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Effect of N- and C-Terminal Functional Groups on the Stability of Collagen Triple Helices

Jasmine Egli,[a] Roman Erdmann,[a,b] Pascal Schmid,[a,c] and Helma Wennemers*[a]

[a] Laboratory of Organic Chemistry, D-CHAB, ETH Zurich,
Vladimir-Prelog-Weg 3,
CH-8093 Zurich, Switzerland.
E-mail: Helma.Wennemers@org.chem.ethz.ch

[b] Current address: Merck Research Laboratories, Kenilworth New Jersey, USA

[c] Current address: Hoffmann-La Roche, Basel, Switzerland

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1) General Aspects

Materials and reagents were of highest commercially available grade and used without further purification. They were purchased from Acros Organics, Aldrich, Fischer, Fluka, Bachem, Biotage, Chem-Impex, IRIS Biotech, Merck Millipore, Protein Technologies *Int.*, and TCI. Water used for peptide preparation and purification was Mili-Q water with resistivity of 18.2 MΩ*cm, prepared by a Sartorius Arium611VF water purification system. Reactions were monitored by TLC (thin layer chromatography) using Merck silica gel 60 F254 plates. Compound were visualized using UV, ninhydrin and $KMnO_4$ staining.

For automated **solid phase peptide synthesis** (SPPS), Rink amide ChemMatrix resin from Biotage and chlorotrityl chloride resin from Fluorochem were used. A Syro I peptide synthesizer (MultiSynTech GmbH, Witten Germany) was employed.

High-resolution **mass spectrometry** (MS) was performed by the MS-service of the Laboratory of Organic Chemistry at ETH Zurich using a Bruker Daltons maXis equipped with an ESI (electrospray ionization) source and a Q-TOF ion analyzer, or a Bruker Daltonics SOLARIX equipped with a MALDI (matrix-assisted laser desorption/ionization)/ESI source and a Q-TOF ion analyzer. A α-cyano-4-hydroxycinnamic (CHCA) was used as MALDI-MS matrix.

Analytical reversed-phase high-performance liquid chromatography (HPLC) was performed on a Dionex UHPLC Ultimate 3000 (Thermo Fisher Scientific, Waltham/USA).

Preparative reverse-phase HPLC purification was carried out on a Dionex UHPLC Ultimate 3000 (Thermo Fisher Scientific, Waltham/USA).

Analytical gel permeation chromatography was performed on a Dionex UHPLC equipped with a size exclusion column (Superdex™ Peptide column).

Circular dichroism (CD) spectra were recorded on a Chirascan plus spectrometer (Applied Photophysics Ltd, Leatherhead/UK) with a Nitropack nitrogen generator (Parker Balston, Haverhill/USA) and a temperature controller TC 125 (Quantum Northwest). The solutions were measured in a quartz cell with a pathlength of 1.0 mm (Hellma 110-QS).

Peptides were dried on a lyophilizer by Christ Alpha 2-4 LD plus (Kuhnner AG, Birsfelden/CH).

2) General Protocols for the Synthesis of CMPs

Protocol A - General procedure for swelling
Before automated peptide synthesis, the resin was swollen in CH_2Cl_2 for 15 min. while shaking, drained, and washed with DMF (dimethylformamide) (3 x 6 mL) and drained again.

Protocol B – First coupling to 2-chlorotrityl chloride resin
2-Chlorotrityl chloride resin (1 equiv., 1.6 mmol/g) was dried under N_2. Fmoc-Pro-Hyp-Gly-OH (1 equiv. 0.2 M) in dry DMF/CH_2Cl_2 (1:4) was added to the resin. Freshly distilled *i*-Pr_2NEt (5 equiv.) was added to the reaction mixture under N_2, which was shaken for 60 min. To cap any remaining reactive groups, CH_3OH (0.8 mL/g) was added and shaken for 15 min. The resin was then washed with DMF (2x), CH_2Cl_2 (2x), CH_3OH (2x), and CH_2Cl_2 (2x).

Protocol C – General procedure for automated peptide synthesis at room temperature
For automated peptide synthesis, a Syro I peptide synthesizer was used. After swelling the resin in DMF on the synthesizer, *i*-Pr_2NEt (9 equiv. as a 3 M solution in NMP (*N*-methyl-2-pyrrolidone)), HCTU (2-(6-Chlor-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium-hexafluorophosphate) (3 equiv., 0.5 M in DMF) and the Fmoc-amino acid/Fmoc-tripeptide (3 equiv., 0.5 M in DMF) were added to the resin. The mixture was allowed to react in intervals of 1 min. agitation and 5 min. rests for 1 h and was then washed with DMF (5x). Fmoc-deprotection was carried out by addition of a solution of 20% (*v/v)* piperidine in DMF and reaction for 5 min. This step was repeated for 10 min. The resin was then washed with DMF (5x). Tripeptide couplings and Fmoc-deprotections were repeated until the desired peptides were obtained.

Protocol D - Acetylation
Acetylation of the solid phase bound peptide was performed manually after the last coupling and deprotection. AcOH (3.0 equiv.) and *i*-Pr_2NEt were dissolved in CH_2Cl_2 (4-5 mL). After pre-activation for 5 min, the coupling mixture was added to the resin. and agitated for 1-2 hrs. The resin was washed with CH_2Cl_2 (3x), DMF (3x), CH_2Cl_2 (3x), and petroleum ether (2x). The reaction was monitored by the qualitative color tests on bead or by LC-MS after cleavage of a small sample (see Protocol F).

Protocol E - Cleavage from the resin
The resin was shaken for 1 h in a mixture of TFA (trifluoroacetic acid)/(*i*-$Pr)_3SiH/H_2O$ (92.5:2.5:2.5). The solution was collected by filtration in a conical flask. Addition of ice-cold Et_2O afforded the peptide as a white precipitate. The solid was isolated by centrifugation followed by decantation. The solid was suspended in Et_2O, the suspension was sonicated, centrifuged again and the supernatant was decanted. The residual white solid was dissolved in water/CH_3CN, frozen, and lyophilized to obtain a white foam.

Protocol F – Purification and analysis by RP HPLC
CH_3CN (A) and H_2O containing 1% CH_3CN and 0.1% TFA (B) were used as eluents. For semi-preparative HPLC a flow rate of 5 mL/min., for analytical HPLC a flow rate of 1 mL/min and for LC-MS a flow rate of 0.5 mL/min. was used. The column oven was heated to 65 °C to prevent triple helix formation, expect for LC-MS where it was at

50 °C. After the semi-preparative HPLC purification all collected fractions were analyzed by analytical HPLC or LC-MS and only pure fractions were combined.

Preparative Columns: Phenomenex, Jupiter 4u, Proteo 90Å, 250 x 10 mm, 4 micron (1); *Analytical Columns*: Phenomenex, Jupiter 4u, Proteo 90Å, 250 x 4.6 mm , 4 micron (2); *LC-MS*: Reprosil Gold C18, 125 x 3mm (3).

Protocol G – Desalting

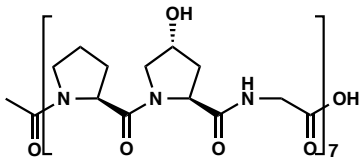
The purified peptides were desalted with VariPure IPE desalting cartridges from Agilent.

3) Synthesis and Analytical Data of Collagen Model Peptides

Ac-[ProHypGly]$_7$-NH$_2$ (CMP 1)

CMP **1** was synthesized according to previously published protocols.[1]

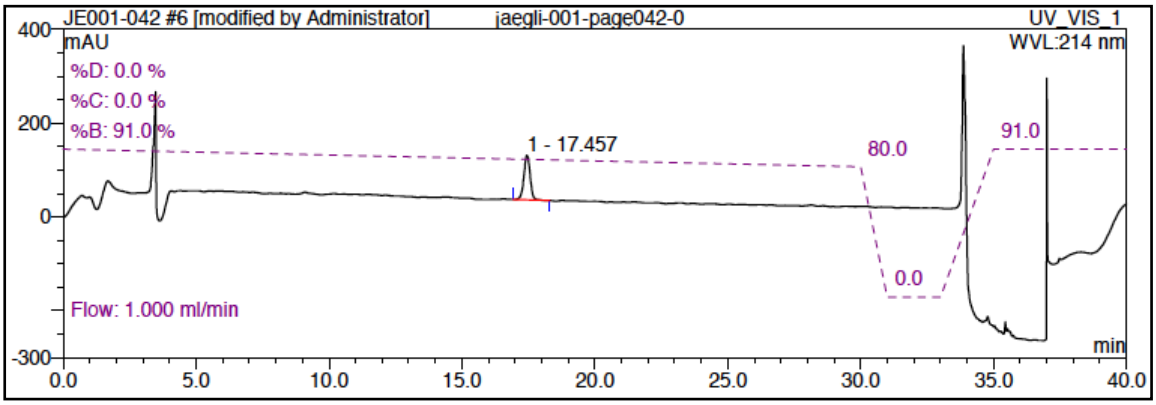
Ac-[ProHypGly]$_7$-OH (CMP 2)



2

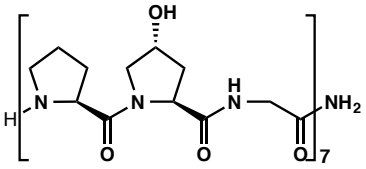
CMP **2** was synthesized on 2-Chlorotrityl chloride resin (1.6 mmol/g). The first tripeptide building block was coupled according to Protocol B. After swelling according to Protocol A, Fmoc-ProHypGly-OH was coupled according to Protocol C. The peptide was then acetylated according to Protocol D. The peptide was cleaved from the solid support according to protocol E, and purified according to Protocol F using a gradient from 93% to 80% B over 30 min. on column (1). After lyophilization CMP **2** was obtained as white foam (7.1 mg, 3.6 μmol)

Analytical reverse-phase HPLC on column (2): 91% to 80% B over 30 min, t_R = 17.46 min.; Purity determined by analytical HPLC using UV detection at 214 nm: >99%.



HRMS (MALDI): *m/z* calcd for [$C_{86}H_{123}N_{21}O_{30}$]: 1929.8817, found $[M+H]^+$ = 1930.8823, found $[M+Na]^+$ = 1952.8641.

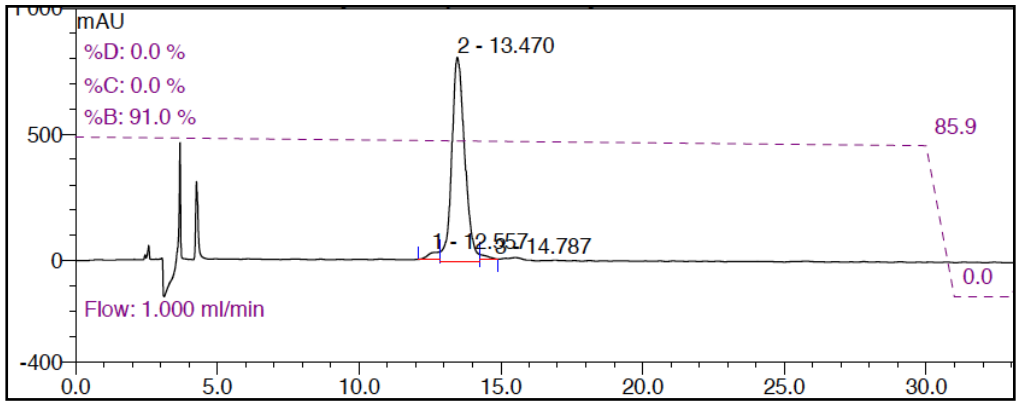
H-[ProHypGly]$_7$-NH_2 (CMP 3)



3

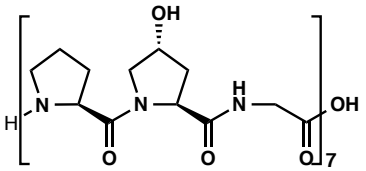
CMP **3** was synthesized on Rink amide ChemMatrix resin (0.47 mmol/g, 100 mg). After swelling according to Protocol A, Fmoc-ProHypGly-OH was coupled according to Protocol C. The peptide was cleaved from the solid support according to protocol E, and purified according to F using a gradient from 91% to 85.9% B over 30 min. on column (1). After desalting according to protocol G and lyophilization CMP **3** was obtained as a white foam (9.4 mg, 4.98 μmol)

Analytical reverse-phase HPLC on column (2): 91% to 85.5% B over 30 min, t_R = 13.4 min.; Purity determined by analytical HPLC using UV detection at 214 nm: 95%.



HRMS (MALDI): *m/z* calcd for $[C_{84}H_{122}N_{22}O_{28}]^+$: 1888.0260, found $[M+H]^+$ = 1889.718.

H-[ProHypGly]$_7$-OH (CMP 4)

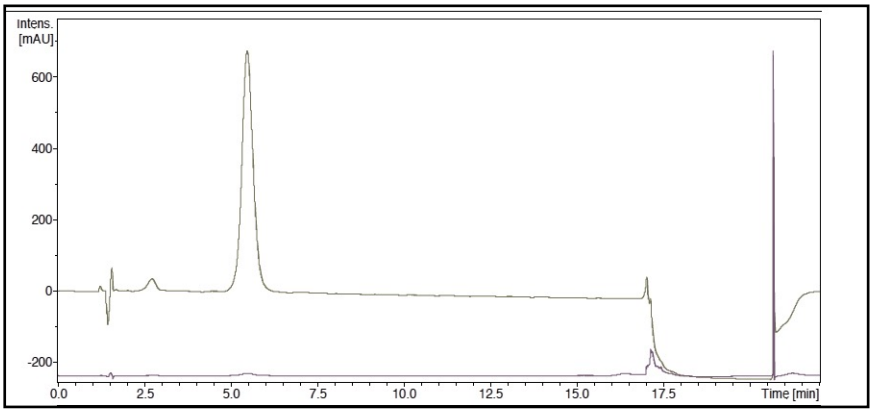


4

CMP **4** was synthesized on 2-Chlorotrityl chloride resin (1.6 mmol/g). The first tripeptide building block was coupled according to Protocol B. After swelling according to Protocol A, Fmoc-ProHypGly-OH was coupled according to Protocol C. The peptide was cleaved from the solid support according to protocol E, and purified according to F using a gradient from 95% to 83% B over 30 min. on

column (1). After desalting according to protocol G and lyophilization CMP **4** was obtained as a white foam (3.5 mg, 1.85 μmol)

Analytical reverse-phase HPLC on column (3): 95% to 80% B over 15 min, t_R = 5.5 min.; Purity determined by analytical HPLC using UV detection at 214 nm: 95%.



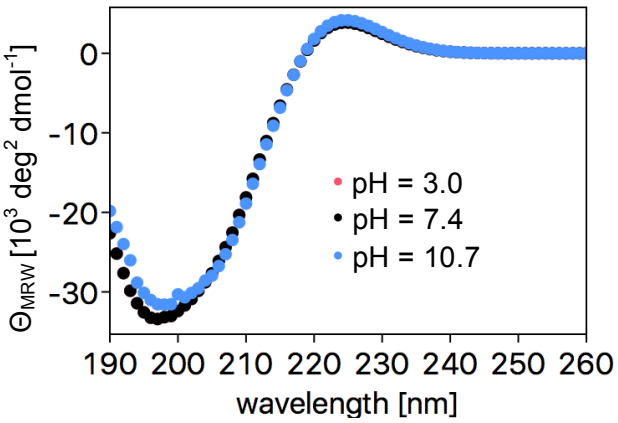
HRMS (MALDI): *m/z* calcd for $[C_{84}H_{122}N_{22}O_{28}]^+$: 1888.0260, found $[M+H]^+$ = 1889.718.

4) CD Spectra and Thermal Denaturation Studies

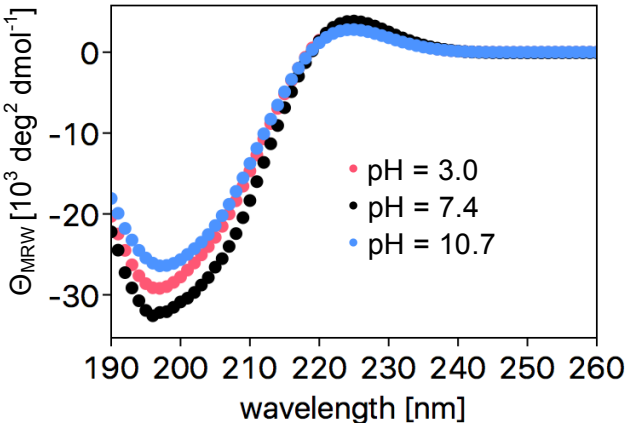
CD Spectra derived from CMPs

The CD spectra for CMP **1-4** of the triple helices were recorded at 7°C as a 0.2 mM solution in miliQ-water. The samples were equilibrated for >24 hrs. The spectra were recorded from 190 nm to 260 nm. Max. ellipticity at 224 nm and min. ellipticity at 195 nm.

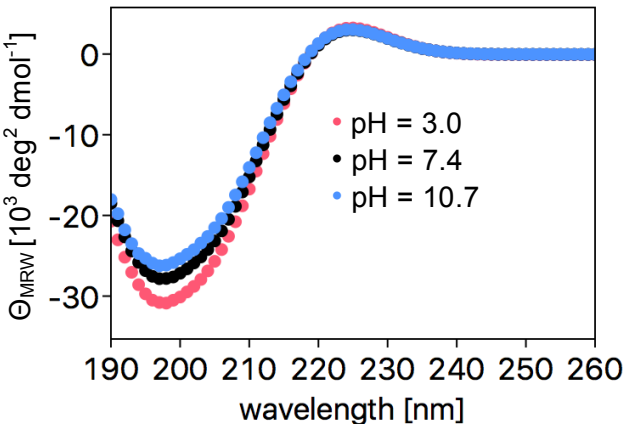
CD spectra of CMP **1** at different pH

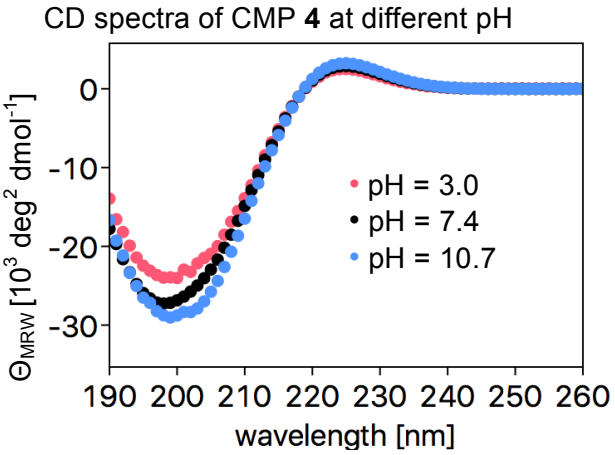


CD spectra of CMP **2** at different pH



CD spectra of CMP **3** at different pH



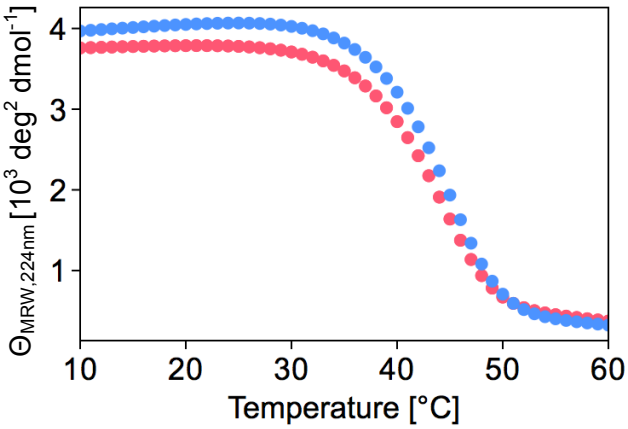


General Procedure for Determination of T_m-values

The midpoints of thermal transitions of triple helices derived from CMP **1**-**4** were determined using 0.2 mM solutions in aqueous AcOH (pH = 3), in PBS buffer (pH = 7.4), and aqueous NaOH/$NaHCO_3$ (pH = 10.7). The data were recorded at a heating rate of 1°C/100 s.
The recorded data were fit to an all or none transition in which three single strands combine to a triple helix as previously reported by Engel et al.[2,3] The fitting was perfomed using Micromath Scientist 3.0 with H=-500'000 and T_m = 40 as initial values. The model used is shown below:

// Model two state
IndVars: TEMP
DepVars: F, CD, K
Params: H, DEU, REFU, DEN, REFN, Tm
R=8.31
K=EXP(H/(R*(TEMP+273.15))*((TEMP+273.15)/(Tm+273.15)-1)-ln(0.75*0.0002^2))
P=1/(3*K*(0.0002^2))
U=(-P/2+(P^2/4+P^3/27)^(1/2))^(1/3)
V=-(P/2+(P^2/4+P^3/27)^(1/2))^(1/3)
F= U+V+1
CDU=REFU+DEU*(TEMP+273.15)
CDN=REFN+DEN*(TEMP+273.15)
CD=F*(CDN-CDU)+CDU

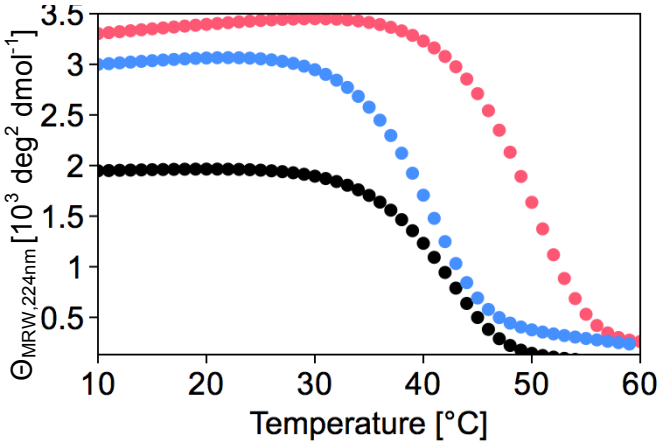
Thermal denaturation curve of Ac-[ProHypGly]$_7$-NH$_2$ (CMP 1) at different pH



- T_m = 43 °C, pH = 3.0
- T_m = 43 °C, pH = 7.4
- T_m = 43 °C, pH = 10.7

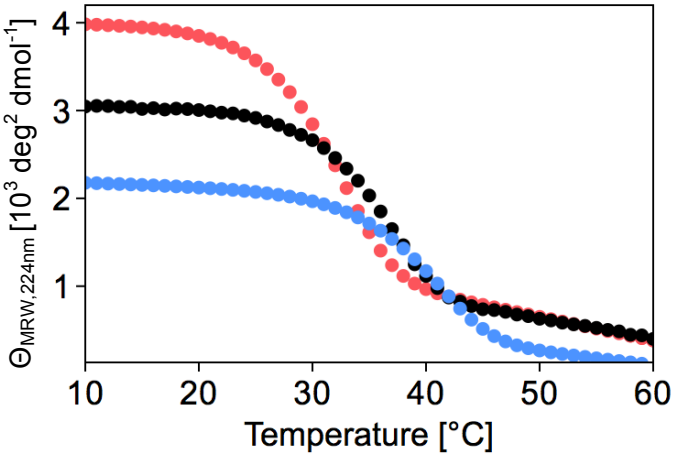
(Note that the data points for pH 7.4 are identical to those at pH 3.0)

Thermal denaturation curve of Ac-[ProHypGly]$_7$-OH (CMP 2) at different pH



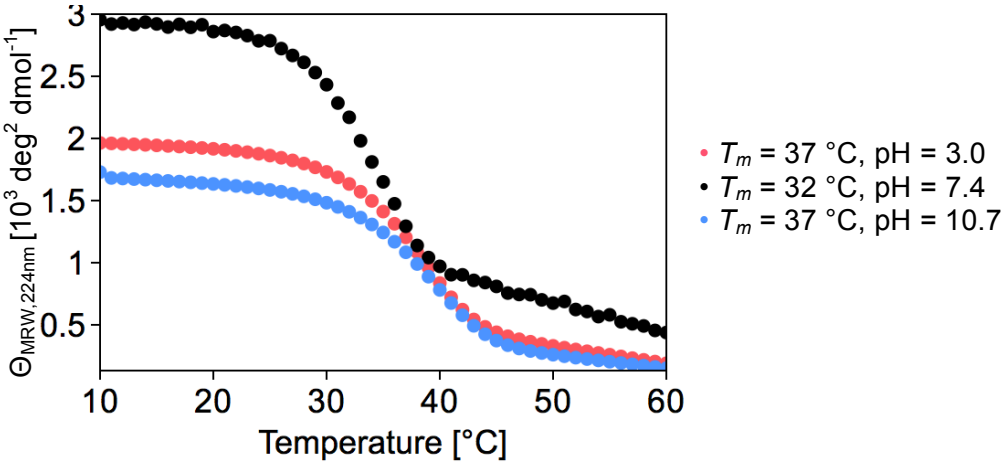
- T_m = 49 °C, pH = 3.0
- T_m = 40 °C, pH = 7.4
- T_m = 40 °C, pH = 10.7

Thermal denaturation curve of H-[ProHypGly]$_7$-NH$_2$ (CMP 3) at different pH



- T_m = 31 °C, pH = 3.0
- T_m = 35 °C, pH = 7.4
- T_m = 41 °C, pH = 10.7

Thermal denaturation curve of H-[ProHypGly]$_7$-OH (CMP 4) at different pH



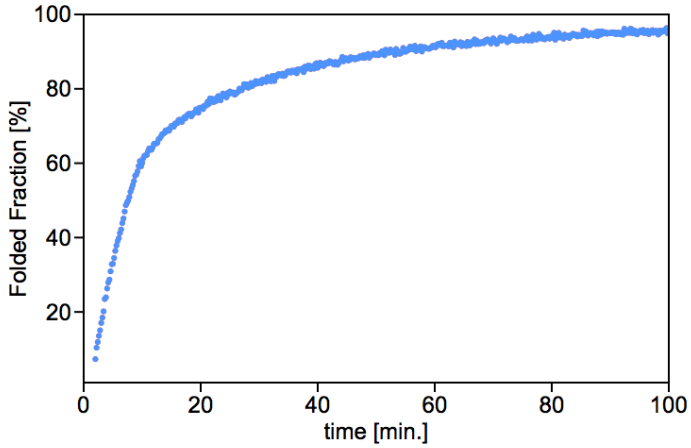
5) Refolding Kinetics – Temperature jump experiment

For the kinetic study of the folding of the triple helix, CD spectroscopy was used as a monitoring tool. A peptide concentration of 0.2 mM in the described buffers was used. During these "temperature jump experiments", the sample was in a 1 mm cuvette and was heated at 90 °C for 20 min., then the instrument was cooled immediately to 7 °C. The ellipticity at 225 nm or 224 nm was monitored over time. After a dead time of 2 min., the data was collected for 120 min. Refolding half-time ($t_{1/2}$) is the time at which half of the peptides have refolded into helices. It was determined when the folded fraction equated 0.5. The folded fraction was calculated by the following equation:

$$F = \frac{\theta_t - \theta_u}{\theta_f - \theta_u}$$

where θ_t the ellipticity at time t, θ_f is the ellipticity of the folded form and θ_u of the unfolded form.

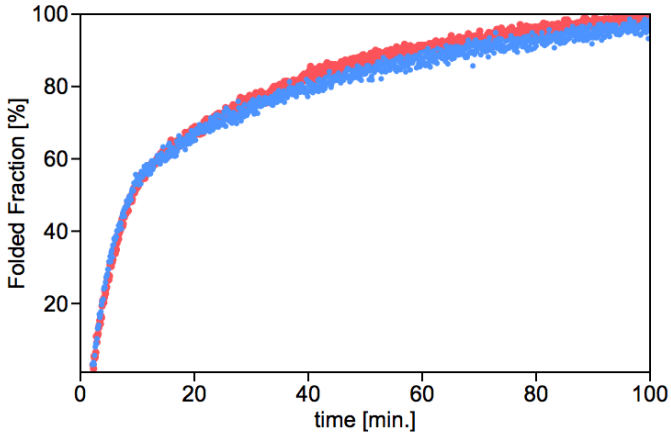
Ac-[ProHypGly]$_7$-NH$_2$ (CMP 1)



- $t_{1/2}$ = 8 min., pH = 3.0
- $t_{1/2}$ = 8 min., pH = 10.7

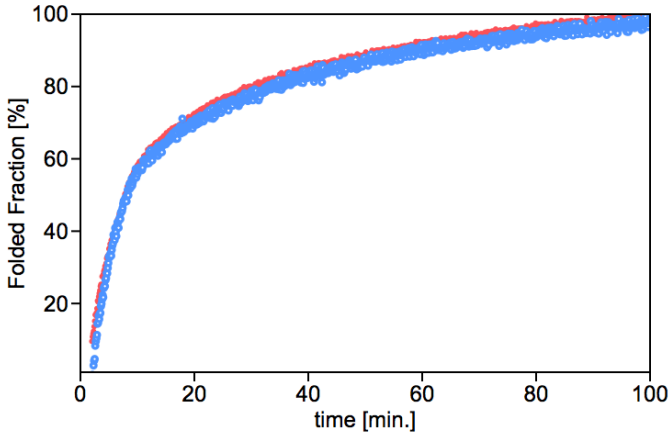
(Note that the data points for pH 10.7 are identical to those at pH 3.0)

Ac-[ProHypGly]$_7$-OH (CMP 2)



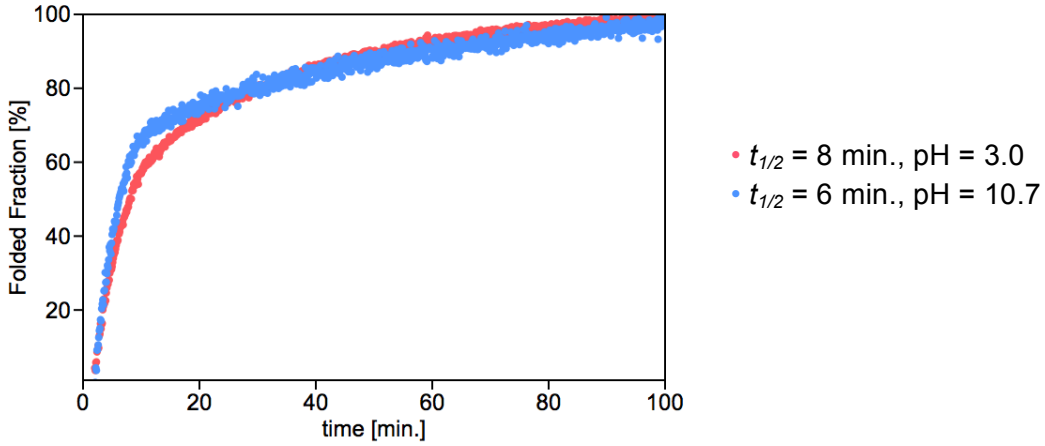
- $t_{1/2}$ = 9 min., pH = 3.0
- $t_{1/2}$ = 9 min., pH = 10.7

H-[ProHypGly]$_7$-NH$_2$ (CMP 3)



- $t_{1/2}$ = 8 min., pH = 3.0
- $t_{1/2}$ = 8 min., pH = 10.7

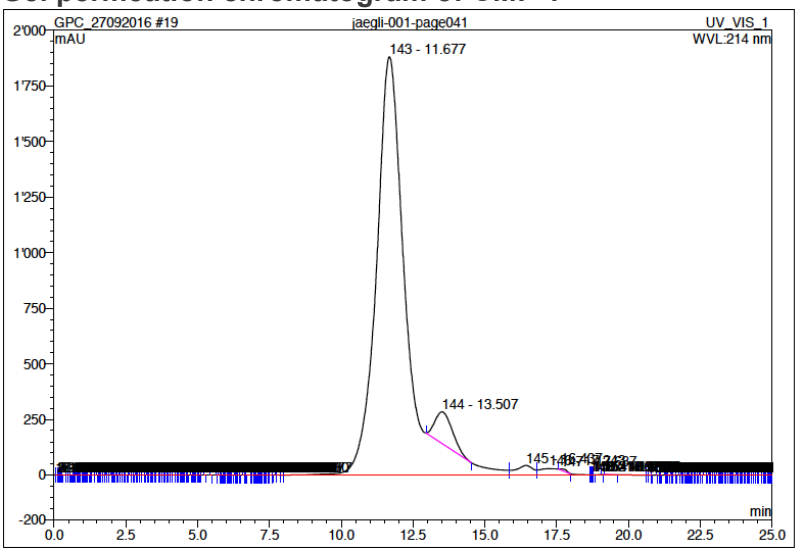
H-[ProHypGly]$_7$-OH (CMP 4)



6) Gel Permeation Chromatography

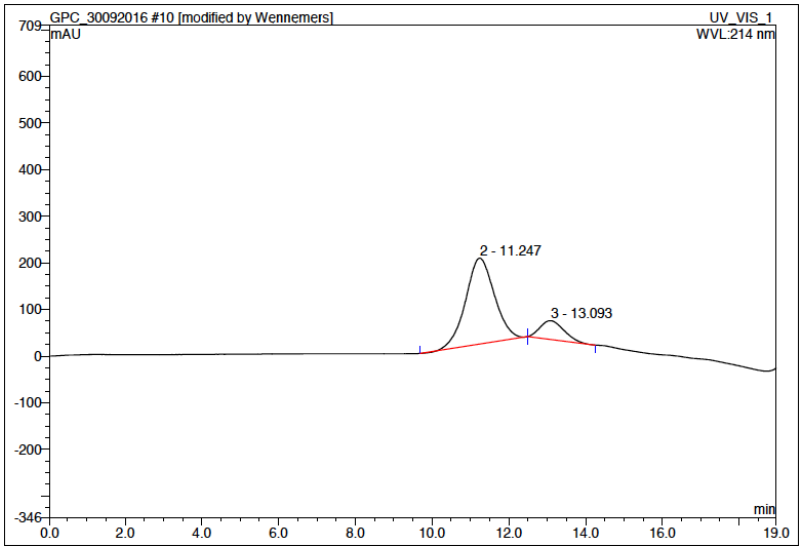
Gel permeation chromatography (GPC) was performed to confirm triple helix formation. 0.2 mM solutions of peptide in mQ water were analyzed by analytical GPC on a Superdex^{TM} Peptide 3.2/300 column. Temperature: room temperature.; flow rate: 0.1mL/min. t_R = 11.6 min. (triple helix), t_R = 13.5 min (single strand) for capped termini.

Gel permeation chromatogram of CMP 1

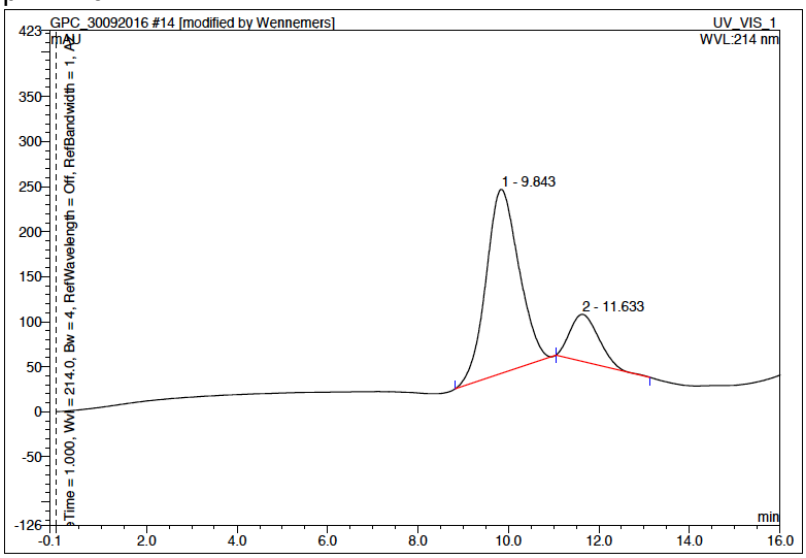


Gel permeation chromatogram of CMP 2

pH = 3

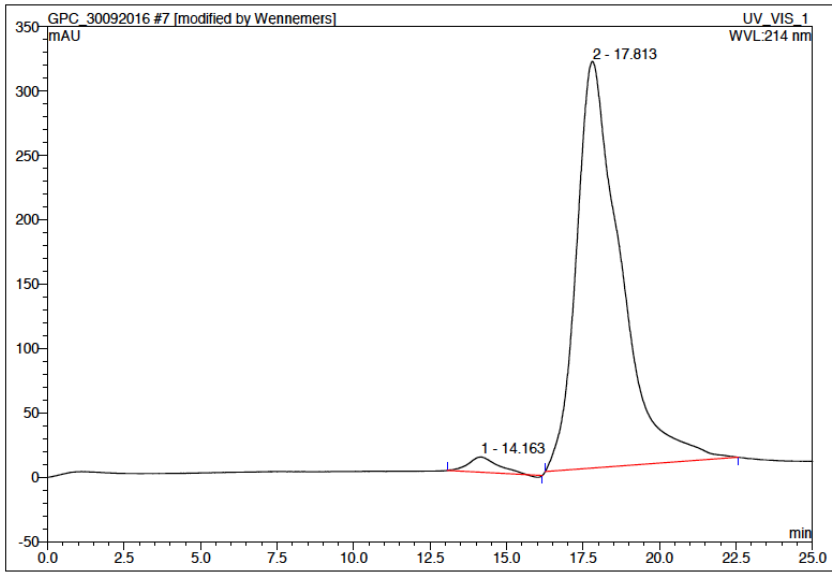


pH = 10.7

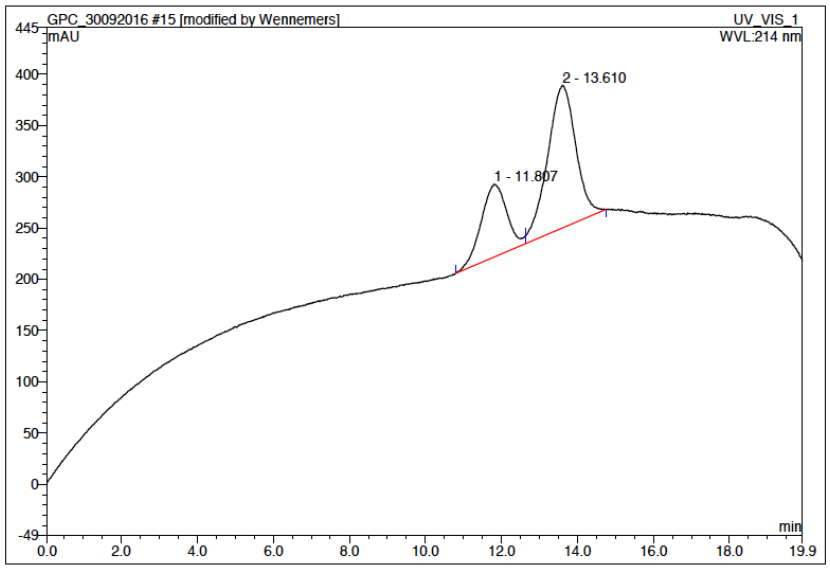


Gel permeation chromatogram of CMP 3

pH = 3

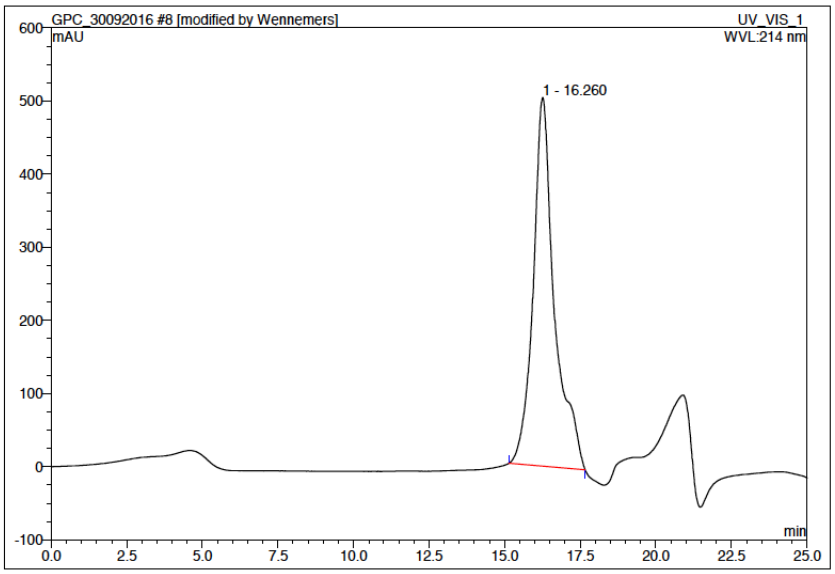


pH = 10.7

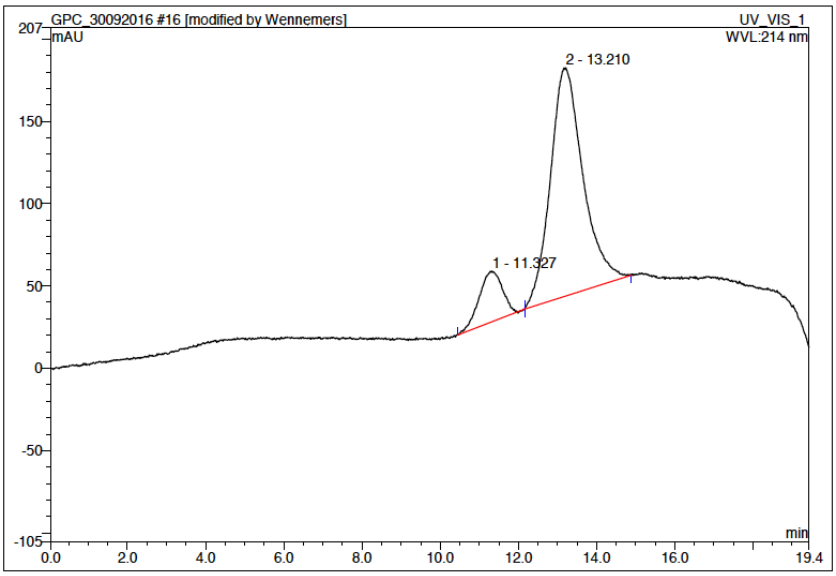


Gel permeation chromatogram of CMP 4

pH = 3



pH = 10.7



7) References

[1] R. S. Erdmann and H. Wennemers, *J. Am. Chem. Soc.* **2010**, *132*, 13957.

[2] J. Engel, H. T. Chen, D. J. Prokop, H. Klump, *Biopolymers* **1977**, *16*, 601-622.

[3] S. Frank, R. A. Kammerer, D. Mechling, T. Shulthess, R. Landwehr, J. Bann, Y. Guo, A. Lustig, H. P. Bachinger, J. Engel, *J. Mol. Biol.* **2001**, *308*, 1081-1089.