing a Gly residue at every third sequence position, with those reported for the collagenous peptides Ac-(Gly-Pro-Hyp)$_n$-NH_2 ($n = 1$, 3, 5, 6, 9) [44], it is evident that the amino acid composition of the adhesion epitope of collagen type IV is not an ideal one for a triple-helical fold.

Dichroic Properties of the Heterotrimers A and B

As reported previously for collagenous heterotrimers related to the collagenase cleavage site of collagen type I [15], *C*-terminal crosslinking of three α chains into heterotrimers leads to a significant stabilization of the triple-helical fold, even when the single chains show low or no tendency to self-associate into triple helices. As shown in Figure 4a, both the heterotrimer A and B exhibit CD spectra characteristic of the collagen triple helix. Although the Rpn values (>0.1) measured at 4 °C are almost identical for the two trimers (Table 1) and not significantly affected by the type of buffer, pH and peptide concentration (Table 2, data shown only for the trimer B), the T_m of the trimer B is markedly lower than that of the trimer A (Figure 4b). Except for their *C*-terminal cystine knots, the chains of the two trimers are identical in the sequence composition. Therefore, the observed different stability of their triple-helical structure has to derive mainly from their different register. The collagen triple helix is known to be stabilized by a very regular hydrogen bonding network with all Gly NH protons of the (Gly-Xaa-Yaa) repeats hydrogen-bonded to Pro carbonyls in position Xaa of the adjacent chain [47–49], whereas the hydration shell apparently does not contribute significantly to the thermal stability of the triple helix [50–52]. This main hydrogen bonding pattern should be fully retained in the trimers A and B, independently of their register, with only the hydration shell being altered by the different spatial display of the side chains. Thus, even the hydration shell has to contribute to the overall stability of the triple helix at least when the chains are built-up non solely by the ideal (Gly-Pro-Hyp) repeats.

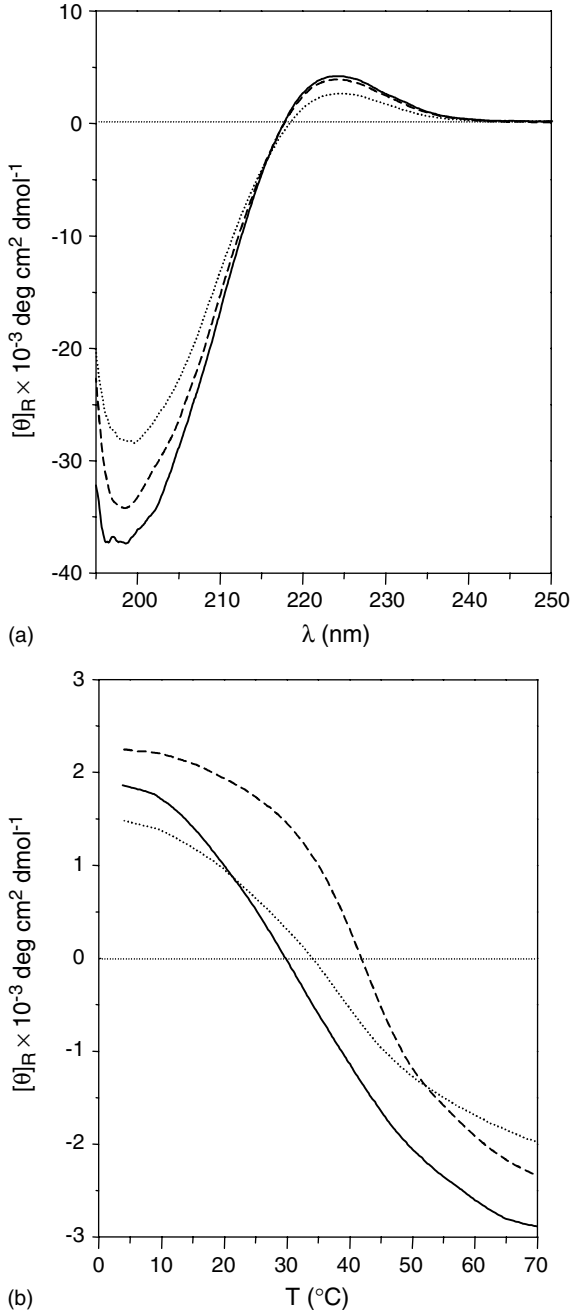


Figure 4 (a) CD spectra of the heterotrimers A (- - - -), B (———) and C (·····) at 4 °C and at 3×10^{-5} M concentration in 50 mM Tris·HCl, 50 mM NaCl, 10 mM $CaCl_2 \cdot 2H_2O$ (pH 7.4). (b) Thermal denaturation curves of the heterotrimers A (- - - -), B (———) and C (·····) at 3×10^{-5} M concentration in 50 mM Tris·HCl, 50 mM NaCl, 10 mM $CaCl_2 \cdot 2H_2O$ (pH 7.4).

As already observed for the non-acetylated α1′(StBu) (A/B/C) chain, and also for the heterotrimer A when constructed with all three chains

Table 1 CD Data of Collagenous Peptides Containing the Adhesion Epitope 457–469 of Collagen Type IV at 3×10^{-5} M Concentration in 50 mM Tris·HCl, 50 mM NaCl,10 mM $CaCl_2 \cdot 2H_2O$ (pH 7.4). The CD Spectra were Recorded at 4 °C, after 12 h of Pre-equilibration at the same temperature. The Thermal Denaturation Curves were Registered with a Heating Rate of 0.2 °C/min

Peptide	max (nm; θ_R)	min (nm; θ_R)	Rpn	T_m (°C)
α1(StBu) (A)	225; 4570	197; −39456	0.116	40
α2(Acm,StBu) (A)	223; 1579	199; −21732	0.072	Monotonic
α1′(StBu) (A/B/C)	226; 1561	199; −28028	0.055	35
Heterotrimer A	224; 3831	198; −34317	0.112	42
Heterotrimer B	225; 4078	199; −37274	0.109	30
Heterotrimer C	225; 2427	199; −28549	0.085	34
Poly(Pro)-II	—	—	0.060	Monotonic
Collagen type IV (fragment F1)	—	—	—	39

Table 2 Cd Data of the Heterotrimer B at 4 °C in Different Buffers and at Different pH Values and Concentrations as well as the Melting Temperatures under these Conditions

Heterotrimer B	max (nm; θ_R)	min (nm; θ_R)	Rpn	T_m (°C)
H_2O, pH 4.4, $c = 3 \times 10^{-5}$ M	224; 4057	198; −37558	0.108	34
Phosphate buffer pH 7.1, $c = 3 \times 10^{-5}$ M	224; 4089	198; −37546	0.109	31
Phosphate buffer pH 5.5, $c = 3 \times 10^{-5}$ M	224; 3908	198; −36979	0.105	29
Tris buffer, pH 7.4, $c = 3 \times 10^{-6}$ M	225; 3560	199; −32076	0.111	33

non acetylated at the *N*-termini (trimer C), a decreased stability of the triple helix is observed as a result of the electrostatic repulsions at the *N*-termini (Table 1 and Figure 4b).

In conclusion, the dichroic properties of the two heterotrimers confirm again the significant effect of the *C*-terminal crosslinking of the three α chains on the conformational preferences. Thereby, the simple artificial cystine knot used for the construction of the heterotrimers apparently not only stabilizes entropically the triple helix, but also acts as a nucleation centre of the supercoiled structure.

Preliminary binding experiments of the synthetic heterotrimers A and B with $\alpha 1\beta 1$ integrin showed a slight preference for the trimer B. These results would indicate that the alignment of the three α chains within the collagen triple helix affects not only the stability and biophysical properties of the molecule, but may also dictate a more or less tight binding to the integrin receptor.

Acknowledgements

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