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Collagen-like triple helix formation of synthetic (Pro-Pro-Gly)$_{10}$ analogues: (4(*S*)-hydroxyprolyl-4(*R*)-hydroxyprolyl-Gly)$_{10}$, (4(*R*)-hydroxyprolyl-4(*R*)-hydroxyprolyl-Gly)$_{10}$ and (4(*S*)-fluoroprolyl-4(*R*)-fluoroprolyl-Gly)$_{10}$

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Abstract: For the rational design of a stable collagen triple helix according to the conventional rule that the pyrrolidine puckerings of Pro, 4-hydroxyproline (Hyp) and 4-fluoroproline (fPro) should be down at the X-position and up at the Y-position in the X-Y-Gly repeated sequence for enhancing the triple helix propensities of collagen model peptides, a series of peptides were prepared in which X- and Y-positions were altogether occupied by Hyp^R, Hyp^S, fPro^R or fPro^S. Contrary to our presumption that inducing the X-Y residues to adopt a down-up conformation would result in an increase in the thermal stability of peptides, the triple helices of (Hyp^S-Hyp^R-Gly)$_{10}$ and (fPro^S-fPro^R-Gly)$_{10}$ were less stable than those of (Pro-Hyp^R-Gly)$_{10}$ and (Pro-fPro^R-Gly)$_{10}$, respectively. As reported by Bächinger's and Zagari's groups, (Hyp^R-Hyp^R-Gly)$_{10}$ which could have an up-up conformation unfavorable for the triple helix, formed a triple helix that has a high thermal stability close to that of (Pro-Hyp^R-Gly)$_{10}$. These results clearly show that the empirical rule based on the conformational preference of pyrrolidine ring at *each* of X and Y residues should not be regarded as still valid, at least for predicting the stability of collagen models in which *both* X and Y residues have electronegative groups at the 4-position. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: collagen model peptides; triple helix; fluroproline; hydroxyproline

INTRODUCTION

The collagen triple helix occurs due to a characteristic sequence involving an extensively repeating tripeptide unit of X-Y-Gly, where X and Y are often imino acids, Pro or Hyp^R (4(*R*)-hydroxyproline) [1]. It is known that the Hyp^R residue plays a role in stabilizing the collagen triple helix because the thermal stability of collagens increases as the total content of Hyp^R increases [2]. By synthesizing the polytripeptides, (Pro-Pro-Gly)$_{10}$ and (Pro-Hyp^R-Gly)$_{10}$, it was demonstrated that both peptides form triple helical structures at low temperature and undergo thermal transition to single random coil states and that the transition temperature of (Pro-Hyp^R-Gly)$_{10}$ is higher than that of (Pro-Pro-Gly)$_{10}$ [3,4]. This is consistent with the relationship found in natural collagens [2]. It had long been believed that (Pro-Hyp^R-Gly)$_{10}$ acquires high thermal stability because of a hydrogen bond possibly formed between the hydroxyl group of Hyp^R and a water molecule. X-ray crystallographic analyses of model peptides containing Pro-Hyp^R-Gly repeated sequences seem to support this consideration [5,6]. However, it was shown that (Pro-Hyp^R-Gly)$_{10}$ is more stable than (Pro-Pro-Gly)$_{10}$ even in a nonaqueous solvent [7], in which hydrogen bonds between the hydroxyl group of Hyp^R and water molecules are unlikely. Later it was reported that the triple helix of (Pro-fPro^R-Gly)$_{10}$ (fPro^R: 4(*R*)-fluoroproline) was much more thermally stable than that of (Pro-Hyp^R-Gly)$_{10}$ [8,9]. Since then, an alternative explanation suggested that not hydrogen bond formation but electronegativity of the substituted group on pyrrolidine ring plays an important role in stabilizing the triple helical structure because a fluorine atom in fluoroalkanes has much less ability to form a hydrogen bond than a hydroxyl group [10–12]. On the other

Abbreviations: Boc, *t*-butoxycarbonyl; CD, circular dichroism; CPK, Corey-Pauling-Koltun; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DSC, differential scanning calorimetry; ESI-MS, electrospray ionization mass spectrometry; fPro, 4-fluoroproline; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-7-azabenzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Hyp, 4-hydroxyproline; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; TEA, triethylamine; TFA, trifluoroacetic acid.

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hand, it was shown that model peptides (X-Y-Gly)$_{10}$ with Hyp^R at the X-position [13] and with non-natural 4(S)-Hyp (Hyp^S) at either position [14] were unable to form the triple helix. In a similar manner to Hyp^S, fPro^S at the Y-position was shown to inhibit triple helix formation [15]. Thus neither hydrogen bond formation nor electronegativity in conjunction with the stereochemistry of Hyp or fPro were sufficient to be the major factor to explain the thermal stability of the triple helix. Alternatively, experimental and computational investigations on various proline derivatives and X-ray analyses on the triple helical structure of some model peptides appeared to suggest empirical rules, which might dictate the propensity of peptides to form the triple helix, as follows: (1) there is the preference of pyrrolidine ring puckering of imino acid residues, i.e. Hyp^R and fPro^R prefer the up form, and Hyp^S and fPro^S prefer the down form [16–22]; (2) the pyrrolidine ring in the X-position tends to take the down form and the up one in the Y-position [23]. Combining these notions, Zagari and co-workers proposed a general rule that 4(*R*)-substitution at the Y-position and 4(S)-substitution at the X-position stabilize the triple helix, whereas 4(*R*)-substitution at the X-position and 4(S)-substitution at the Y-position destabilize it. Since it was demonstrated that (fPro^S-Pro-Gly)$_{10}$ forms a triple helix, whereas (fPro^R-Pro-Gly)$_{10}$ does not [24,25], their rule has proved successful for interpreting the triple helix propensity of all the single-substituted model peptides except for (Hyp^S-Pro-Gly)$_{10}$. If this rule is still valid in the case of double-substituted model peptides and further allows us to elaborate the additivity of the effect of the substitution by Hyp or fPro residue on the thermal stability of the triple helix, it should be possible to design collagen model peptides with higher thermal stability than those of (Pro-Hyp^R-Gly)$_{10}$ and (Pro-fPro^R-Gly)$_{10}$. In particular, it would be expected that peptides containing Hyp^S or fPro^S at the X-position and Hyp^R or fPro^R at the Y-position would acquire quite high thermal stability. To corroborate such an additivity, (Hyp^S-Hyp^R-Gly)$_{10}$ and (fPro^S-fPro^R-Gly)$_{10}$ were synthesized and characterized. (Hyp^R-Hyp^R-Gly)$_{10}$ which had been synthesized in this context and showed unexpectedly to form a triple helix [26,27], was also investigated for comparison. This study reports the stabilities of triple helices obtained by the thermodynamic analysis of these model peptides, which eventually have provided some contradictory results to the assumption. Employing the same rule predicting their preference being the up-up pucker, a possible interpretation is suggested about the difficulty in preparing (Hyp^R-Hyp^R-Gly)$_{10}$ and (fPro^R-fPro^R-Gly)$_{10}$ by fragment condensation between Gly and Hyp^R-Hyp^R or fPro^R-fPro^R units.

MATERIALS AND METHODS

Peptide Synthesis and Purification

(Hyp^S-Hyp^R-Gly)$_{10}$ and (Hyp^R-Hyp^R-Gly)$_{10}$ were synthesized by solid-phase chemistry using Fmoc-Hyp^R(Bu^t)-OH, Fmoc-Hyp^S(Bu^t)-OH and Fmoc-Gly-OH. Fmoc-Hyp^R(Bu^t)-OH was obtained from Bachem, whereas Fmoc-Hyp^S(Bu^t)-OH was synthesized from Boc-Hyp^R-OH as described in the supporting information of reference [28]. Fmoc-Gly-OH was available from the Peptide Institute Inc. and (fPro^S-fPro^R-Gly)$_{10}$ was synthesized by solid-phase coupling of the Fmoc-fPro^S-fPro^R-Gly-OH unit. Synthesis of Fmoc-fPro^S-fPro^R-Gly-OH was carried out by solution-phase techniques. The derivatives of fPro were synthesized by our developed method [16]. Couplings were carried out on an Alko-PEG resin (Watanabe Chemical Industries, Ltd) using an Applied Biosystems 433A peptide synthesizer. The synthesis scale was 0.1 mmol and Fmoc-amino acids or Fmoc-tripeptide unit were activated with HATU (4.0 eq)/DIEA (6.0 eq) in DMF. Cleavage of the peptide resin proceeded for 1 h using a TFA/water/triisopropylsilane mixture (95 : 2.5 : 2.5). The peptides were purified by HPLC on an YMC-Pack C-18 reversed-phase column (20 × 250 mm). The eluting system was 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) applying a linear gradient from 10% to 50% B over 20 min at a flow rate of 9.5 ml/min. The purity and identity of each peptide were checked by HPLC and MALDI-TOF mass spectrometry, respectively.

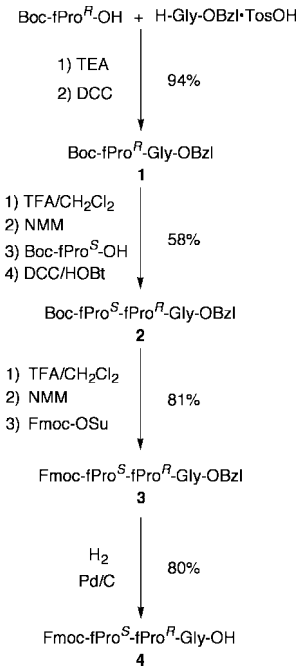
Fmoc-fPro^S-fPro^R-Gly-OH was synthesized using a standard solution phase method. Boc-fPro^R-OH and Boc-fPro^S-OH were synthesized by our developed method [16]. The coupling reaction of Boc-fPro^R-OH and H-Gly-OBzl with DCC gave a dipeptide derivative, Boc-fPro^R-Gly-OBzl. Removal of the Boc group by treatment with TFA/CH_2Cl_2 and subsequent coupling with Boc-fPro^S-OH using the DCC/HOBt method gave a tripeptide derivative, Boc-fPro^S-fPro^R-Gly-OBzl. Removal of the Boc group and reaction with Fmoc-OSu gave Fmoc-fPro^S-fPro^R-Gly-OBzl, which was converted to Fmoc-fPro^S-fPro^R-Gly-OH by hydrogenolysis of the benzyl group (Scheme 1).

Boc-fPro^R-Gly-OBzl (1)

To a solution of H-Gly-OBzl • TosOH (2.61 g, 7.74 m mol), TEA (0.78 g, 7.74 mmol) and Boc-fPro^R-OH (1.99 g, 8.51 mmol) which was prepared by our developed method in dichloromethane (50 ml), DCC (1.76 g, 8.51 mol) was added at 0 °C, and then the mixture was allowed to stir at room temperature overnight. The precipitated DCU was removed by filtration and the solvent was evaporated *in vacuo*. The resulting oil was extracted with ethyl acetate (100 ml) and washed with 10% citric acid in water (2 × 50 ml), saturated aqueous $NaHCO_3$(2 × 50 ml) and water (250 ml). The solution was dried over Na_2SO_4, and evaporated to dryness. The residue was purified by column chromatography over silica gel eluting with ethyl acetate/hexane (1 : 2, v/v). Yield: 2.76 g (93.8%). ESI-MS (*m*/*z*): $[M + Na]^+$ calcd for $C_{19}H_{25}FN_2O_5Na$, 403.17; found, 403.00.

Boc-fPro^S-fPro^R-Gly-OBzl (2)

A solution of **1** (2.60 g, 6.84 mmol) in 15 ml of TFA/CH_2Cl_2 (1 : 2, v/v) was stirred at room temperature for 1 h, and



Scheme 1 Synthesis of Fmoc-fPro^S-fPro^R-Gly-OH.

then concentrated under reduced pressure. The residual oil was precipitated with diethyl ether, filtered and dried *in vacuo*. To the crude product of H-fPro^R-Gly-OBzl • TFA (2.02 g, 5.12 mmol) in dichloromethane (50 ml) was added *N*-methylmorpholine (0.518 g, 5.12 mmol), HOBt (0.761 g, 5.63 mmol), Boc-fPro^S-OH (1.31 g, 5.63 mmol) which was prepared by our developed method, and DCC (1.16 g, 5.63 mol) at 0 °C, and then the mixture was allowed to stir at room temperature overnight. The precipitated DCU was removed by filtration. The filtrate was subjected to the same work-up procedure described above. The product was purified by column chromatography over silica gel eluting with ethyl acetate/hexane (1 : 2, v/v). Yield: 1.95 g (57.5%). ESI-MS (*m*/*z*): $[M + H]^+$ calcd for $C_{24}H_{32}F_2N_3O_6$, 496.23; found, 496.05.

Fmoc-fPro^S-fPro^R-Gly-OBzl (3)

The solution of **2** (1.80 g, 3.63 mmol) in 10 ml of TFA/CH_2Cl_2 (1 : 2, v/v) was stirred at room temperature for 1 h, and then concentrated under reduced pressure. The residual oil was precipitated with diethyl ether, filtered and dried *in vacuo*. To the crude product of H-fPro^S-fPro^R-Gly-OBzl • TFA (1.76 g, 3.46 mmol) in CH_2Cl_2 (40 ml) was added *N*-methylmorpholine (0.349 g, 3.46 mmol) and Fmoc-OSu (1.28 g, 3.80 mmol) at room temperature, and then the mixture was stirred for 6 h. After the solvent was removed *in vacuo*, the residual oil was triturated with water and the powdery product was collected by filtration. The crude product was purified by the recrystallization from ethanol. Yield: 1.81 g (80.8%). ESI-MS (*m*/*z*): $[M + H]^+$ calcd for $C_{34}H_{34}F_2N_3O_6$ Na, 618.24; found, 618.05.

Fmoc-fPro^S-fPro^R-Gly-OH (4)

Compound **3** (1.75 g, 2.83 mmol) was dissolved in methanol (40 ml) and hydrogenated in the presence of 5% Pd-on-charcoal catalyst (0.2 g). After stirring overnight in an atmosphere of hydrogen, the catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was purified by silica-gel column chromatography eluting with ethyl acetate/hexane (1 : 2, v/v). The oily product was triturated with diethyl ether and the powdery product was collected by filtration. Yield: 1.20 g (80.3%). ^{1}H NMR (400 MHz, d_6-DMSO, 22 °C) 12.60 (br, s, 1H), 8.80 (m, 0.1H), 8.61 (m, 0.05H), 8.19 (m, 1H), 7.89 (m, 2H), 7.67 (m, 1H), 7.57 (m, 1H), 7.45–7.31 (m, 4H), 5.49–5.21 (m, 2H), 4.85 (m, 0.5H), 4.68 (m, 0.5H), 4.58 (m, 0.5H), 4.46 (m, 0.5H), 4.30–4.20 (m, 4H), 3.81–3.51 (m, 4H), 2.65–1.98 (m, 4H) ppm; ^{13}C NMR (100 MHz, d_6-DMSO, 22 °C) 171.10, 170.99, 169.05, 168.76, 153.71, 153.56, 143.73, 143.59, 143.52, 140.64, 140.48, 140.44, 127.61, 127.10, 125.22, 125.17, 125.06, 120.07, 119.96, 93.51, 93.43, 92.47, 91.76, 91.69, 91.46, 90.71, 89.70, 67.07, 66.64, 58.03, 57.92, 57.13, 56.90, 53.58, 53.33, 53.14, 53.04, 52.92, 52.82, 52.58, 46.68, 46.45, 40.52, 35.82, 35.62, 35.44, 34.73, 34.51 ppm; ESI-MS (*m*/*z*): $[M + H]^+$ calcd for $C_{27}H_{27}F_2N_3O_6$, 528.19; found, 528.30.

Sample Preparation

The peptides were dissolved in 100 mM AcOH, or 100 mM AcOH and 100 mM NaCl. After keeping a stock solution of a peptide for 1 h at 90 °C to complete the dissociation into the monomer, the solution was gradually cooled to room temperature and equilibrated at 4 °C for 3 days so that the triple helices could be formed properly. The precise concentration of a peptide in solution was determined based on the amino acid analysis.

CD Spectroscopy

CD measurements were carried out on an Aviv Model 202 spectropolarimeter. Spectra were obtained with a cell of either 1 or 5 mm pathlength by averaging eight scans from 190 to 260 nm. The peptides were dissolved in 100 mM AcOH at a concentration of 0.043 mM with the exception of (Hyp^S-Hyp^R-Gly)$_{10}$ at 1.7 mM. Thermal transition curves were obtained by recording the molar ellipticity [θ] at 225 nm, while the temperature was continuously increased in the range 4°–100 °C at a heating rate of 0.1 K/min. The fraction of trimer was obtained from the ratio of the difference between the pre-transition baseline and the observed data to the difference between the pre- and post-transition baselines.

Analytical Ultracentrifugation

Sedimentation equilibrium studies were performed with a Beckman-Coulter Optima XL-I analytical ultracentrifuge at 4 °C with absorption optics. In the case of (Hyp^S-Hyp^R-Gly)$_{10}$, the sedimentation equilibrium experiments were also carried out at 37 °C. The rotor speeds for (Hyp^S-Hyp^R-Gly)$_{10}$, (Hyp^R-Hyp^R-Gly)$_{10}$ and (fPro^S-fPro^R-Gly)$_{10}$ were 45 000, 40 000 and 40 000 rpm, respectively. (Hyp^S-Hyp^R-Gly)$_{10}$ and (Hyp^R-Hyp^R-Gly)$_{10}$ were dissolved in 100 mM NaCl, 100 mM AcOH at concentrations of 0.18–1.8 mM. (fPro^S-fPro^R-Gly)$_{10}$ was dissolved in 100 mM AcOH at concentrations of

0.18–0.35 mM. Data were collected taking the average of eight measurements at each radial distance. The partial specific volumes were determined by the concentration dependencies of the density of the sample solution, which were measured using a vibrational density meter; Anton Paar DMA 5000 at 10 °C (within ±0.01 °C) and collected by taking the average of three measurements (within $\pm 3 \times 10^{-5}$ g cm^{-3}).

Differential Scanning Calorimetry

DSC measurements were carried out on a VP-DSC calorimeter (Microcal Inc.). The peptides were dissolved in 100 mM AcOH at concentrations of 1.7 mM for (Hyp^S-Hyp^R-Gly)$_{10}$ and (Hyp^R-Hyp^R-Gly)$_{10}$ and 0.69 mM for (fPro^S-fPro^R-Gly)$_{10}$. A degassed peptide solution was loaded into a calorimeter cell and heated from 10° to 110 °C at a heating rate of 0.1 K/min. To confirm the reproducibility of the measurements, the transition curves of the same sample solution were recorded three times at a time interval of 2 h. Buffer baselines were subtracted from the data. To assess whether the thermal transition of each peptide involves only two states (a triple helical structure and a single coil state) or include the presence of intermediates, the data were analysed for both the three-state and two-state models [29,30]. The thermodynamic parameters were estimated by considering trimer-monomer association-dissociation equilibrium and the change in the heat capacity between trimer and monomer states.

RESULTS AND DISCUSSION

Unlike the case of synthesizing Hyp-containing peptides, the coupling between the carboxyl group of Gly and the amino group of fPro proceeded ineffectively; the stepwise elongation of the peptide chain to prepare (fPro^S-fPro^R-Gly)$_{10}$ and (fPro^R-fPro^R-Gly)$_{10}$ was unsuccessful. Therefore the reaction steps were reduced by employing the Fmoc-fPro^S-fPro^R-Gly-OH unit for the synthesis, resulting in an appreciably improved yield. However, (fPro^R-fPro^R-Gly)$_{10}$ could not be obtained by the same procedure. An extremely strenuous reaction condition with prolonged reaction times of 3 × 24 h and 1 × 48 h was required for the synthesis of peptides using fPro^S- or fPro^R-Hyp(Bu^t)-Gly unit [31]. The common structural feature of fPro^S-fPro^R-Gly and fPro^S-Hyp^R(Bu^t)-Gly units is that the penultimate fPro or Hyp residue has a relatively bulky substituent at the 4-position of the pyrrolidine ring with the *R*-configuration. According to the preference of ring puckering suggested by Zagari and co-workers [20,23], fPro^R tends to take the up-pucker more than does Hyp^R(Bu^t), owing to the more electronegative fluorine atom than the *t*-butyloxy group. The up-up pucker of the fPro^R-fPro^R moiety could orient one or two of the δ-hydrogen atoms and a fluorine atom such that the approach of the activated carbonyl group of the *C*-terminal Gly residue to the *N*-terminal α-imino group of fPro^R is severely *restricted*; the down-up pucker of fPro^S-fPro^R somewhat relieves the terminal imino nitrogen atom from such steric hindrance as illustrated in Figure 1. Although the *t*-butyloxy group in Hyp(Bu^t) appears to have far more bulkiness than the fluorine atom in fPro, the conformation of Hyp^R(Bu^t)-Hyp^R(Bu^t) moiety is less likely to be restricted to the up-up pucker, thus reducing the steric hindrance to the coupling reaction at the *N*-terminal imino group. To alleviate the difficulty in the coupling using fPro^R-fPro^R unit as an amine component, (fPro^R-fPro^R-Gly)$_{10}$ is being prepared using Fmoc-fPro^R-Gly-fPro^R-OH unit, the results of which will be reported elsewhere.

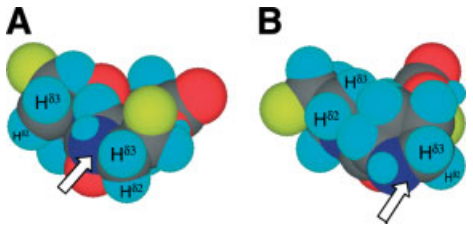


Figure 1 CPK models of (A) H-fPro^R-fPro^R-Gly-OH with the up (fPro^R)-up (fPro^R) pucker and (B) H-fPro^S-fPro^R-Gly-OH with the down (fPro^S)-up (fPro^R) pucker. The models were created using the molecular geometries of fPro^S and fPro^R presented in ref [21], and drawn with Chem3D Pro (CambridgeSoft). Atoms are colored in blue (N), green (F), grey (C), red (O) and pale blue (H). Each terminal imino-nitrogen atom is indicated by an arrow.

Triple Helical Structure and Conformational Transition

Each CD spectrum of these three peptides shown in Figure 2 has a positive peak around 225 nm and a negative peak around 198 nm, which are the characteristics of a collagen triple helix. The amplitudes of the peaks for (Hyp^S-Hyp^R-Gly)$_{10}$ are smaller than those for (fPro^S-fPro^R-Gly)$_{10}$ and (Hyp^R-Hyp^R-Gly)$_{10}$. The sigmoidal temperature dependencies of ellipticities for (fPro^S-fPro^R-Gly)$_{10}$ and (Hyp^R-Hyp^R-Gly)$_{10}$ indicate that there are cooperative conformational transitions (Figure 3). Assuming two-state transition for all these

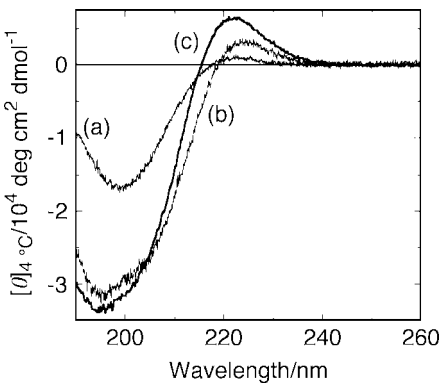


Figure 2 CD spectra of (a) (Hyp^S-Hyp^R-Gly)$_{10}$, (b) (Hyp^R-Hyp^R-Gly)$_{10}$ and (c) (fPro^S-fPro^R-Gly)$_{10}$ at 4 °C.

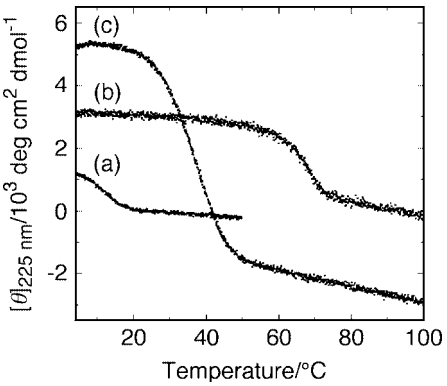


Figure 3 Temperature dependencies of molar ellipticity at 225 nm for (a) (Hyp^S-Hyp^R-Gly)$_{10}$, (b) (Hyp^R-Hyp^R-Gly)$_{10}$ and (c) (fPro^S-fPro^R-Gly)$_{10}$.

three cases, the transition temperatures were determined as a midpoint of transition and are listed in Table 1.

The densities of the solution of the double-substituted model peptides were measured at various concentrations. The concentration dependency of each peptide fell in a linear relation (Figure 4). Its slope represents the partial specific volume. The resulting partial specific volumes of these peptides in triple helical state are also listed in Table 2.

Using these values of partial specific volumes, the molecular weights of these three peptides were obtained as weight-averaged molecular weights by extrapolating the apparent molecular weights at various concentrations to an infinite dilution as shown in Figures 5 and 6. The weight-averaged molecular weights of (fPro^S-fPro^R-Gly)$_{10}$ and (Hyp^R-Hyp^R-Gly)$_{10}$ were determined at 4 °C to be $(7.94 \pm 0.40) \times 10^3$ and $(7.77 \pm 0.39) \times 10^3$, respectively. They correspond nicely to the values that are expected when these peptides exist in their trimeric states calculated from their chemical compositions, i.e. 2890.8 and 2850.8, respectively. There were no indications of further aggregation higher than trimeric states for both peptides at 4 °C.

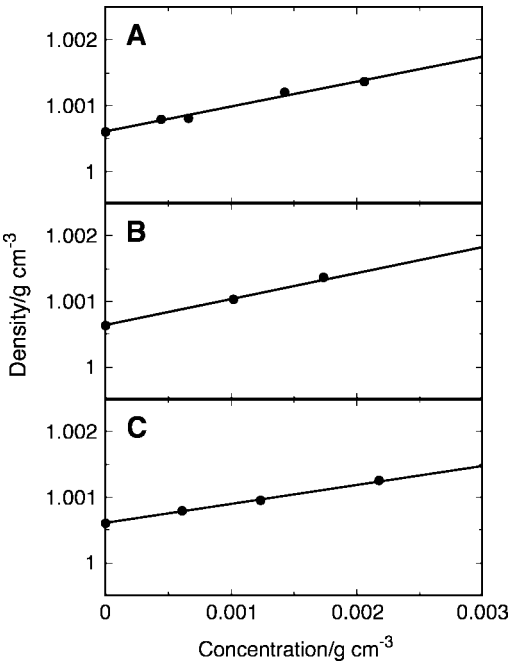


Figure 4 Concentration dependencies of the density for (A) (Hyp^S-Hyp^R-Gly)$_{10}$, (B) (Hyp^R-Hyp^R-Gly)$_{10}$ and (C) (fPro^S-fPro^R-Gly)$_{10}$ at 10 °C.

Table 2 Partial Specific Volume of the Double-substituted Model Peptides at 10 °C

Peptide	$\bar{v}$/cm^3 g^{-1}
(Hyp^S-Hyp^R-Gly)$_{10}$	0.6139
(fPro^S-fPro^R-Gly)$_{10}$	0.7068
(Hyp^R-Hyp^R-Gly)$_{10}$	0.6024

Combining the results of the CD and analytical ultracentrifugation experiments, it is concluded that (fPro^S-fPro^R-Gly)$_{10}$ takes the triple helical structure at 4 °C and undergoes thermal transition to single coil states as does (Hyp^R-Hyp^R-Gly)$_{10}$ [26,27].

Table 1 Thermodynamic Parameters of the Transition of the Double-substituted Model Peptides along with those of (Pro-Pro-Gly)$_{10}$ and the Single-substituted Model Peptides

Peptide	$T_{1/2}$ (°C)	T° (°C)	$\Delta H(T^\circ_{PPG})$ (kJ mol^{-1})	$-T^\circ_{PPG}\Delta S(T^\circ_{PPG})$ (kJ mol^{-1})	$\Delta C_p(T^\circ_{PPG})$ (J mol^{-1} K^{-1})	$\Delta G(T^\circ_{PPG})$ (kJ mol^{-1})	ref
(Hyp^S-Hyp^R-Gly)$_{10}$	9.0	39.9	112.6	−124.3	−380.3	−11.7	This work
(Hyp^R-Hyp^R-Gly)$_{10}$	64.6	110.7	102.4	−91.6	154.3	10.7	This work
(fPro^S-fPro^R-Gly)$_{10}$	29.9	93.0	69.6	−65.0	961.3	4.6	This work
(Pro-Pro-Gly)$_{10}$	31.4	71.9	108.1	−108.1	681.7	0	28
(Pro-Hyp^R-Gly)$_{10}$	62.2	92.3	147.1	−138.8	96.6	8.3	28
(Pro-fPro^R-Gly)$_{10}$	77.0	126.7	85.7	−72.0	545.7	13.7	28
(fPro^S-Pro-Gly)$_{10}$	54.5	116.7	65.9	−58.0	139.5	7.9	28

T°_{PPG} refers to $T^\circ = 71.9$ °C for (Pro-Pro-Gly)$_{10}$.

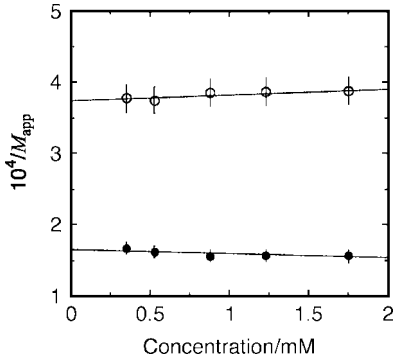


Figure 5 Concentration dependencies of the apparent molecular weights of (Hyp^S-Hyp^R-Gly)$_{10}$ at 4 °C (closed circles) and 37 °C (open circles).

In the case of (Hyp^S-Hyp^R-Gly)$_{10}$, the weight-averaged molecular weight values were found to be $(5.37 \pm 0.27) \times 10^3$ at 4 °C and $(2.50 \pm 0.12) \times 10^3$ at 37 °C. The former value is larger than the value calculated from the chemical composition (2850.8), whereas the latter is comparable. According to the CD experiment, it is evident that the thermal stability of (Hyp^S-Hyp^R-Gly)$_{10}$ is much lower than those of (fPro^S-fPro^R-Gly)$_{10}$ and (Hyp^R-Hyp^R-Gly)$_{10}$; with the concentration used in the ultracentrifugation experiment, (Hyp^S-Hyp^R-Gly)$_{10}$ is unable to form the triple helix completely even at 4 °C and is in the monomer-trimer equilibrium. It exists in the single coil at 37 °C.

Thermodynamic Parameters for the Transitions

For these three double-substituted model peptides, the heat capacity curves at the second and third scan completely overlapped for all peptides without refilling the solution. This indicates that these transitions are reversible. Their heat capacity curves were fit to a two-state model, which assumed no intermediate state between the trimer and the monomer, being in an association-dissociation equilibrium as shown in Figure 7. Therefore it was concluded that these double-substituted model peptides including (Hyp^S-Hyp^R-Gly)$_{10}$ form the triple helix and undergo a transition to the single coil without any significant intermediate as the temperature increases. Enthalpy change (ΔH), entropy change (ΔS), Gibbs free energy change (ΔG) and heat capacity change (ΔC_p) associated with the transitions of each peptide were obtained from the fitting processes employing statistical thermodynamic analysis [28]. These thermodynamic parameters are summarized in Table 1 and Figure 8 along with the corresponding data of (Pro-Pro-Gly)$_{10}$ and the single-substituted model peptides which have been shown to form the triple helix. Note that transition temperature ($T_{1/2}$) is defined as the temperature at which the transition is half-completed and does not correspond to the temperature at which ΔG equals zero (T°). $T^\circ\Delta S$ and ΔG in Table 1 were normalized at $T^\circ_{PPG} = 71.9$ °C for T° of (Pro-Pro-Gly)$_{10}$ as a reference. Contrary to our assumption that conformational propensity can be evaluated cumulatively with respect to those of the X and Y residues, the transition temperatures of (Hyp^S-Hyp^R-Gly)$_{10}$ and (fPro^S-fPro^R-Gly)$_{10}$ were much lower than those of (Pro-Hyp^R-Gly)$_{10}$ and (Pro-fPro^R-Gly)$_{10}$, respectively. Furthermore, the thermal stability of (Hyp^R-Hyp^R-Gly)$_{10}$ is comparable to that of (Pro-Hyp^R-Gly)$_{10}$ although, judging from Zagari's rule, (Hyp^R-Hyp^R-Gly)$_{10}$ was not expected to form a thermally stable triple helix. The similar results of CD measurements on (Hyp^R-Hyp^R-Gly)$_{10}$ have been reported recently by two other groups [26,27]. It is known that (Hyp^S-Pro-Gly)$_{10}$ does not form a triple helix, a violation of the rule which regards this case as an exception because of the steric hindrance of hydroxyl group of Hyp^S. However, this explanation causes another contradiction by showing evidence that (fPro^S-Pro-Gly)$_{10}$ forms a triple helix. The difference in volumes between the hydroxyl group and a fluorine atom is too small to provide a steric effect with such an appreciable distinction.

To conclude, it is unlikely that the relationship between the thermal stability of a triple helix and the puckering propensity of a proline analogue at the X- or Y- position of X-Y-Gly unit is applicable to double-substituted model peptides. In other words, it should be emphasized that the assumption of the additivity of the effects of substitution is not acceptable.

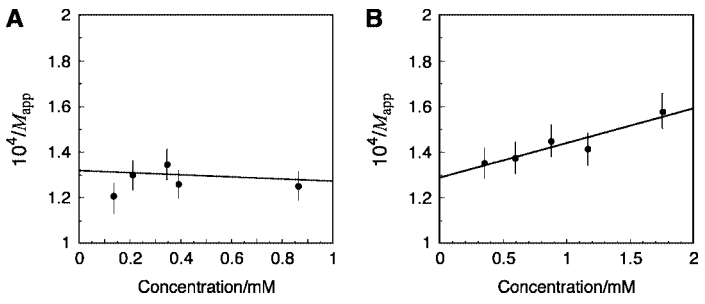


Figure 6 Concentration dependencies of the apparent molecular weights of (A) (fPro^S-fPro^R-Gly)$_{10}$ and (B) (Hyp^R-Hyp^R-Gly)$_{10}$ at 4 °C.

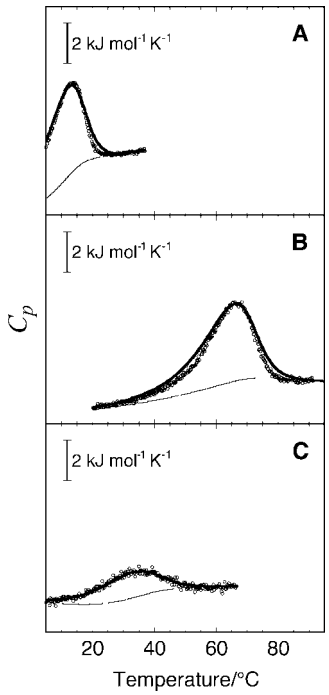


Figure 7 Molar heat capacity curves and fitting functions of (A) $(Hyp^S\text{-}Hyp^R\text{-}Gly)_{10}$, (B) $(Hyp^R\text{-}Hyp^R\text{-}Gly)_{10}$ and (C) $(fPro^S\text{-}fPro^R\text{-}Gly)_{10}$.

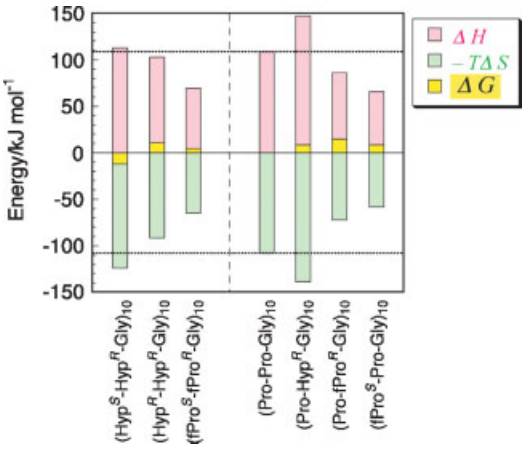


Figure 8 Comparison of thermodynamic parameters of the transition of the double-substituted model peptides along with those of $(Pro\text{-}Pro\text{-}Gly)_{10}$ and the single-substituted model peptides.

As shown in Table 1 and Figure 8, the absolute values of ΔH and ΔS of $(fPro^S\text{-}fPro^R\text{-}Gly)_{10}$ are much smaller than those of $(Hyp^S\text{-}Hyp^R\text{-}Gly)_{10}$ or $(Hyp^R\text{-}Hyp^R\text{-}Gly)_{10}$. In the series of fPro-containing single-substituted model peptides, it has been found that they have smaller ΔH and ΔS values than those of a Hyp-containing peptide [28,32]. The present experiments show that a similar tendency was also found in double-substituted peptides. In order to explain the double-substitution effects on the thermal stability of collagen triple helix, it necessary to evaluate the hydration states of the double-substituted model peptides because the contrasting trend of thermodynamic parameters of the single-substituted model peptides may be ascribed to a difference in the degree of hydration in our last paper [28]. To determine the degree of hydration, it is essential to determine the structure of double-substituted model peptides in the triple helix and single coil states as well as their partial molar volumes. The detailed structures of triple helices would also provide information about hydrogen bond formation which is assumed by Zagari and co-workers to be a major factor in making $(Hyp^R\text{-}Hyp^R\text{-}Gly)_{10}$ take a triple helix, violating the propensity based model [27]. With the values of partial molar volumes, it would be possible to discuss the *trans*/*cis* ratios of peptide bonds that reflect the molecular expansion in the single coil state. This could be related to the stability of the triple helix as pointed out by Bächinger and co-workers [26]. Detailed structural analysis of double-substituted model peptides is being performed with X-ray crystallography and sufficient peptides are being synthesized for the determination of their partial molar volumes in the single coil state.

CONCLUSIONS

In addition to $(Hyp^R\text{-}Hyp^R\text{-}Gly)_{10}$ it was demonstrated that double-substituted model peptides, $(Hyp^S\text{-}Hyp^R\text{-}Gly)_{10}$ and $(fPro^S\text{-}fPro^R\text{-}Gly)_{10}$, form a triple helix and undergo thermal transition to single coil states. Actually their thermal stabilities were quite different from predicted according to the conformational preference of pyrrolidine ring puckering at the X- or Y-position of $(X\text{-}Y\text{-}Gly)_{10}$. It is concluded that the empirical rule about the effect of substitution on the thermal stability of the triple helical structure is not at all additive with respect to the X,Y-doubly-substituted collagen models, but requires another elaboration based on, for example, detailed structural analyses as well as precise thermodynamic investigations to address various problems of the collagen triple helix.

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