Synthesis and Folding of Native Collagen III Model Peptides

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ABSTRACT: Solid-phase synthesis of triple-helical peptides, including native collagen III sequences, was started with a trimeric branch, based upon the lysine dipeptide [Fields, C. G., Mickelson, D. J., Drake, S. L., McCarthy, J. B., and Fields, G. B. (1993) *J. Biol. Chem. 268,* 14153−14160]. Branch synthesis was modified, using TentaGel R as resin, *p*-hydroxybenzyl alcohol (HMP) as linker, Dde as N^{ϵ}-protective group, and HATU/HOAT as coupling reagent. Three homotrimeric sequences, each containing the Gly 606−Gly 618 portion of human type III collagen, were added to the amino functions of the branch. The final incorporation of GlyProHyp triplets, stabilizing the collagen III triple helix, was performed by using protected GlyProHyp tripeptides, each containing *tert*-butylated hydroxyproline [P*(tBu)] instead of hydroxyproline (P*). Among the protected tripeptides FmocP*(tBu)PG, FmocPP*(tBu)G, and FmocGPP*-(tBu), prepared manually on a chlorotrityl resin, incorporation of FmocPP*(tBu)Gly was best suited for synthesis of large and stable peptides, such as PP*G(8), containing 8 $(PP*G)_3$ trimers (115 residues, 10 610 Da). The structures of five peptides, differing from each other by the type and number of the triplets incorporated, were verified by MALDI-TOF-MS. Their conformations and thermodynamic data were studied by circular dichroism and differential scanning calorimetry. Except for P*PG(8), containing 8 $(P*PG)_3$ trimers with hydroxyproline in the X position and adopting a polyproline II structure, all peptides were triple-helical in 0.1 M acetic acid and their thermal stabilities ranged from $t_{1/2} = 39.4$ to $t_{1/2} = 62.5$ °C, depending on the identity and number of the triplets used. Similar values of the van't Hoff enthalpy, ΔH_{vH}, derived from melting curves, and the calorimetric enthalpy, ΔH_{cal}, obtained from heat capacity curves, indicate a cooperative ratio of $CR = \Delta H_{vH}/\Delta H_{cal} = 1$, establishing a two-state process for unfolding of THP(III) peptides. The independence of the transition temperatures $t_{1/2}$ on peptide concentration as well as equilibrium centrifugation data indicate monomolecular $dimer_f$ to $dimer_u$ ($F_2 \leftrightarrow U_2$) transitions and, in addition, bimolecular $dimer_f$ to $monomer_u$ transitions ($F_2 \leftrightarrow 2U$). The dominance of the concentration-independent monomolecular reaction over the concentration-dependent bimolecular reaction makes thermal unfolding of THP(III) peptides appear to be monomolecular. If one designates the molecularity described by the term pseudomonomolecular, unfolding of the dimeric peptides PP*G(6−8) follows a two-state, pseudomonomolecular reaction.

Cell−collagen interactions contribute decisively to the regulation of cellular activities, influencing normal physiological processes, such as maintenance of tissue integrity in blood vessel walls and collagen degradation by matrix metalloproteinases, and pathological conditions, such as metastasis in cancer and platelet aggregation in arteriosclerosis (*2*).

The triple-helical conformation of collagens has been shown to be important for binding of connective tissue cells to fiber-forming collagens: Fibroblast adhesion is promoted by triple-helical collagen I derived sequences (*3*), platelet reactivity bases on the native collagen III fragment α1(III)-CB4[1] (*4*), and human fibrosarcoma cells adhere to the triple-helical CB3 fragment of type IV collagen but not to the denatured form (*5*).

To study amino acid sequence motifs on native collagen, adhering to cell surface proteins, the preparation of collagen peptides in the native triple-helical conformation is of special interest. Native sequences can be prepared by renaturation of CNBr-derived peptides by stepwise cooling (*6*) or solid-phase synthesis of associated peptides. The sequence under investigation is flanked on each side by stretches of GlyPro-Hyp repeats, inducing a triple-helical conformation within the putative epitope region (*7*). A third approach, used in this work, is the synthesis of 3-fold-bridged peptides, modeled on the disulfide linked C-terminus of type III

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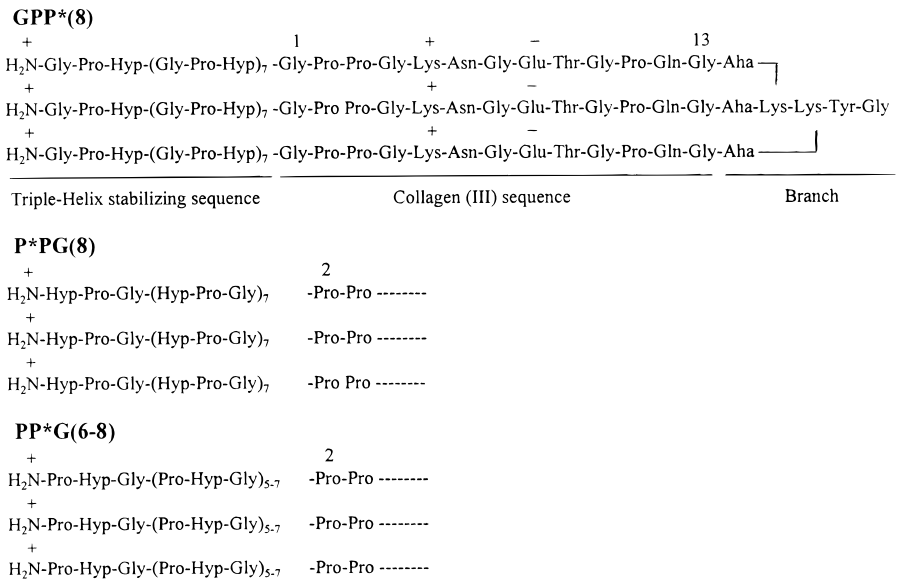

FIGURE 1: Schematic models of the peptides GPP*(8), P*PG(8), and PP*G(6−8) at neutral pH, indicating the positions of the charged residues. The three chains of the triple helix are represented in a parallel arrangement, facilitating direct comparison of the peptides. Amino acid positions within the collagen III sequence are denoted by numbers. Regions of P*PG(8) and PP*G(6−8), identical with the corresponding area of GPP*(8), are marked by broken lines (- - -). Blanks within P*PG(8) and PP*G(6−8) indicate the absence of glycine in position 1, ensuring a continuous triplet sequence.

collagen (*8*) and indicating an enhanced thermal stability compared with associated triple-helical peptides (*9*).

Here we describe the solid-phase synthesis of triple-helical collagen III epitopes, containing 10−20 amino acid residues per chain, using a modified methodology of Fields (*10*). The collagen III sequences are transferred into a homotrimeric strand by starting the synthesis on a trimeric branch, containing a lysine dipeptide with three functional amino groups, known to constrain the homotrimer into the correct raster. After completion of the trimeric collagen III sequence, $(GlyProHyp)_n$ triplets are added, inducing triple helicity and enhancing thermal stability within the collagen III homotrimer (Figure 1). Since the synthesis of this stabilizing segment was strongly impeded on the Synergy 432 A instrument, incorporation of GPP* triplets required a different synthetic approach. T-butylation of hydroxyproline and its impact on THP(III) synthesis was studied in detail. Conformational analysis of the peptides was performed by CD spectroscopy and extraction of their thermodynamic data by analysis of temperature-induced equilibrium melting curves.

Studies were also focused on the unfolding mechanism of 3-fold-branched peptides, using circular dichroism, microcalorimetry, and equilibrium centrifugation. There are different opinions about the molecularity of the melting transitions, important for calculation of van't Hoff enthalpies and entropies: For the peptide Col 1−3 of bovine type III procollagen, consisting of three chains cross-linked by a cystine knot, a first-order transition has been established (*11*), whereas, for the synthetic THP peptides, characterized by an analogous triple-branched structure, trimolecular melting transitions have been assumed (*10, 12, 13*). Moreover, there are no experimental data available, concerning the transition states of THP melting. Comparison of the calorimetric transition enthalpy, ΔH_{cal}, and the corresponding van't Hoff enthalpy, ΔH_{vH}, allows one to evaluate whether the transition conforms to a two-state model or involves intermediate states. In the present work we describe a modified THP(III) synthesis, compatible with the Synergy 432 A instrument, and report on the conformations, thermodynamic parameters and the folding/unfolding behavior of THP(III) peptides.

MATERIALS AND METHODS

Materials. 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT) in DMSO/NMP (51.6:48.4 vol %), *N*,*N*-diisopropylethylamine (DIEA), piperidine, tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), and trifluoromethanesulfonic acid (TFMSA) were from Applied Biosystems, Inc. (Foster City, Ca). 1-Hydroxy-7-azabenzotriazole (HOAT) and 0-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from PerSeptive Biosystems GmbH (Framingham, MA). Boc-Gly-PAM resin (substitution level: 0.65 mmol/g), FmocGlyProHyp, Fmoc-6-aminohexanoic acid (Fmoc-Ahx), Fmoc-Lys(Dde)-OH, and all other Fmoc-amino acids were products from Bachem (Heidelberg, Germany). Fmoc-Gly-TentaGel R PHB resin (substitution level: 0.18 mmol/g) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were from Rapp Polymere GmbH (Tübingen, Germany). Materials for tripeptide syntheses as H-Gly-2-Cl Trt resin (substitution level: 0.9 mmol/g), 2-chlorotrityl

[1] Abbreviations: CD, circular dichroism; $T_{1/2}$ and $t_{1/2}$, melting temperature in K and °C, respectively; RP-HPLC, reversed-phase high-performance liquid chromatography; SE, size exclusion; PAM, phenylacetamidomethyl; HMPA, 4-(hydroxymethyl)phenoxyacetic acid; Trt, trityl; Dcb, 2,6-dichlorobenzyl; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; Fmoc, fluorenylmethoxycarbonyl; Boc, *tert*-butyloxycarbonyl; tBu, *tert*-butyl. Hydroxyproline is denoted by P* (one-letter code) or Hyp (three-letter code). α1(III)CB4, the cyanogen bromide peptide of human type III collagen, comprises the sequence region from position 579 to 727 of the α1(III) chain (*1*). THP(III), collective name for triple-helical peptides containing a collagen III specific sequence. The single THP(III) peptide is designated according to its triple-helix stabilizing sequence: P*PG(8) denotes a peptide, the stabilizing sequence consists of $[P*PG]_3$ trimers, repeating eight times. PP*G(6−8) denotes peptides with either 6, 7, or 8 $[PP*G]_3$− repeats.

chloride resin (substitution level: 1.05 mmol/g), Fmoc-Hyp-(tBu)-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, and 1-hydroxybenzotriazole (HOBT) were purchased from Novabiochem (Bad Soden, Germany). Hydrazine hydrate, thioanisole, ethanedithiol (EDT), trifluoroethanol (TFE), 1,4-dioxane, and 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) were from Sigma (St. Louis, MO). Acetic acid (HOAc), trifluoroacetic acid (TFA), dichloromethane (DCM), dimethyl sulfoxide (DMSO), *N*-methylpyrrolidone (NMP), diethyl ether, petroleum ether, methyl *tert*-butyl ether, and *tert*-butanol were purchased from Fluka (Buchs, Switzerland), acetonitrile, methanol, and water from Baker (Deventer, Holland), and chloroform, benzene, and thin layer chromatography (TLC) plates (5 × 10 cm), precoated with silica gel 60 F_{254}, from Merck (Darmstadt, Germany).

Synthesis of Tripeptides. (i) Synthesis of *FmocHyp(tBu)-ProGly* was done manually in a shaker. Either 4.555 g of FmocPro (13.5 mmol) or 5.528 g of FmocHyp(tBu) (13.5 mmol) was coupled to 5 g of H-Gly-2-ClTrt-resin (4.5 mmol) with 4.335 g of TBTU (13.5 mmol), 1.824 g of HOBT (13.5 mmol), and 37 mL of 12.5% DIEA/DMF (27 mmol of DIEA) in 45 mL for 1 h. A concentration of 0.3 mol/L was applied for the Fmoc amino acid, TBTU, and HOBT. After coupling, the peptide resin was washed three times with both DMF and then DCM. Coupling was monitored by the qualitative Kaiser test. Fmoc deprotection was performed in piperidine/DMF (25:75) for 5 and 15 min. After deprotection, the peptide resin was washed three times with DMF, once with dioxane/H_2O (3:1), and three times with H_2O, DMF, and DCM. The FmocHyp(tBu)ProGly resin was washed with diethyl ether and dried by high vacuum. Peptide cleavage was achieved with DCM/TFE/acetic acid (7:2:1) for 1 h at room temperature, applying 100 mL of cleavage solution/5 g of resin. After P2 filtration, the cleavage solution was evaporated and the oily residue taken up in 10 mL of DCM. The tripeptide was precipitated by slowly adding the solution to 200 mL diethyl ether. Precipitation was completed by adding 200 mL of petroleum ether. After centrifugation (600*g*; 5 min) the precipitate and the evaporated supernatant were washed several times with petroleum ether, dissolved in *tert*-butanol/H_2O (4:1), and lyophilized. The homogeneity of the peptide, occurring in both the precipitate and the supernatant, was verified by thin-layer chromatography (TC) and analytical RP-HPLC. TC was performed in chloroform/methanol/benzene/H_2O (40:40:40:5) on a plate, coated with silica gel 60, containing the fluorescence indicator F_{254}. Detection of the peptide was by irradiation of the plate at 254 nm, yielding a dark spot on a fluorescent background. In the analytical RP-HPLC (see below) as elution gradients 1−90% B in 89 min and 45% B in 20 min were used.

(ii) FmocGlyProHyp(tBu). Attachment of FmocHyp(tBu)-OH to the 2-chlorotrityl chloride resin was effected by esterification, carried out in a shaker. A 5 g amount of chlorotrityl resin (5.25 mmol) and 2.15 g of FmocHyp(tBu)-OH (5.25 mmol) were mixed with dry DCM (20 mL), just enough to dissolve the Fmoc amino acid and agitate the resin. A total of 2 equiv of diisopropylethylamine (1.848 mL = 10.5 mmol) was added in drops for 5 min and the suspension shaken for 45 min. Then 4 mL of methanol (approximately 0.8 mL/g of resin) was added to covalently block those chlorotrityl groups on the resin, where formation of ester bonds had failed. After shaking of the sample for 10 min, the resin was washed three times with 50 mL of DCM and dried in vacuo over KOH. The substitution of the resin was measured by spectrophotometric determination of the Fmoc residue at 290 nm, following treatment of 1 g of resin with 20% piperidine in DMF. A solution of 0.1 mM FmocGly in methanol was used as standard. The level of first residue attachment was 0.56 mM/g of resin. Coupling of FmocPro and FmocGly to the FmocHyp(tBu) resin was performed with a 3-fold excess of Fmoc amino acids (see above). After deprotection, either 3.036 g of FmocPro (9 mmol) or 2.676 g of FmocGly (9 mmol) was coupled to 5 g of H-Hyp(tBu)-2-Cl-Trt resin (3 mmol) with 2.89 g of TBTU (9 mmol), 1.378 g of HOBT (9 mmol), and 24.05 mL of 12.5% DIEA/DMF (18 mmol DIEA) in 30 mL for 1 h.

(iii) FmocProHyp(tBu)Gly. Preparation of the peptide followed the procedure as described in (i). Either 4.177 g of FmocHyp(tBu) (10.2 mmol) or 3.442 g of FmocPro (10.2 mmol) was coupled to 5 g of H-Gly-2-Cl-Trt resin (3.4 mmol) with 3.276 g of TBTU (10.2 mmol), 1.562 g of HOBT (10.2 mmol), and 28.5 mL of 12.5% DIEA/DMF (20.4 mmol DIEA).

THP(III) Synthesis. Microscale synthesis (25 μmol) of THP(III) peptides was performed by Fmoc solid-phase methodology on the Continuous Flow Synthesizer Synergy 432 A (Appl. Bios.), using modified cycles:

(i) Preparation of the trimeric branch $(FmocAhx)_3$LysLys-Tyr(Dcb)Gly first was carried out using the BocGly-PAM resin (substitution level: 0.65 mmol/g). Manual removal of the N-terminal Boc protecting group was accomplished by placing the resin into a flask and treating it with 50% (v/v) TFA in DCM for 30 min at room temperature with constant shaking. After washing three times with DCM, the deprotected resin was neutralized with DIEA/DCM (1:9) for 30 min, washed several times with DCM, and then dried overnight under high vacuum over KOH. A 38 mg amount of the dried resin, corresponding to 25 μM equiv of H-GlyPAM, was transferred to the synthesis column. 39.5 mg of FmocTyr(Dcb) (75 μmol), 36.5 mg of FmocLys(Boc) (75 μmol), and 36.5 mg of FmocLys(Boc) (75 μmol) were incorporated on the H-GlyPAM resin, using the standard Fmoc chemistry of the synthesizer and coupling times of about 2 h. After coupling of the second FmocLys(Boc) and the following N-α-Fmoc removal, cleavage of the N-ϵ-Boc protecting groups from H_2N-Lys(Boc)Lys(Boc)Tyr(Dcb)-GlyPAM resin was performed off-column as described above. One-third of the dried resin, the origin for 25 μM peptide chains, was refilled into the synthesis column, and 26.5 mg of FmocAhx(75 μmol) was double-coupled for 2 h to the free N^{α}- and N^{ϵ}-amino groups. Completion of the coupling reactions was controlled by the Kaiser test (*14*).

(ii) Alternatively, branch synthesis was started with the FmocGlyTentaGel R PHB resin (substitution level: 0.18 mmol/g). 34.5 mg of FmocTyr(tBu) (75 μmol), 40 mg of FmocLys(Dde) (75 μmol), and 40 mg of FmocLys(Dde) (75 μmol) were sequentially coupled to 46.4 mg (25:3 = 8.34 μmol) of H-GlyPHB TentaGel R resin. After N-α-Fmoc removal, cleavage of the Dde protecting groups was performed off-column by two 5-min treatments of the peptidyl resin with 2% hydrazine hydrate in DMF. Coupling of 26.5 mg of FmocAhx (75 μmol) to the H_2N-Lys(NH_2)Lys(NH_2)-Tyr(tBu) TentaGel R PHB resin, containing 25 μM

(3 × 8.34 μmol) free amino groups, was performed as outlined above.

Incorporation of the type III collagen-specific sequence GlyProProGlyLysAsnGlyGluThrGlyProGlnGly onto the branched peptide resin was achieved by single couplings for residues 13−7 and double couplings for residues 6−1. Coupling times applied were 90 and 180 min, respectively.

Formation of the triple-helix stabilizing sequence by incorporation of either FmocGlyProHyp, FmocGlyProHyp-(tBu), FmocHyp(tBu)ProGly, or FmocProHyp(tBu)Gly was done with a 3-fold excess of Fmoc tripeptides. 42.3 mg of the Hyp(tBu) tripeptides (75 μmol) and 38.6 mg of the Hyp tripeptide (75 μmol) were coupled to 25 μM peptide chains. Coupling times of 3−4 h and double deprotections were used.

Cleavage and side-chain deprotection of THP(III) peptides, coupled to the H-GlyPAM resin, were performed by treatment with 1 mL of TFMSA/TFA (1:9) for 2.5 h in the presence of 100 μL of thioanisole and 50 μL of EDT. THP-(III) peptides, attached to the H-GlyTentaGel R PHB resin, were cleaved by a mixture of 1 mL of TFA/thioanisole/EDT (900:50:50) for 1 h. The crude THP(III) was precipitated with methyl *tert*-butyl ether, lyophilized, and dissolved in 0.1% TFA or sodium acetate buffer (0.02 mol; pH 4.8; 4 mol of urea) for purification by RP- and SE-HPLC, respectively.

The correctness of THP(III) synthesis was checked by preparation of the "branch" and a second intermediate peptide, containing the "branch + the collagen III specific sequence". After cleavage, precipitation and purification the intermediate peptides were analyzed by MALDI-TOF-MS.

Azabenzotriazole-based Coupling. The peptides PP*G(6−8) were synthesized using both HBTU/HOBT and in addition HATU/HOAT as coupling reagents. HOAT and its uronium salt analogue HATU were used as direct substitutes for HOBT and HBTU, respectively. Peptide synthesis with the Synergy 432 A instrument was performed using a solution of HATU instead of HBTU. A 3.042 g amount of HATU (8 mmol) was dissolved in 40 mL of 0.2 M HOAT, prepared by dissolving 1.089 g of HOAT in 20.1 mL of DMSO and 18.8 mL of NMP.

Purification of THP(III) Peptides. Analytical RP-HPLC was performed on a Beckman System Gold liquid chromatograph. The peptides were applied to a high-pore C-18 column (Biorad RP-318, 5 μm particle size, 300 Å pore size, 25 cm × 4.6 mm), equilibrated in solution A (0.1% TFA). Elution was carried out at room temperature with different gradients of solution B (0.1% TFA in acetonitrile) at a flow rate of 1 mL/min. Diode array detection was at 220 nm. Semipreparative RP-HPLC was also done on the Beckman System using a Vydac 218 TP column (ODS C-18, 250 × 10 mm, 10 μm particle size, 300 Å pore size). Isocratic gradients at a flow rate of 3 mL/min were applied for purification of the branch (11% B) and the peptide composed of the branch and the specific residues (15% B). A and B were the same as for analytical HPLC. Detection was at 220 nm. SE-HPLC was performed on a TSK G 2000 SW column (LKB, 7.5 × 600 mm), eluted with sodium acetate buffer (0.02 mol; pH 4.8; 4 mol of urea) at a flow rate of 50 μL/min. Monitoring was at 220 nm.

Mass Spectrometry. High-resolution mass spectrometry (*15*) of THP(III) peptides was performed on a Lamma 1000 instrument using matrix-assisted ultraviolet laser desorption/ionization and time-of-flight mass analysis (MALDI-TOF-MS). The method allows a fast and very sensitive identification of the peptides. Good sample homogeneity has been obtained by applying the following preparation procedure: A 0.5 μL volume of peptide solution (10^{-5} mol of peptide/l 0.1 mol acetic acid) was mixed with 2 μL of the matrix solution [9 volumes of 2,5-dihydroxybenzoic acid (20 g/L matrix solvent) and 1 volume of 2-hydroxy-5-methoxybenzoic acid (20 g/L matrix solvent)] on a sample holder, consisting of inert metal. Matrix solvent was a mixture of 0.1% TFA (2 parts) and acetonitrile (1 part). Solvents were removed using a constant flow of cold air. The dried probe was irradiated with a N_2 laser at 337 nm, using an irradiance of about 10^7 W cm^{-2}, pulses of 4 ns in duration, and beams of 10−30 μm in diameter. Between 10 and 30 spectra were run with the same sample, summed, and avaraged.

Circular Dichroism Measurements. CD measurements were performed with a CD6 Dichrograph (Jobin-Yvon), using CD cuvettes (Hellma) with optical path lengths ranging from 0.01 to 1 cm. CD spectra reported are the avarage of four scans in the wavelength range of 190 to 260 nm with an increment of 0.5 nm and an integration time of 1s. For measurements below 210 nm the instrument and the sample compartment were purged thoroughly with purified nitrogen (10 L/min). All spectra were buffer-corrected and smoothed. Temperature readings were done by a Teflon-coated 100-Ω platinium resistance probe, immersed in the sample solutions above the light path. Peptide solutions were prepared at concentrations of 10, 1, 0.1 and 0.01 mg/mL and equilibrated at 4 °C for 24 h prior to analysis. Melting curves of the peptides were recorded by monitoring the mean residue ellipticity, Θ_{MRE}, at 225 nm, while the sample temperature was increased from 5 to 80 °C at a rate of 1 °C/min. Integration time was 4 s.

Sample Preparation. To measure CD spectra as a function of solvent pH, 30 μg of the peptide was dissolved in 300 μL of solvent, adjusted to pH values of 1.9, 2.35, 3.2, 4.35, and 5.0 by adding 15 vol % of 0.1 N sodium citrate/HCl buffer (pH 1.1, 1.5, 2.0, 2.5, and 3.0) to 85 vol % of methanol. Solvent pH values above 6 are unsuitable as THP-(III) precipitates from the solution. To determine the influence of solvent composition on CD spectra, different portions of methanol in 0.1 M acetic acid were prepared by increasing the content of methanol in steps of 10 vol % A 30 μg amount of THP(III) was dissolved in 300 μL of each solvent mixture.

Calculation of Thermodynamic Parameters. The extraction of thermodynamic data was done according to van't Hoff analysis (*16*). A two-state first-order model was assumed,

$$F \leftrightarrow U$$

where F is the fraction folded and U is the fraction unfolded. The equilibrium constant K of the unfolding reaction is

$$K = \frac{U}{1 - U}$$

The fraction unfolded (U) was calculated using the equation

$$U = \frac{\Theta_{Obs} - \Theta_F}{\Theta_U - \Theta_F}$$

where Θ_{Obs} is the observed mean residue ellipticity, Θ_F is the ellipticity of the peptide in the folded and Θ_U in the unfolded state. Θ_U ellipticities of the transition region were determined by extrapolating the posttransition baseline to lower temperatures and Θ_F values by linear extrapolation of the premelting baseline. *U* values, obtained at different temperatures, allow calculation of *K* as a function of temperature and thus determination of the van't Hoff enthalpy, ΔH_{vH}, according to the equation

$$\ln k = \frac{\Delta H_{vH}}{R}\frac{1}{T}$$

Melting temperatures, $T_{1/2}$, of THP(III) peptides were obtained from the van't Hoff plot by determination of the temperature at $\ln k = 0$, where 50% of the peptides are folded ($F = 0.5$).

The entropy $\Delta S(T_{1/2})$ was calculated by the equation

$$\Delta S(T_{1/2}) = \frac{\Delta H_{vH}(T_{1/2})}{T_{1/2}}$$

Differential Scanning Calorimetry. DSC scans were performed with an electronically modified Privalov-type DASM 4 microcalorimeter (*17*), having a cell volume of 0.474 mL. The sample run was preceded by a baseline run with solvent-filled cells. Electrical calibration of the instrument was performed using 50-μW power signals. Concentration of the peptide PP*G(8) was 0.94 mg/mL. The C_p values were calculated using a value of 0.735 mL/g for the partial specific volume, v, of PP*G(8) (*18*). Heat capacity and temperature data were routinely taken every 0.1 K using a Keithley DMM 192 voltmeter and stored on a personal computer. Integration of the transition curve, representing excess heat capacity as a function of temperature, was done numerically to obtain the calorimetric enthalpy, ΔH_{cal}. ΔH_{vH} values were calculated from the heat capacity curve using the appropriate factor 4 for a two-state monomolecular reaction of the type $F \leftrightarrow U$ (*16*):

$$\Delta H_{vH} = 4R(T_{1/2})^2\frac{C_{p,max}}{\Delta H_{cal}}$$

where $T_{1/2}$ represents the temperature at half-completion (50% area) of the C_p transition curve, $C_{p,max}$ the molar excess heat capacity at $T_{1/2}$, and ΔH_{cal} the molar enthalpy resulting from integration of the area under the transition peak. *R* is the gas constant [$R = 8.3144$J/(mol·K^{-1})].

Equilibrium Centrifugation. Apparent molar masses were determined from sedimentation−diffusion equilibrium experiments in a Beckman XL-A analytical ultracentrifuge equipped with absorption optics. Concentration gradients were measured at 280 and 230 nm depending on the concentration of peptides in the cell. The gradients were evaluated by nonlinear least-squares fitting using the program package AKKUPROG (Björn Kindler (1997), Thesis, University Hannover, Germany). In all cases the equilibrium concentration gradient was described by

$$c(x) = c(m)\mathrm{e}\left(\frac{M_{app}(1-\nu\rho)}{2RT}\right)\omega^2((x^2 - x(m)^2)$$

where $c(x)$ and $c(m)$ designate the concentrations at position x and the meniscus ($x(m)$), respectively, ν is the partial specific volume of the solute (assumed to be 0.735×10^{-3} m^3/kg), ρ is the density of the solvent, and M_{app} is the apparent molar mass for the species.

Prior to sedimentation, lyophilized peptides were dissolved in 0.1 M acetic acid. Seven different concentrations per peptide were used, ranging from 2 to 0.1 mg/mL. Runs were carried out on all peptides at 4, 25, and 35 °C. The weight-average molar masses of the peptides at different temperatures were obtained by extrapolating the apparent molar masses to the concentration $c = 0$, using linear least-squares fitting.

RESULTS

Synthesis of the Intermediate Peptides. Synthesis of the trimeric branch $(Ahx)_3$LysLysTyrGly was started first with the BocGly-PAM resin (see Materials and Methods) by using a branching chemistry, which has been reported to provide high yields of THP peptides (*10*). After cleavage from the PAM resin with TFA/TFMSA (9:1) and purification by RP-HPLC the mass of the branch was confirmed by MALDI-TOF-MS (not shown), indicating signals at 834.7, 856.9, and 872.9 Da, representing the additions of H^+, Na^+, and K^+ to the branch, having the apparent mass of 833.9 Da (calculated 834.1). The structure of another intermediate peptide, comprising the branch and the three homotrimeric sequences from α1(III)CB4, was verified by the mass of 4370 Da (calculated 4365.9) (not shown). The final coupling of 8 $(GlyProHyp)_3$ trimers by incorporation of GlyProHyp tripeptides was very incomplete, as shown by MALDI-TOF-MS and amino acid sequence analysis, although coupling times of 3−4 h were used.

THP(III) synthesis was altered by applying a different synthetic scheme. A modified branching system was used coupled to a TentaGel R resin. N^{α}-amino protection was achieved by Fmoc, Lys N^{ϵ}-amino side-chain protection by Dde, and Tyr side-chain protection by tBu, and HMP was used as linker attached to the TentaGel R resin. Tripeptides, containing *tert*-butylated hydroxyproline instead of hydroxyproline, were used to form triple-helix inducing sequences.

Synthesis of the Protected GlyProHyp Tripeptides. The impact of *tert*-butylated hydroxyproline on THP(III) synthesis was studied in detail. Three different tripeptides FmocHyp(tBu)ProGly, FmocProHyp(tBu)Gly, and FmocGlyProHyp(tBu), each containing a Hyp(tBu) residue, were prepared manually on a chlorotrityl resin, substituted with either FmocGly (5 g of resin; substitution level = 0.76 mmol/g or 0.68 mmol/g) or FmocHyp(tBu) (6.136 g of resin; substitution level = 0.555 mmol/g). Yields of the tripeptides were 2.062, 1.87, and 1.85 g, corresponding to 96%, 97%, and 96% of theoretical yield, respectively. Homogeneity of the peptides was confirmed by analytical RP-HPLC, using an isocratic gradient of 41% B, and thin-layer chromatography, yielding a single spot with a R_F value of 0.3. Peptide masses were established by MALDI-TOF-MS (not shown), indicating signals at 586.5 and 602.4 Da, representing the additions of Na^+ and K^+ to the tripeptides, having the mass of 563.5 Da (calculated 563.6).

*Synthesis and Properties of the THP(III) Peptides. (a) P*PG(8).* Incorporation of FmocHyp(tBu)ProGly proceeded

Table 1: Yields and Thermodynamic Data for THP(III) Peptides for the Coil ↔ Triple-Helix Transition in Different Solvent Systems

	yield[a]								
peptide	%	mg	solvent	$t_{1/2}$ (°C)	$t_{1/2}$/triplet	ΔH_{vH} (kJ/mol)	ΔH_{vH}/triplet	ΔS_{vH} (J/mol·K)	ΔS_{vH}/triplet
P*PG(8)	6.1	5.4	0.1 M HA						
HBTU			85% Met	58.5	1.77	−116	−3.52	−350	−10.61
GPP*(8)	2.9	2.6	0.1 M HA	62.5	1.74	−122	−3.39	−363	−10.08
HBTU									
PP*G(8)									
HATU	4.1	3.6	0.1 M HA	50.7	1.54	−123	−3.73	−380	−11.52
HBTU	2.8	2.5							
PP*G(7)									
HATU	4.9	4.0	0.1 M HA	44.4	1.48	−102	−3.4	−321	−10.7
HBTU	4.8	3.9							
PP*G(6)									
HATU	4.3	3.2	0.1M HA	39.4	1.46	−158	−5.85	−505	−18.7
HBTU	2.7	2.0							

[a] Yield was determined by relating the peptide amount, purified by SE-HPLC, to the theoretical amount, calculated from resin substitution.

smoothly, and the arising peptide, containing 8 $(P^*PG)_3$ trimers and designated as P*PG(8), was purified by RP-HPLC using a combination of two different gradients (not shown). The yield of P*PG(8) was 5.4 mg, corresponding to 6.1% of the theory (Table 1). The apparent molar mass was 10 601 Da (calculated 10 609.6 Da) by MALDI-TOF-MS (Figure 2a). The CD spectrum of the peptide in 0.1 M acetic acid at a concentration of 1 mg/mL is characterized by a large negative peak around 200 nm and a positive one around 225 nm. The ratio of positive peak intensity over negative peak intensity, denoted as the Rpn parameter (*19*), was used as criterion to judge whether the peptides synthesized are present as polyPro-II-like or collagen-like triple-helical structures (*20, 21*). The CD spectrum of P*PG(8) at 20 °C with Rpn = 0.05 (Table 2) clearly excludes the occurrence of a triple helix in 0.1 M acetic acid and points to the presence of a polyPro-II-helix (Figure 3d). This is supported by the thermal denaturation curve, characterized by a linear decrease of the mean residue ellipticity $[\Theta]_{MRE}$ with increasing temperatures, demonstrating the lack of a cooperative melting transition (Figure 4b, solid line).

Low-molecular weight alcohols, known to stabilize triple-helical structures, were used to amplify and detect possible weak triple-helical propensities of the peptide. Increasing the proportion of methanol in 0.1 M acetic acid increased the Rpn values of the peptide (Figure 3a, Table 3). A solvent, containing 85 vol % methanol, was best suited for the association of the polyPro-II-like peptide chains to triple-helical structures, as indicated by a Rpn value of 0.1 at 10 °C. Evidence of triple helicity is confirmed by the melting curve, marked by a cooperative transition and a melting temperature of $t_{1/2}$ = 58.5 °C (Table 1). The van't Hoff reaction enthalpy, calculated from the van't Hoff plot, was $\Delta H°$ = 116 kJ/mol (Table 1). The stabilizing effect of the methanolic solvent on the triple-helical structure was confirmed also for the other peptides synthesized (Figure 3c, Table 2).

The CD spectrum of P*PG(8) in 85 vol % methanol/15 vol % 0.1 N sodium citrate/HCl is modulated by the pH of peptide solution (Figure 3b). At pH 1.9 a decreased Rpn value is observed, which has a maximum at pH 2.35 and 3.2 and decreases at pH values of 4.35 and 5.04 (Table 3). As the pH becomes more neutral, the peptide precipitates from the solution.

The instability of P*PG(8) in aqueous solvent systems is obviously caused by the triple-helix inducing sequence, containing GlyHypPro repeats with hydroxyproline in the X position, destabilizing collagen triple helices (*22*). On the other hand, the instability of the peptide structure does favor peptide synthesis and considerably simplifies peptide purification, carried out exclusively by RP-HPLC.

(b) GPP(8).* Incorporation of FmocGlyProHyp(tBu) into the triple-helix inducing sequence is possible yet strongly impeded, as shown by monitoring traces. The complete peptide, containing 8 $(GPP^*)_3$ trimers and denoted as GPP*(8), could only be enriched but not purified by size-exclusion HPLC, using TSK SW columns. The yield of peptide GPP*(8) is clearly reduced compared to that of peptide P*PG(8) (Table 1). The signal at 11 442 Da corresponds to the mass of GPP*(8), containing at the N-terminus 3 N-α-Fmoc groups, indicating impeded deprotections in the final stage of synthesis (Figure 2b). The CD spectrum in 0.1 M acetic acid at 20 °C with Rpn = 0.10 as well as the temperature-induced transition curve with $t_{1/2}$ = 62.5 °C demonstrate the stability of GPP*(8) in 0.1 M acetic acid under physiological conditions (Figures 3d and 4b). Incorporation of FmocGlyProHyp(tBu) tripeptides obviously provides THP(III) peptides of high stability, caused by GlyProHyp repeats with hydroxyproline in the Y position. However, the stable peptide structures strongly impede coupling and deprotection. Coupling efficiency in addition is reduced by the sterically hindered Hyp(tBu) residue within FmocGlyProHyp(tBu).

*(c) PP*G(6−8).* Both stable and pure THP(III) peptides were obtained by incorporation of FmocProHyp(tBu)Gly. Coupling of the tripeptide, in contrast to incorporation of FmocGlyProHyp(tBu), proceeded more smoothly, due to the small glycine at the C-terminus, sterically less hindered. A peptide, containing 8 $(PP^*G)_3$ trimers and denoted as PP*G(8), was synthesized and purified by SE-HPLC under denaturing conditions. Its mass was 10 634 Da (calculated 10 610 Da) (Figure 2c). CD spectra in 0.1 M acetic acid at 20 °C (Rpn = 0.104) and a cooperative transition curve with a melting point of $t_{1/2}$ = 51 °C indicate the stability of the peptide on physiological conditions (Figure 4b). The van't Hoff enthalpy of PP*G(8) was 123 kJ/mol, corresponding to the value of peptide GPP*(8) (Table 1).

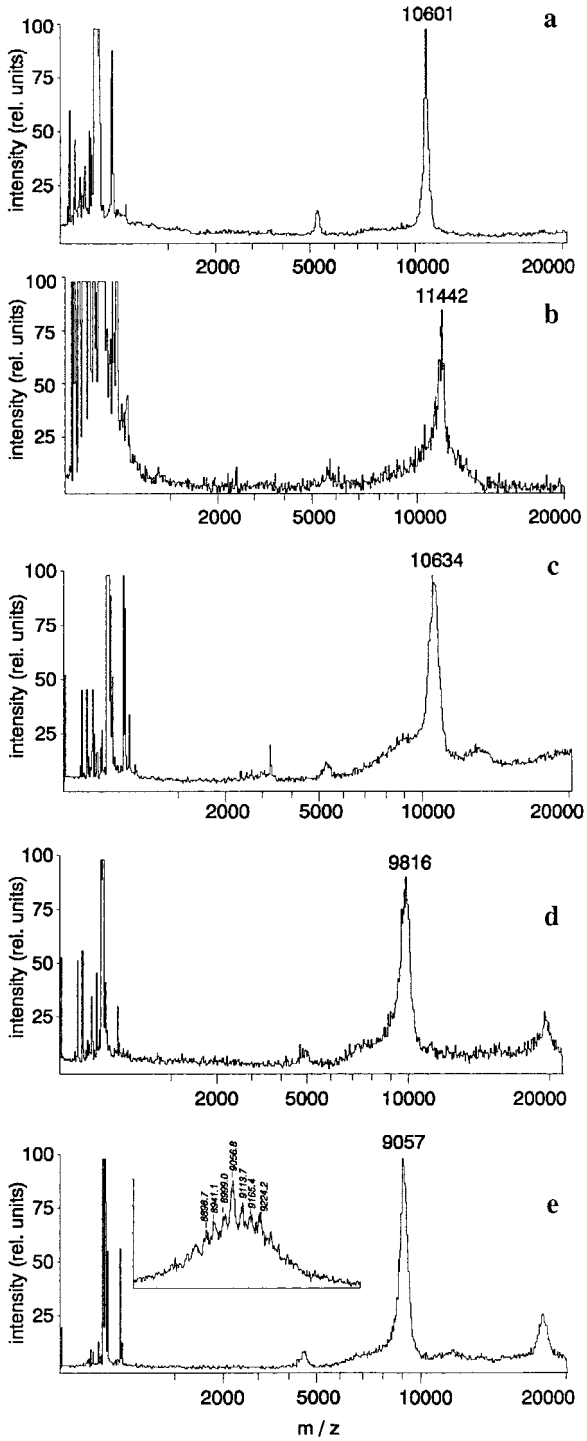

FIGURE 2: MALDI-TOF mass spectra of THP(III) peptides: (a) P*PG(8), (b) GPP*(8); (c) PP*G(8); (d) PP*G(7); (e) PP*G(6). For peptide concentration and matrix composition, see Materials and Methods.

The incorporation of *tert*-butylated hydroxyproline as FmocProHyp(tBu)Gly proved to be best suited for THP(III) synthesis, taking advantage of the two procedures mentioned above and avoiding their difficulties. The yield of PP*G(8) was 2.5 mg of pure peptide and could be increased to 3.6 mg (4.1% of the theory), applying the new coupling reagent HOAT/HATU instead of HOBT/HBTU (Table 1).

According to the altered synthesis protocol, two further THP(III) peptides, homologous to PP*G(8), were synthesized and designated as PP*G(6) and PP*G(7), containing 6 or 7 $(PP*G)_3$ trimers. Purification of the peptides was performed on a TSK SW 2000 column (not shown).Yields were 3.2 and 4 mg (4.3% and 4.9% of the theory) (Table 1). Their masses were verified by MALDI-TOF-MS, showing signals at 9057 Da (calculated 9006 Da) and 9816 Da (calculated 9808 Da) (Figure 2e,d). CD spectra with Rpn values of about 0.04 and 0.1 indicate a decrease in triple helicity for peptide PP*G(6) in 0.1 M acetic acid at 20 °C compared to peptide PP*G(7) (Table 2). The thermal transition curves are indicative of monophasic melts with $t_{1/2} = 39.4$ °C for PP*G(6) and $t_{1/2} = 44.4$ °C for PP*G(7) (Table 1). Considering also $t_{1/2} = 50.7$ °C for PP*G(8), the melting temperatures demonstrate the effect of chain length of $(GPP*)_n$ on the triple-helical stability of THP(III). The van't Hoff enthalpy of PP*G(6), $\Delta H_{vH} = 158$ kJ/mol, is increased compared to the values of the other THP(III) peptides (Table 1).

The results described indicate that the preparation of native collagen(III) epitopes is feasable on a Synergy 432 A instrument (25 μmol scale), using TentaGel R resins, a modified branching chemistry, the tripeptide FmocProHyp-(tBu)Gly, and HOAT/HATU as coupling reagent. The synthetic protocol developed allows one to study collagen III binding regions interacting with specific receptors of the cell surface.

Unfolding Mechanism of THP(III) Peptides. To gain insight into the transition states of the melting process, the enthalpy change, obtained from the van't Hoff analysis (ΔH_{vH}), was compared for the first time with the one measured directly using calorimetry (ΔH_{cal}). For PP*G(8) a high sensitivity measurement of heat capacity C_p as a function of temperature was performed, using a DASM4 scanning microcalorimeter. The first scan indicates a heat absorption peak in the temperature range 27−65 °C (Figure 5a), corresponding to the transition region of the melting curve. A second scan, taken on the same conditions, shows a high degree of identity to the first one, proving the reversibility of the thermal unfolding reaction. Therefore, the area under the transition peak is a direct measure of the transition enthalpy. A calorimetric enthalpy of $\Delta H_{cal} = 140.1$ kJ/mol, a van't Hoff enthalpy of $\Delta H_{vH} = 139.4$ kJ/mol, a melting temperature of $t_{1/2} = 49.9$ °C, and an entropy change of $\Delta S = 422.5$ J/(mol·K) were obtained, being similar to the corresponding values derived from the melting curve which are marked by an error of ±15% (*23*).

The molecularity of the melting process of 3-fold-bridged peptides was analyzed by unfolding studies where the influence of peptide concentration on the transition temperature $T_{1/2}$ was investigated. Molecularity is an important parameter for calculation of thermodynamic data from equilibrium melting curves (*16*). Experiments were performed with PP*G(7), applying concentrations of 0.01, 0.1, 1.0, and 10.0 mg/mL. With monitoring of the thermal unfolding reaction by the ellipticity change at 225 nm, characteristic of the loss in triple-helical structure, four nearly identical melting curves were obtained (Figure 5b). The plot of the melting temperatures versus the logarithm of the corresponding peptide concentrations demonstrates that the transition temperatures are independent of peptide concentration (Figure 5b, inset).

Aggregation Analysis of THP(III) Peptides. To elucidate aggregation of 3-fold-bridged peptides and to obtain supporting information on the molecularity of their melting

Table 2: Circular Dichroism Data for the Peptides P*PG(8), GPP*(8), PP*G(8), PP*G(7), and PP*G(6) in 0.1 M Acetic Acid and 85 vol % Methanol/15 vol % 0.1 M Acetic Acid[a]

	0.1 M acetic acid			85 vol % methanol		
peptides	min (nm)	max (nm)	Rpn[b]	min (nm)	max (nm)	Rpn
P*PG(8)	-1.6×10^4 (200)	0.8×10^3 (225)	0.05	-2.1×10^4 (198)	2.0×10^3 (226)	0.10
GPP*(8)	-1.6×10^4 (198)	1.6×10^3 (225)	0.10	-2.2×10^4 (199)	2.7×10^3 (224)	0.12
PP*G(8)	-1.7×10^4 (197)	1.7×10^3 (225)	0.10	-1.7×10^4 (199)	2.2×10^3 (225)	0.13
PP*G(7)	-1.9×10^4 (198)	1.7×10^3 (225)	0.09	-1.9×10^4 (199)	1.8×10^3 (225)	0.10
PP*G(6)	-2.5×10^4 (199)	0.7×10^3 (226)	0.04	-1.5×10^4 (199)	1.8×10^3 (225)	0.12

[a] CD spectra were obtained at 20 °C, using a peptide concentration of 1 mg/mL. [b] Rpn represents the ratio of positive peak intensity over negative peak intensity (absolute values).

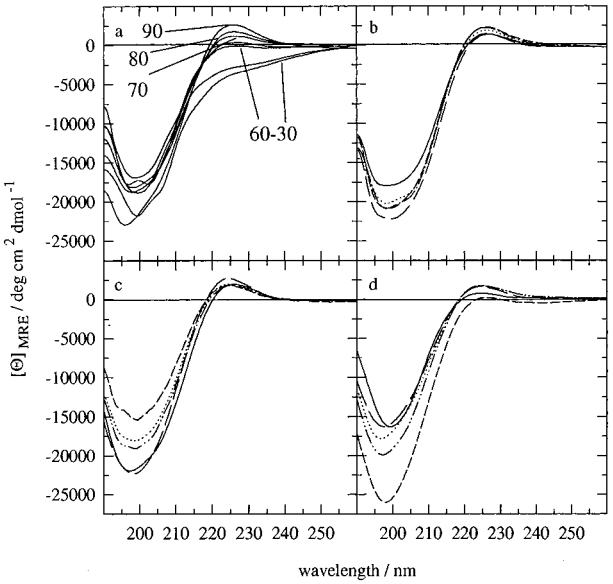

FIGURE 3: Circular dichroism spectra of THP(III) peptides. Spectra of P*PG(8) are shown as a function of (a) the methanol content of the solvent (vol % methanol in 0.1 M acetic acid) and (b) the pH of the solvent (85 vol % methanol/15 vol % 0.1 N sodium citrate/HCl). pH values of 1.90 (···), 2.35 (-·-), 3.20 (---), 4.35 (− −) and 5.04 (—) were used. Spectra are shown of the peptides P*PG(8) (—), GPP*(8) (− −), PP*G(8) (···), PP*G(7) (- ·· -), and PP*G(6) (---) in (c) 85 vol % methanol/15 vol % 0.1 M acetic acid and (d) 0.1 M acetic acid.

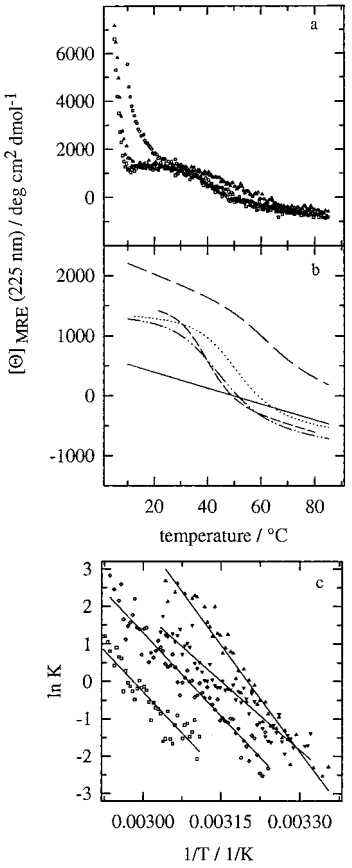

FIGURE 4: Thermal equilibrium transitions of THP(III) peptides in 0.1 M acetic acid. (a) Plot of experimental mean residue ellipticities (Θ_{MRE}) vs temperature for the peptides PP*G(6) (○), PP*G(7) (□), and PP*G(8) (△). (b) Plot of calculated Θ_{MRE} values vs temperature for the peptides P*PG(8) (—), GPP*(8) (− −), PP*G(8) (···), PP*G(7) (- ·· -), and PP*G(6) (---). For calculation of Θ_{MRE} values see under Materials and Methods. (c) van't Hoff plots of the peptides GPP*(8) (□), PP*G(8) (◇), PP*G(7) (▽), and PP*G(6) (△), derived from the thermal transition curves in (b).

transitions, the peptides PP*G(6−8) were studied by equilibrium centrifugation in 0.1 M acetic acid at different temperatures (Figure 6, Table 4). At 4 °C, the peptides in 0.1 M acetic acid are completely associated into the dimer form. At 25 and 35 °C, the dimeric nature of PP*G(7) and PP*G(8) is preserved. There is no aggregation of 3-fold-bridged peptides, increasing temperature from 4 to 35 °C. An average molar mass of 14 000 Da for PP*G(6) at 35 °C indicates, in agreement with the melting curve of the peptide (Figure 4), unfolding of PP*G(6), leading to monomerization of peptide aggregates.

DISCUSSION

Several methods for synthesizing covalent branched collagen model peptides have been proposed, based upon the Lys-Lys dipeptide (*10*). One of these methods we have used in a slightly modified form, and the principle is briefly described. For preparation of the branch, the N^{α}-amino function was protected by the Fmoc group, the N^{ϵ}-amino function was protected by the Boc group, and the C^{α}-carboxyl group of glycine was attached to the polystyrene resin by the PAM linker. The synthesis of the branch, the incorporation of the three homotrimeric collagen III sequences, and the addition of the $(GlyProHyp)_n$ triplets were adapted to the continuous flow synthesizer Synergy 432 A (Appl. Bios.), working on a microscale level (25 μmol). The main problem of THP(III) synthesis is the incorporation of the (GlyProHyp) triplets, leading to growing stabilities of the triple-helical structures that is desired but at the same time impedes the progress of synthesis to an increased extent.

To facilitate THP(III) synthesis on the Synergy 432 A instrument, the initial protocol was modified in some respects: TentaGel R was used as resin, HMP as linker, and HOAT/HATU as coupling reagent, and N^{ϵ}-protection was achieved by the Dde group. Tripeptides, containing *tert*-

Table 3: Circular Dichroism Data for the Peptide P*PG(8) as a Function of Methanol Content (vol % Methanol in 0.1 M Acetic Acid) and pH of the Solvent (85 vol % Methanol/15 vol % 0.1 N Sodium Citrate/HCl)

vol % methanol				pH			
vol %	min (nm)	max (nm)	Rpn	pH	min (nm)	max (nm)	Rpn
50	-1.9×10^4 (199)	-0.1×10^3 (227)	0.01	1.9	-2.0×10^4 (198)	2.1×10^3 (225)	0.10
60	-1.7×10^4 (198)	0.3×10^3 (227)	0.02	2.35	-2.1×10^4 (199)	2.5×10^3 (225)	0.12
70	-1.8×10^4 (199)	1.1×10^3 (227)	0.06	3.2	-2.1×10^4 (199)	2.5×10^3 (225)	0.12
80	-1.8×10^4 (198)	1.7×10^3 (225)	0.09	4.35	-2.2×10^4 (200)	1.6×10^3 (225)	0.07
90	-2.1×10^4 (196)	2.6×10^3 (226)	0.12	5.04	-1.8×10^4 (199)	1.7×10^3 (225)	0.09

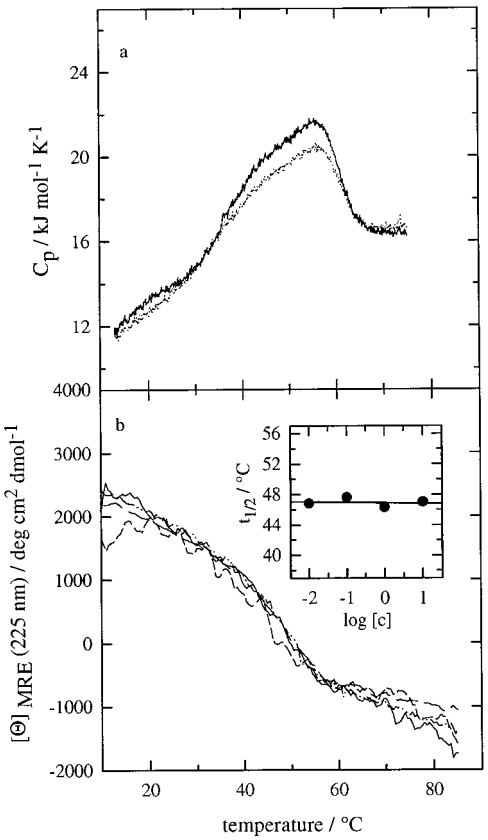

FIGURE 5: Thermal unfolding of 3-fold-bridged peptides is a two-state monomolecular process. (a) DSC scans of PP*G(8) in 0.1 M acetic acid, pH 2.4. The peptide concentration was 0.94 mg/mL and the heating rate 1 °C/min over a temperature range of 10−100 °C. The solid curve refers to the first scan, and the dotted one to the second scan of the same sample. (b) Thermal equilibrium transitions of PP*G(7) in dependence on peptide concentration. The mean residue ellipticity Θ_{MRE} of PP*G(7) at 225 nm was followed as a function of temperature. The peptide was dissolved in glycine buffer (10 mM; pH 2.4), and concentrations used were 0.01 (—), 0.1 (— —), 1.0 (---), and 10 mg/mL (- ·· -). The inset represents the dependence of the unfolding temperature $t_{1/2}$ on the logarithm of peptide concentration c. The solid line is the result of a linear least-squares fit.

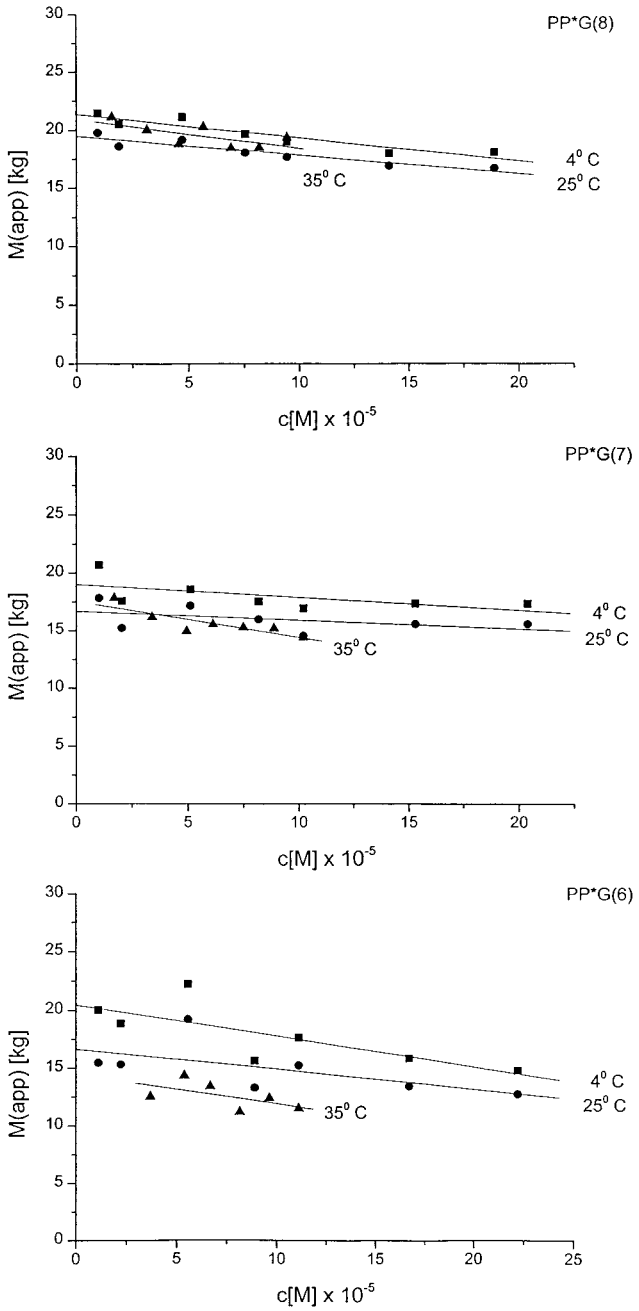

FIGURE 6: Plot of the apparent molar masses (M_{app}) of the peptides PP*G(6−8) vs the molar concentration of the peptides. Values of M_{app} were determined in 0.1 M acetic acid at 4, 25, and 35 °C. The molar masses of the peptides as a function of temperature were obtained by extrapolating M_{app} to the concentration $c = 0$.

butylated hydroxyproline, were applied throughout to form triple-helix inducing sequences.

TentaGel R resin favors the assembly of peptides that are prone to form secondary structures and aggregations, due to the low cross-linked polystyrene backbone, the low substitution of the resin, and the spacer effect of the poly(ethyleneglycol) chains grafted onto the polystyrene.The structures of the poly(ethyleneglycol) chains of TentaGelR and related poly(ethyleneglycol)−polystyrene resins (*10*) are different, caused by different methods of preparation (Rapp, personal communication). Incorporation of monofunctional poly(ethyleneglycol) units (TentaGel R) instead of bifunctional units (*10*) leads to non-cross-linked chains; the terminal reaction sites are distinguished by an outstanding solvation and an unimpeded access of reagents. The linkers, *p*-hydroxybenzyl alcohol (HMP) used here or *p*-(hydroxy-

Table 4: Molecular Weights of the Peptides PP*G(6−8) in 0.1 M Acetic Acid at Different Temperatures, Determined by Equilibrium Centrifugation

peptide	MW(4 °C)	MW(25 °C)	MW(35 °C)	MW_{Dim}	MW_{Mon}
PP*G(6)	20 000	17 000	14 000	18 012	9 006
PP*G(7)	19 000	17 000	18 000	19 615	9 808
PP*G(8)	21 000	19 000	21 000	21 219	10 610

methyl)phenoxyacetic acid (HMPA) (*10*), both allow peptide cleavage from the resin by TFA instead of the very strong acid TFMSA. Deprotection of Dde, different from that of Boc, proceeds rapidly under mild basic conditions (*10*, *24*). Yields of THP(III) peptides were increased, using the coupling reagent HATU/HOAT (*25*) instead of HBTU/HOBT (*10*). Finally, as earlier attempts had failed to assemble THP(III) peptides by incorporation of Gly-Pro-Hyp tripeptides, derivatization of hydroxyproline was applied to decrease association of growing peptide chains.

Hydrogen bonds, originating from hydroxyproline, are assumed to be the root cause of the impeded synthesis, leading to the formation of stable triple-helical structures and its aggregates. So, the idea is to prevent formation of hydrogen bonds by derivatization of hydroxyproline, resulting in destabilization of triple-helical structures which is thought to have a benificial effect on peptide synthesis. Suppression of stable structures has been realized in solid-phase peptide synthesis, using protection of peptide bonds, inhibiting formation of hydrogen bonds and thus of stable secondary structures (*26*). The collagen synthesis on the ribosomes of the rough endoplasmic reticulum starts with the formation of pro-α-chains, in which the prolines are not hydroxylated, preventing formation of stable triple-helical structures (*27*).

Derivatization of hydroxyproline is also believed to reduce formation of THP(III) aggregates during synthesis: Conformational energy computations on poly(GlyProHyp)$_n$ triple helices have shown that Hyp in the Y position contributes directly to the interaction between triple helices. The hydroxyl functions of hydroxyprolines are thought to strengthen the attractive interactions by formation of hydrogen bonds with backbone C═O groups of adjacent molecules (*28*). More recently X-ray crystallographic studies on a collagen-like triple-helical peptide have indicated that adjacent triple helices are linked by water networks, interconnecting the Hyp side chains of one molecule and the main-chain carbonyls of another molecule. The critical role of the Hyp residues is emphasized which are the keystones supporting the water network (*29*). Recent investigations on (ProFlpGly)$_{10}$ peptides, where Flp is a 4(*R*)-fluoroproline residue, demonstrate that the inductive effect of hydroxyproline is an important factor for aggregation and stability of collagen triple-helical peptides (*30*).

In our experiments, we have destabilized the THP(III) peptides by incorporation of tripeptides, containing a hydroxyproline residue, in which the hydroxyl function is blocked by the *tert*-butyl protective group. Among the different *tert*-butylated tripeptides prepared, PP*(tBu)G was best suited for the assembly of stable THP(III) peptides. Side-chain protection of Hyp has been described previously to improve the synthesis of peptide α1(I)772−786 (*3*). Instead of protected tripeptide blocks, used here and requiring less coupling steps, individual FmocGly, FmocPro, and Fmoc-Hyp(tBu) residues were incorporated. The destabilizing effect of Hyp(tBu), whether explained by a decrease of hydrogen bonds or a reduction of the inductive effect, should be comparable to the O-acetylation of hydroxyproline which has been reported to reduce the thermal stability of (Pro-HypGly)$_{10}$ by about 33 °C from $t_{1/2} = 58$ °C to $t_{1/2} = 25$ °C (*31*).

Purification of the intermediate peptides was achieved by RP-HPLC solely, which is also the method of choice for the large but instable peptide P*PG(8). In contrast, RP-HPLC was unsuitable for purification of the large and stable THP(III) peptides GPP*(8) and PP*G(6−8), which were separated from higher molecular weight species and deletion peptides by SE-HPLC on TSK columns. As described for the 3-fold-bridged peptide [α1(III)CB9B]$_3$ from type III collagen, containing a cystine knot at the C-terminus (*32*), TSK gel filtration was performed under denaturing conditions (4 mol of urea, 35 °C) in order to reduce peptide aggregation. Yields of THP(III) peptides, after purification by SE-HPLC, were in the range of 2.7−6.1% of the theory, and they were increased by using the new coupling reagent HATU/HOAT instead of HBTU/HOBT. The reagent, not disturbing monitoring of peptide synthesis, has been reported to enhance the yields of difficult peptides (*25*), which indeed were improved at best by about a factor of 1.5.

MALDI-TOF-MS was used to determine the molar masses of the rechromatographed peptides. Peptides were detected as molecular ions, even though they are present as dimers in 0.1 M acetic acid, shown by equilibrium centrifugation. A similar phenomenon has been observed for the ROP protein, which is a dimer where subunits are interconnected by noncovalent bonds to give a coiled-coil structure. Applying MALDI-TOF-MS, only the mass signal of the monomeric subunit is indicated (*33*). The large excess of matrix molecules relative to the THP(III) molecules, separating the noncovalently bonded THP(III) molecules and limiting their aggregation (matrix isolation), and the nature of the used matrix may cause the dominant occurrence of monomers already in solution. The precision of mass determination is in the range of 0.02−0.25%, as typically for proteins (*15*). The base peak of PP*G(6) at 9056.8 Da may indicate *tert*-butylation of the peptide. It is assumed that after cleavage the *tert*-butyl cations partly recombine with the peptide, causing mass differences of 57 Da (Figure 2e, inset).

CD spectroscopy of THP(III) peptides indicates that peptide P*PG(8) occupies a special position among the triple-branched peptides. The typical properties of the peptide are determined by the (P*PG)$_n$ triplets at the N-terminus of the molecule, as shown by comparing the covalent branched peptide P*PG(8) with the associated peptide (P*PG)$_{10}$ (*22*). Associated peptides are triple-helical structures, and the single chains are held together by noncovalent bonds. Both peptides, containing Hyp in the collagen-nonspecific X position of the GlyXY triplet, indicate several common features. At 5 °C the peptides, solubilized in diluted acetic acid, display the characteristic CD spectrum of the polyproline II conformation: a strong negative band at 200 nm (−16 250 and −20 000, respectively) and a weak positive maximum at 225 nm (+771 and +1400, respectively), yielding Rpn values of 0.05 and 0.07. Raising the temperature from 5 to 80 °C the polyproline II structures of both peptides

are transformed to disordered forms, marked by less intense bands at 200 and 225 nm, leading to lowered Rpn values of 0.02. The melting curves of both peptides indicate a linear decrease of ellipticities at $\lambda = 225$ nm, reflecting unfolding of the polyproline II structure in a noncooperative manner. Finally, both peptides do not form triple-helical structures in 0.1 M acetic acid.

Nevertheless, the presence of Hyp in the X position does not exclude the possibility that the single peptide strands of either the associated or the branched peptide can fold into a triple helix. X-ray diffraction data, obtained from (GlyHyp-Pro)$_n$ polymers, exhibited triple-helical structures (*34*). As we have shown by CD spectroscopy, the branched peptide P*PG(8) adopts a triple-helical conformation in solvents containing high proportions of methanol. The triple-helical structure of the alcohol-solubilized peptide is confirmed by its thermodynamic data, corresponding approximately to those of the triple-helical peptide PP*G(8) in diluted acetic acid.

The CD spectra of P*PG(8) in 85 vol % methanol/15 vol % 0.1 N sodium citrate/HCl under the influence of different pH values might reflect a modulation of peptide stability. At the lowest pH values the interchain repulsion between both the charged N-terminal imino groups of hydroxyproline (pK 8−9) and the ϵ-amino groups of lysine (pK about 10.5) at position 5 must be destabilizing for P*PG(8). As the pH is increased, the triple-helical conformation is stabilized by the ionization of the Glu residues (pK 3.5−4.5) at position 8, which become capable of forming intrachain ion pairs with the charged lysine residues (*35*). However, with approximation of the solvent pH to the isoelectric pH of the peptide (pH 5−6), solvation of the P*PG(8) molecules is decreased, leading to destabilization, aggregation, and finally precipitation of the peptide at pH 6.0. To support the occurrence of triple-helical structures, THP(III) peptides were dissolved in 0.1 M acetic acid (pH 2.4) or 85 vol % methanol/15 vol % 0.1 M acetic acid (pH 3.5).

CD spectra of GPP*(8), PP*G(8), and PP*G(7) with Rpn values of 0.1 indicate a triple-helical fold of the peptides in 0.1 M acetic acid at 20 °C. More stable triple-helical structures were obtained in the alcoholic solvent 85 vol % methanol/15 vol % 0.1 M acetic acid, as shown by increased Rpn values in the range of 0.10−0.13. The CD spectrum of PP*G(6), marked by a decreased Rpn value of 0.04, points to the occurrence of the unfolded conformation at 20 °C, indicating a sharp decrease of triple-helicity, reducing the number of (GPP*)$_3$ trimers from 6 to 5. The loss of triple-helicity at a specific chain length has also been reported of other triple-branched peptides containing Kemp triacid as template (*19*).

The thermodynamic parameters of THP(III) peptides were derived from thermal melting curves.Triple-branched peptides, based on the lysine dimer, exhibit broad melting transitions, whereas peptides, containing disulfide bonds (*11*) or Kemp triacid (*19*) as templates, indicate triple helix ↔ coil transitions in a narrow temperature range. The sigmoidal shapes of the melting curves appear to be dependent on the templates involved. Melting curves in addition are distinguished by a steep positive increase of $[\Theta]_{MRE}$ between 12 and 4 °C, which may be attributed to a structural change of THP(III) dimers but not to peptide aggregation and solvent effects, considering equilibrium centrifugation data and CD-buffer spectra. Melting temperatures of the THP(III) peptides PP*G(6), PP*G(7), PP*G(8), and GPP*(8) were 39.4, 44.4, 50.7, and 62.5 °C, respectively, which can be correlated to the GlyProHyp content of the peptides, containing 5, 6, 7, and 8 (GPP*)$_3$ trimers, respectively (see Figure 1). Each incorporation of a trimer contributes to a distinct increase of the melting temperature. $T_{1/2}$ values, normalized for chain length ($T_{1/2}$/triplet = 1.46−1.77), correspond to those of other 3-fold-bridged peptides (*10*). The melting temperatures of the peptides PP*G(8) and GPP*(8), containing 7 and 8 (GPP*)$_3$ trimers, respectively, differ from each other by 12 °C instead of the expected 5−6 °C. The enhanced thermal stability of GPP*(8) may be due not only to the additional (GPP*)$_3$ trimer but also to the N-terminus of the peptide; the glycine amino functions are protected by Fmoc groups, as derived from mass spectrometric analysis (see above). Derivatization of the N-terminal amino groups, suppressing charge repulsion, has been described to increase the melting temperature by 4 °C (*36*). For the associated peptide (ProProGly)$_{10}$ it has been shown that elimination of an end group effect increases the thermal stability of the peptide by about 10 °C (*37*). The thermal stability of PP*G(6) is the lowest one among the PP*G(6−8) peptides, even though the enthalpy of folding is larger than that of PP*G(7) or PP*G(8), indicating that the thermal destabilization of PP*G(6) is due to a larger negative entropic change.

The van't Hoff enthalpies of THP(III) peptides were calculated from cooperative thermal transition curves, assuming a two-state monomolecular melting procedure (see below). van't Hoff enthalpies (ΔH_{vH}) were in the range of 102−158 kJ/mol or 3.39−5.85 kJ/triplet. For comparison, a calorimetric determination of the transition enthalpy (ΔH_{cal}) was performed for peptide PP*G(8), measuring directly the thermodynamic parameters without any model assumptions. A value of $\Delta H_{cal} = 140$ kJ/mol or 4.24 kJ/triplet was obtained, which nearly corresponds to the van't Hoff enthalpy $\Delta H_{vH} = 123$ kJ/mol of the same peptide. The van't Hoff enthalpy $\Delta H_{vH} = 139.4$ kJ/mol, derived from calorimetric data, excellently agrees with ΔH_{cal} yielding a cooperative ratio of CR = $\Delta H_{vH}/\Delta H_{cal} = 1$ that demonstrates for the first time the validity of a two-state model for the melting transitions of 3-fold-bridged peptides; that is, the reaction proceeds by an all-or-none mechanism and does not involve intermediate species (*38*).

Noteworthy are also the low $\Delta H°$/triplet values of the Lys-Lys-based THP(III) peptides (3.4−5.8 kJ/(mol·triplet)), whereas associated peptides, such as (PP*G)$_{10}$ and (PP*G)$_4$-EKG(PP*G)$_5$, the melting transitions follow a trimolecular reaction of the type F_3 ↔ 3U, exhibit values of 22 and 25 kJ/(mol·triplet), respectively (*36*). With interpretation of the enthalpy as the sum of the intra- and intermolecular interactions, the greater enthalpy difference, $\Delta H°$, of the associated peptides may be attributed to a high level of interactions in the high-ordered native state, decreasing to low values in the unfolded state, marked by single chains separated from each other. In contrast, the small enthalpy change of the Lys-Lys-based peptides may be due to a smaller level of interactions in a less ordered native conformation and the occurrence of a residual structure in the unfolded state, forced by the trimeric branch. The $\Delta H°$ values of other C-terminally branched triple-helical peptides can be divided into two different classes. The $\Delta H°$ values of Heidemann (*8*) were in

the range of 5.06−6.88 kJ/(mol·tripeptide), and the value of Bruckner (*11*) was 12.5 kJ/(mol·tripeptide), based on a two-state monomolecular transition, the enthalpy change of which can be calculated by the van't Hoff equation:

$$\Delta H^\circ = 4R(T_{1/2})^2 \cdot (\mathrm{d}F/\mathrm{d}T)_{F=0.5}$$

In contrast, the laboratories of Fields (*10*) and Tanaka (*13*) calculated the enthalpy values by assuming a two-state trimolecular transition and applying the van't Hoff relationship described by Engel et al. (*23*):

$$\Delta H^\circ = 8R(T_{1/2})^2 \cdot (\mathrm{d}F/\mathrm{d}T)_{F=0.5}$$

As the melting temperatures $t_{1/2}$ of branched peptides are independent of peptide concentration (see above), ΔH° values of both groups were corrected by dividing them in half. Values from 5.21 to 7.68 and 11.76 to 13.95 kJ/(mol·tripeptide) were obtained for the laboratories of Fields and Tanaka, respectively, leaving the values of Heidemann and Fields in good agreement and similar to our values and the values of Tanaka and Bruckner considerably higher than those of the other groups. The increased values of the peptides $(GlyProHyp)_n$[N] (*13*) may be due to the composition of their triple-helical structures, containing exclusively $(GlyProHyp)_3$ trimers, and the linkage of the trimers to the branch via flexible thioether bindings instead of peptide bonds. The trimers, less influenced by the branch, can be regarded as a more independent structural unit, the properties of which appear to approximate those of the associated peptides $(GPP^*)_n$, resulting in sharp transitions and high enthalpies. The steep transition of Col 1−3 (*11*) could be explained by the arrangement of the three interchain disulfide bonds, providing an effective nucleus for triple-helix formation. Calculating the enthalpy per residue, the small values of THP(III) peptides (1.0−1.6 kJ/mol·res) agree well with the average value of $\Delta H = 1.1$ kJ/(mol·res) of 12 monomeric globular proteins at 25 °C; the melting transitions follow a monomolecular reaction (*39*). Low enthalpy changes per residue might point to monomolecular melting transitions.

Direct evidence for the significance of monomolecular melting transitions was provided by the dimer PP*G(7); the melting transition can be described in terms of three species $dimer_{folded}$, $dimer_{unfolded}$, and $monomer_{unfolded}$. Within the temperature range from 4 to 25 °C the folded dimer is the stable species, probably formed by an antiparallel alignment of two native helices, linked to each other by water networks, in homology to the crystal structure of the collagen-like peptide $(X\text{-}Y\text{-}Gly)_n$ (*29*). During melting a monomolecular $dimer_f$ to $dimer_u$ transition occurs ($F_2 \leftrightarrow U_2$), demonstrated by the melting temperature of PP*G(7), not influenced by peptide concentration; that is, thermal unfolding of the dimer is characterized by an equilibrium between folded and unfolded dimers and the position is independent of peptide concentration. In addition, a bimolecular reaction ($F_2 \rightarrow 2U$) becomes detectable, comparing equilibrium centrifugation data at 4 (19 kD) and 35 °C (18 kD). There is a small decrease of the average molecular weight of dimeric PP*G(7) at 35 °C, pointing to a monomerization of the dimeric species, just starting at the early stage of transition. The bimolecular transition is shown very clearly by the dimer PP*G(6); the average molecular weight decreases from 20 to 14 kDa by increasing temperature from 4 to 35 °C. A monomerization of the dimer PP*G(8) at 35 °C cannot be observed yet is assumed at elevated temperatures within the transition zone. Equilibrium centrifugation data of the peptides PP*G(6−8) exclude trimolecular reactions ($F_3 \leftrightarrow 3U$), as indicative of triple-helix ↔ coil conversions of collagen-like polytripeptides (*23*). The melting transitions of the dimeric peptides PP*G(6−8) appear to be determined by mono- and bimolecular reactions, competing with each other.

For the long and stable peptides PP*G(7) and PP*G(8), indicating a lack of concentration dependence of $t_{1/2}$, the dimer to monomer enthalpy change is small relative to the unfolding enthalpy change; that is, the monomolecular concentration-independent reaction dominates the bimolecular concentration-dependent reaction, which would make the thermal unfolding appear to be monomolecular, even though reaction molecularity is greater than one. This kind of molecularity can be described by the term pseudomonomolecular, as defined by Marky et al. (*16*). van't Hoff enthalpies of pseudomonomolecular transitions are calculated according to the rules of monomolecular reactions (*16*). For the short and instable dimer PP*G(6) the bimolecular reaction becomes more significant, as suggested from equilibrium centrifugation data. The greater enthalpy value of dimeric PP*G(6) compared to the values of dimeric PP*G(7) and PP*G(8) might be explained by the increasing importance of the $F_2 \rightarrow 2U$ transition, requiring unfolding of the dimer and, in addition, disruption of the intermolecular bonds between the subunits of the dimer. Thus, unfolding of the dimers PP*G(6−8), as documented by the cooperative part of the melting curve, obviously follows a two-state, pseudo-first-order reaction.

The melting behavior of the dimers PP*G(6−8) is similar to that of associated oligonucleotides, where the presence of mono- or bimolecular transitions depends on the chain length of the oligomers (*16*). In the case of long oligomers, where the monomolecular helix growth dominates the bimolecular helix formation, a strongly reduced concentration dependence of the melting temperature or a pseudo-first-order reaction has been observed. For relatively short oligomers, forming predominantly the bimolecular species, concentration dependence of the melting temperature and bimolecular transitions have been established.

As reported previously, there is another case of a branched triple-helical peptide that aggregated. At least three molecules had complexed to form aggregates, as shown by SE chromatography (*12*) and rotary shadowing microscopy (*40*). The cooperative melting transition of this peptide seems to be determined by a molecularity greater than 2.

Other reports have described the transition of branched triple-helical peptides as being from monomeric triple-helical species to monomeric denatured species (*10, 13*). There is one case where the molecularity of the transition has been established experimentally and the melting transition was of first-order. For the branched triple-helical peptide Col 1−3 of type III procollagen, containing at the C-terminus a cystine knot, transition curves were independent of peptide concentration, and kinetics of triple-helix refolding was of first-order (*11*).

Equilibrium centrifugation was applied to shed light on the aggregation of covalent branched peptides in solution.

Aggregation of the peptides PP*G(6−8) was studied in dependence on temperature, using 0.1 M acetic acid as solvent. The data demonstrate that at 4 °C the peptides are present as dimers, and there is no aggregation of the peptides, increasing temperature from 4 to 35 °C. Another branched triple-helical peptide, comprising the α1(IV) 1263−1277 sequence, was shown to aggregate (*40*), and this aggregation, in contrast to the aggregation of the PP*G(6−8) peptides, was induced by increasing temperature (*12*). SE chromatography at 4 and 35 °C yielded molecular weights of 11.6 and 36 kD, respectively, indicating at 4 °C the monomeric molecule and at 35 °C the association of three molecules to a trimer. Aggregations of individual triple-helical molecules at temperatures lower than 5 °C have been observed for the associated peptides $(PPG)_{15}$ and $(PPG)_{20}$, applying equilibrium centrifugation (*41*) and electron microscopy (*42*), respectively.

There was no aggregation of the PP*G(6−8) molecules, increasing peptide concentration to a value of 2 mg/mL. The results agree well with those of associated peptides (*36*), indicating no larger molecular weight forms, even at the highest concentration of 3 mg/mL. The decreasing values of M_{app} with increasing peptide concentrations could be a sign that solute−solvent interactions are favored over solute−solute or solvent−solvent interactions.

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