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Synthesis of heterotrimeric collagen models containing Arg residues in Y-positions and analysis of their conformational stability

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Abstract—An Arg residue incorporated into the Y-position of collagenous host–guest peptide Ac-(Gly-Pro-Hyp)$_3$-Gly-Pro-Y-(Gly-Pro-Hyp)$_4$-Gly-Gly-NH_2 is reported to stabilize the triple helical structure as well as a 4(*R*)-hydroxyproline (Hyp) residue. Here, we synthesized heterotrimeric collagen models containing Arg in Y-positions utilizing the cystine knot strategy. Analysis of their thermal transition temperatures using circular dichroism spectrometry demonstrated unexpected decrease in the triple helical stability as the number of Arg increased. The obtained results indicated that an Arg residue in a Y-position is not always an equivalent of a Hyp residue, and that it possesses a potential helix destabilizing effect.

Collagen triple helix is a unique secondary structure consisting of tandem repeats of Gly-X-Y sequences. It is a right-handed supercoiled structure with one residue stagger. In the triple helix, individual polypeptide chains take left-handed polyproline II-like helices. In vertebrate collagens, Pro and 4(*R*)-hydroxyproline (Hyp) residues are most frequently found at X- and Y-positions, respectively. Therefore, peptides based on repeating Gly-Pro-Hyp triplets are widely used for structural and biochemical studies of collagen. There is a growing interest in the origin of the triple helical stability. Previous studies have suggested that important factors affecting triple helix stability are water-mediated hydrogen bond networks involving the hydroxyl groups of Hyp at Y-positions.[1,2] Recent evidence obtained using collagen models containing 4(*R*)-fluoroproline in Y-positions provided another stabilizing mechanism based on the electron-inductive effect controlling imino acid ring puckerings.[3–5] The triple helix propensity of naturally occurring amino acid residues was exhaustively studied using host–guest models, in which a Gly-X-Y guest triplet was incorporated into the repeating Gly-Pro-Hyp host tripeptides, such as Ac-(Gly-Pro-Hyp)$_3$-Gly-X-Y-(Gly-Pro-Hyp)$_4$-Gly-Gly-NH_2.[6] Among the amino acid incorporated into the Y-position, Arg was found to be as effective as Hyp residue in stabilizing the triple helical conformation, showing the same melting temperature of the triple helix.[7] Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that plays crucial roles in collagen biosynthesis. Interestingly, HSP47 also interacts with collagen triple helix recognizing Arg residues at Y-positions.[8] Although naturally occurring collagens are often composed of different polypeptide chains (i.e., types I, IV and V), most of the previous studies were performed using self-assembling collagen model peptides to form homotrimers, and there was no information about the effect of asymmetrically introduced Arg residues on triple helical stability or on HSP47-binding. Here, we synthesized heterotrimeric collagen models that contain 0–3 Arg residues by taking advantage of Moroder's cystine knot strategy.[9] Contribution of the Arg residues to the triple helical stability was reappraised using the heterotrimers. Analysis of the conformational stability using circular dichroism (CD) spectrometry demonstrated an unexpected destabilizing effect of Arg residues.

Structures of heterotrimeric collagen models **1**–**4** are shown in Figure 1. Each peptide was designed to have the similar context of (Gly-Pro-Hyp)$_n$-Gly-Pro-Y-(Gly-Pro-Hyp)$_n$ as the previously reported host–guest peptides.[6] A cystine knot was introduced to the N-termini of the peptides rather than the C-termini for the convenient construction of the peptide chains. A flexible

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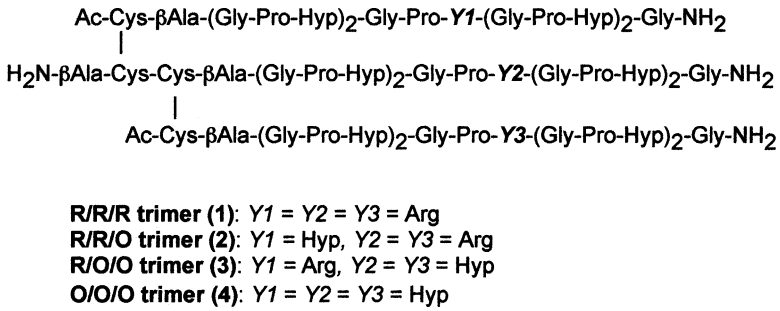


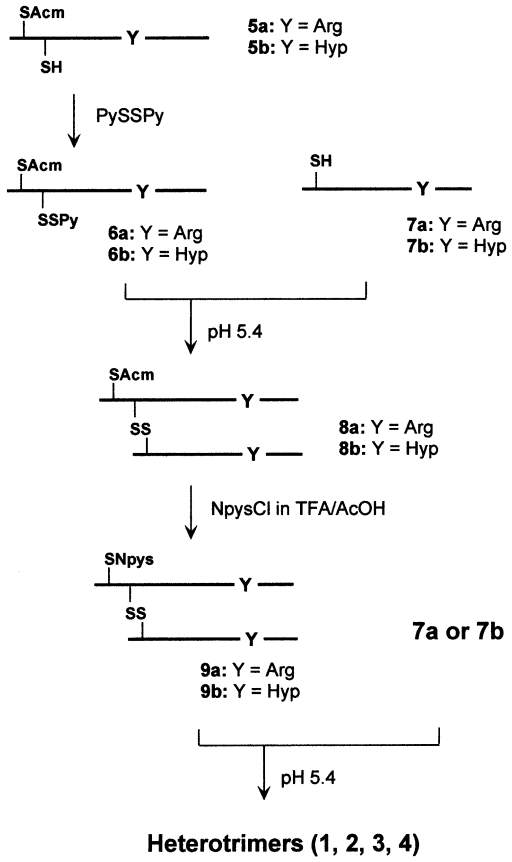
Figure 1. Structure of heterotrimeric collagen models synthesized in this study.

β-Ala linker was introduced between the helical portion and the cystine knot expected to facilitate triple helix formation with one residue stagger.

Single-chain precursors of trimeric peptides were synthesized by the standard *N*-(9-fluorenyl)methoxycarbonyl (Fmoc)-based solid-phase protocol on Rink-amide resins. t*ert*-Butyl, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl and trityl groups were used for the protection of Hyp, Arg and Cys residues, respectively. Only the N-terminal Cys side chains in the biscysteinyl fragments were protected with acetamidomethyl (Acm) groups. After deprotection/cleavage by treatment with a trifluoroacetic acid (TFA)–scavenger system,[10] the main products were purified by reversed-phase HPLC (RP-HPLC).

As summarized in Scheme 1, heterologous trimerization was achieved by stepwise disulfide bond-forming reactions. RP-HPLC profiles of crude mixtures in each step for the synthesis of R/R/R trimer **1** are shown in Figure 2. At each step, main products were purified by RP-HPLC and characterized by electron spray ionization mass spectrometry (ESI-MS). First, the free thiol groups of the biscysteinyl peptides (**5a**,**b**) were activated with 2-pyridylthio (PyS) group by the treatment with PySSPy (22 equiv) in 1:1 mixture of buffer A[11] and 2-propanol (Fig. 2a). The purified SPy activated peptides (**6a**,**b**) were then mixed with 1–1.3 equiv of monocysteinyl peptides (**7a**,**b**) in buffer A to yield corresponding heterodimeric peptides (**8a**,**b**, Fig. 2b). In this reaction, small amounts of homodimers were also generated. The *S*-Acm groups in the heterodimers were converted to *S*-3-nitro-2-pyridinesulfenyl (Npys) groups by treating them with 1.1 equiv of NpysCl in the solvent of TFA/AcOH (1:2 v/v) (Fig. 2c). Even under optimized conditions, this reaction generated some by-products, including homodimers as reported earlier.[12] Finally, the Npys-activated dimers (**9a**,**b**) were mixed with 1.1–1.3 equiv of monocysteinyl peptides (**7a**,**b**) in buffer A to yield desired heterotrimers (Fig. 2d). ESI-MS: **1** $m/z = 5137.7$ [M], calcd 5137.7; **2** $m/z = 5095.0$ [M], calcd 5094.6; **3** $m/z = 5052.0$ [M], calcd 5051.5; **4** $m/z = 5009.2$ [M], calcd 5008.5.

CD spectra of the refolded trimers (1 mg/mL) in 20 mM bis-Tris–HCl (pH 7.4) containing 150 mM NaCl were recorded at 4 °C to characterize the conformational state (Fig. 3a). The spectra were similar one another, showing positive signal around 225 nm, indicating the



Scheme 1. Synthesis of the heterotimeric collagen models (**1**–**4**).

formation of the collagen triple helix. Contribution of the N-terminally introduced cystine knot to the stability of the triple helix was demonstrated by comparing the thermal transition curve of trimeric R/R/R (**1**) with that of the corresponding reduced form. The CD_{225} values of trimeric R/R/R monitored with increasing temperature showed sharp decrease around 35–40 °C indicating cooperative triple helix ↔ random coil transition. When the disulfide bridges were reduced with dithiothreitol (DTT) prior to the measurement, mean residue ellipticity at 225 nm at 4 °C decreased to about a half, and no apparent thermal transition was observed. This result indicates that the peptide sequence cannot completely fold into triple helical conformation without the cystine knot, even at 4 °C.

To clarify the effect of Arg residues at Y-positions on triple helical stability, thermal transition curves of the

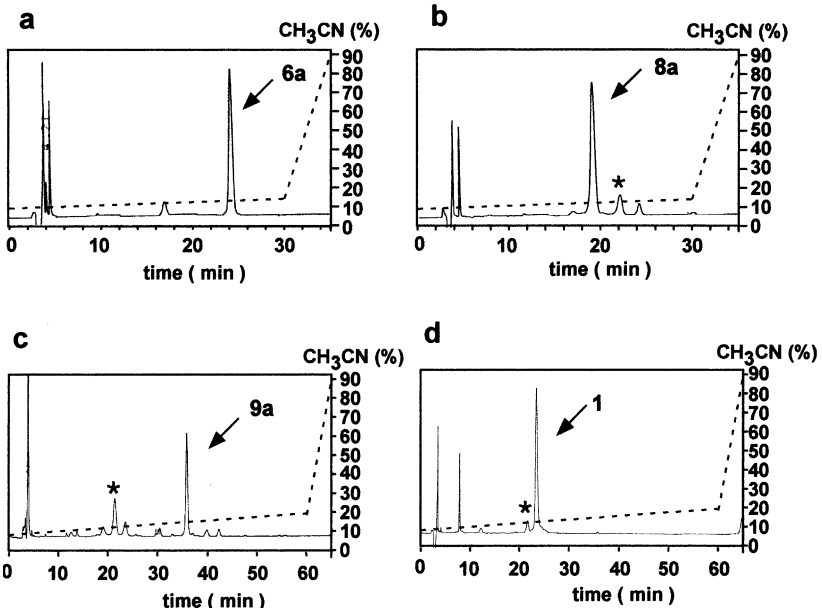


Figure 2. RP-HPLC profiles of the crude products in each steps for the synthesis of R/R/R heterotrimer **1**: (a) pyridylthiolation of **5a**; (b) dimerization of **6a** with **7a**; (c) exchange of the SAcm group of **8a** to an SNpys group; (d) trimerization to make **1**. HPLC was conducted on a Cosmosil 5C18-AR II (4.6 i.d.×250 mm) with linear gradients of CH_3CN in water both containing 0.05% (v/v) TFA at 42 °C. Arrows indicate desired products. Asterisks indicate by-products identified as the dimer of **7a**.

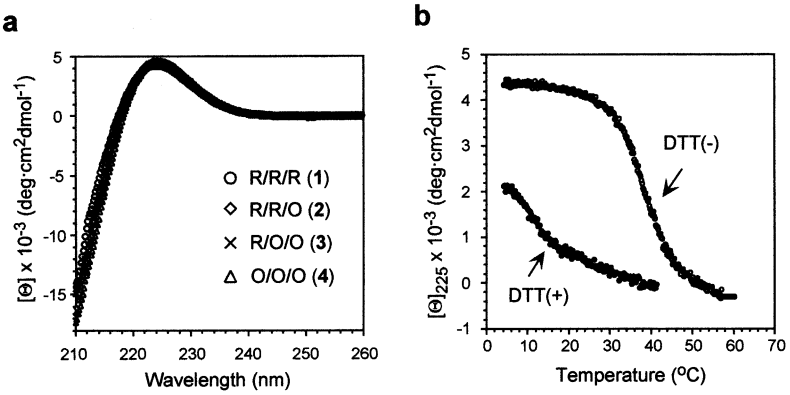


Figure 3. (a) CD spectra of heterotrimers **1**–**4** recorded at 4 °C; (b) thermal equilibrium curves for R/R/R trimer **1** in the presence and absence of DTT as monitored by CD spectroscopy at 225 nm. Temperature was increased at the rate of 0.3 °C/min.

heterotrimers were obtained by measuring CD_{225} with increasing temperature (Fig. 4). The thermal transition curves shifted lower as the Arg number increased (Fig. 4). Melting temperatures (T_m) of the R/R/R (**1**), R/R/O (**2**), R/O/O (**3**) and O/O/O (**4**) triple helices were estimated to be 37.5, 40.8, 44.5, 47.2 °C, respectively. The destabilizing effect of the Arg residues appeared additively. While, the earlier analysis by using host–guest peptides estimated the identical T_m value of 45.5 °C for Ac-(Gly-Pro-Hyp)$_8$-Gly-Gly-NH_2 and Ac-(Gly-Pro-Hyp)$_3$-Gly-Pro-Arg-(Gly-Pro-Hyp)$_3$-Gly-Gly-NH_2,[13] and authors concluded that Arg residues at Y-positions possess triple helix-stabilizing effect in the context of (Gly-Pro-Hyp)$_n$. Our result indicates that Arg in Y-positions are not always as stabilizing as Hyp residues even in the same context of (Gly-Pro-Hyp)$_n$. We propose that the origin of the destabilizing effect would not be a simple charge repulsion because only one residue incorporation of Arg destabilized the triple helix.

As demonstrated here, the peptide system in which cystine knots are introduced into the N-termini of collagenous peptides together with β-Ala linkers serves as an effective stabilizer of collagenous triple helices as well as other knotting systems.[9,14–18] It should be noted that only N-terminal and C-terminal[9] cystine knot systems make it possible to investigate heterotrimeric collagen helix to date. Quite recently, the stabilizing effect of a tripeptide unit was shown to largely depend on sequence environment by comparing host–guest peptides and repeating tripeptides.[19] The present study is the first report implying another factor affecting stability contributions of a tripeptide unit. Further biophysical study using the Arg-containing heterotrimers is required for unveiling the enigmatic mechanisms of triple helix stabilization. In addition, these heterotimeric collagen models were also used for elucidating the client-binding mechanism of collagen-specific molecular chaperone HSP47. Results of HSP47-binding study will be described in a separate paper.

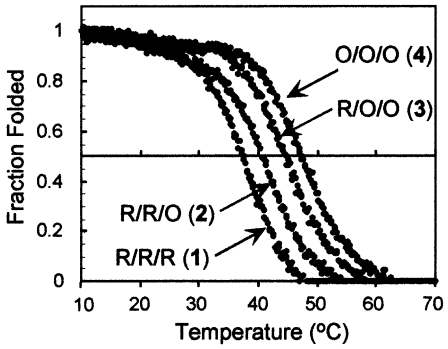


Figure 4. Thermal stability of the heterotrimers **1**–**4**. CD thermal transition profiles recorded at 225 nm were normalized to the fraction folded for the peptides. Melting temperatures were taken at a fraction folded of 0.5.

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