Polymer Journal, Vol. 38, No. 4, pp. 400–403 (2006)

SHORT COMMUNICATIONS

Threonine in Collagen Triple-helical Structure

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(Received October 4, 2005; Accepted December 5, 2005; Published April 15, 2006)

KEY WORDS Threonine / Collagen / Triple-helical Structure / Host-guest Peptide / Side-chain Conformation / Hydration Pattern /
[DOI 10.1295/polymj.38.400]

Collagen is the most abundant proteins found in the extracellular matrix of multicellular animals, and has a unique triple-helical structure, which is composed of the three polypeptides. The three chains form a right-handed supercoiled triple-helix. Each polypeptide chain requires Gly at every third residue, which generates -Xaa-Yaa-Gly- repeating sequence. The glycine residues in every third position are packed in the center of the triple-helix. The residues in the Xaa and the Yaa positions are exposed to the molecular surface. High contents of imino acids in the Xaa and the Yaa positions are required to the stability of the structure. Collagen family includes more than thirty proteins in vertebrates.[1,2] Similar collagens and much more diverse collagen proteins are also presented throughout invertebrates including a few giant molecules found in the cuticles of several worm species.[3,4] For example, the *Riftia pachyptila* cuticle collagen has a low Hyp content but the Thr content is much higher than those found in other collagens.[5] The mechanism of the stability of the collagen helix is still unknown. The Thr of the collagen is highly glycosylated.[4] Several model peptides were synthesized to analyze its thermal stability and property.[5–9] The O-galactosylation of Thr increases the thermal stability (the helix-coil transition temperature) of Ac-(Gly-Pro/4(*R*)Hyp-Thr)$_{10}$-NH$_2$ peptides.[6] The CD experiments of Ac-(Gly-4(*R*)Hyp-Yaa)$_{10}$-NH$_2$ peptides with various amino acids in the Yaa position (Thr, Ser, Val, Ala, and *allo*Thr) suggested that the methyl group, hydroxyl group and stereo configuration of Thr are important for the stability.[9] The methyl group of Thr was hypothesized to shield the inter-chain hydrogen bond between the amide of Gly and carbonyl of Xaa residues from water molecules by energy-minimization method.[9] Although several studies have challenged to rationalize the experimental data, the mechanism of the stability in cuticle collagen is still ambiguous.

In order to understand the stabilization mechanism of Thr in the Yaa position, we attempted to crystallize the peptides Ac-(Gly-4(*R*)Hyp-Thr)$_{10}$-NH$_2$ and H-(Gly-4(*R*)Hyp-Thr)$_{10}$-OH. Despite their ability to form a triple-helical structure,[6] we could not succeed in the formation of the single crystals of these peptides yet. The host-guest peptide system is an alternative way to get single crystals of the peptide with interesting sequence. Therefore, 4(*R*)Hyp-Thr-Gly tripeptide unit was inserted into the stable host peptide (Pro-Pro-Gly)$_9$.[10] The host-guest peptide H-(Pro-Pro-Gly)$_4$-(4(*R*)Hyp-Thr-Gly)-(Pro-Pro-Gly)$_4$-OH (OTG) contains 4(*R*)Hyp-Thr-Gly tripeptide unit that is abundant in the *Riftia pachyptila* cuticle collagen. The single crystal analysis of the OTG peptide provided the first insight into the unique 4(*R*)Hyp-Thr-Gly tripeptide unit conformation. Here, the Thr conformation and the observed hydration patterns around Thr residue in triple-helical structure were revealed.

EXPERIMENTAL

Crystallization was performed by hanging-drop diffusion method at 4 °C. Sample solution was prepared at concentrated 10 mg mL^{-1}. Reservoir solution contained 0.1 M Hepes buffer pH 7.5 and 23% (w/v) PEG1000. Drop mixture made up of sample solution 2 µl and reservoir solution 2 µl. Rod-like crystals appeared in about 3 weeks. A single crystal was measured at 100 K on the beamline BL6A at the Photon Factory in Tsukuba. Intensity data was processed by CrystalClear.[11] Crystal belongs to monoclinic space

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group $P2_1$ with unit cell parameters $a = 26.0$, $b = 26.5$, $c = 80.2$ Å, $\beta = 90.4°$. The structure of OTG was determined by molecular replacement method using (Pro-Pro-Gly)$_9$ peptide (PDB code 1ITT)[12] as a search model. Positional refinement was performed by X-PLOR[13] and structure refinement was carried out by SHELX-L.[14]

RESULTS AND DISCUSSION

Thr residues are at the central tripeptide unit of the molecule. To describe side-chain conformation of Thr, χ_1 dihedral angle is defined by N-C_α-C_β-$O_{\gamma1}$ or N-C_α-C_β-$C_{\gamma2}$. The different conformations of the side-chain as a function of χ_1 values of $-60°$, $180°$, and $60°$ are referred to *gauche*$^+$, *trans*, and *gauche*$^-$, respectively. Thus, Thr side-chain in OTG structure, the $O_{\gamma1}$ takes *gauche*$^+$ conformation, whereas the $C_{\gamma2}$ takes *trans* conformation to amide group (Figure 1). Both the $O_{\gamma1}$ and the $C_{\gamma2}$ are directed toward adjacent chains. This kind of Thr side-chain conformation is the same as two out of three Thr in T3-785 peptide.[15] The average main-chain dihedral angles (ϕ/ψ) of Thr in this study are $-61°$ and $145°$, which are consistent with those values in the Yaa position of collagen-like peptides.[10,16] In T3-785 structure,[15] water-mediated hydrogen bond was reported between the Thr OH group and the Gly carbonyl in the same chain *via* one water molecule. For Thr, not only the above water-mediated pattern, but also diverse water-mediated patterns are observed in the OTG structure in Figure 2a. In the first case, two water molecules make hydrogen bonds with the OH group of Thr114 and the

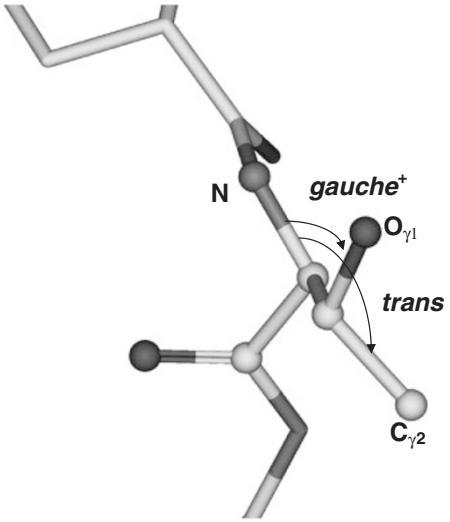
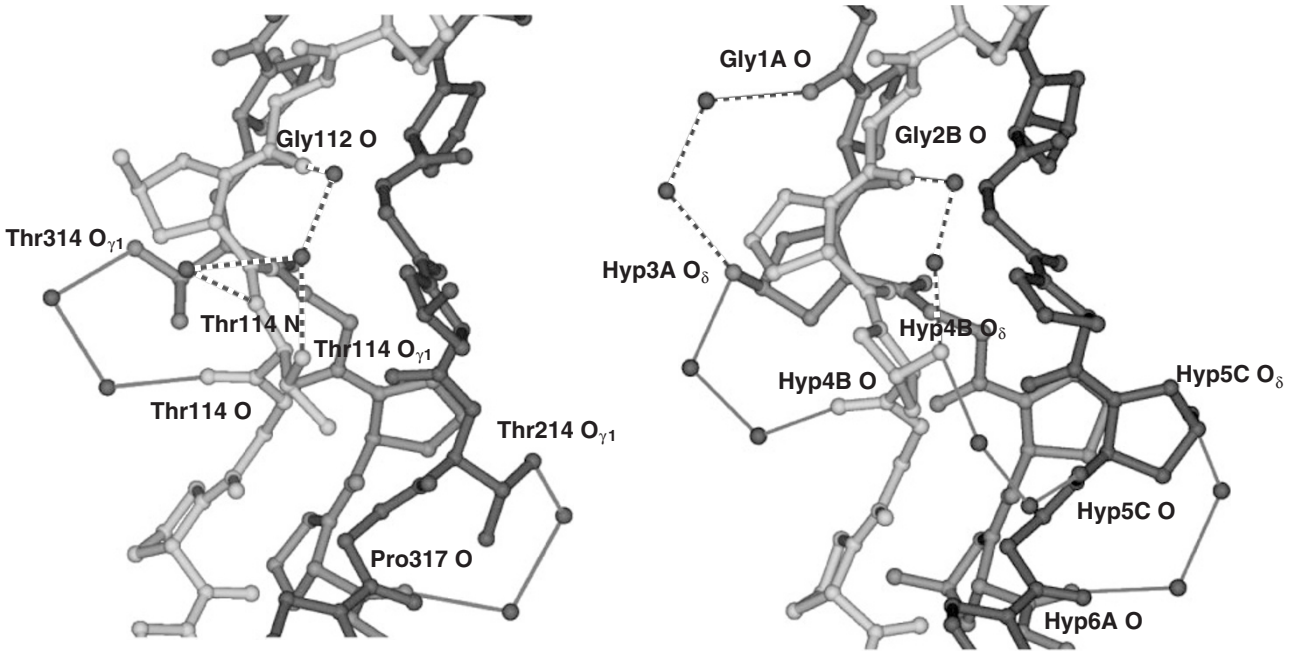


Figure 1. *gauche*$^+$/*trans* conformation of Thr in the OTG structure.



(a) **(b)**

Figure 2. (a) Central region of the OTG molecule shows three cases of water-mediated hydrogen bonds at OH group of Thr. Case 1: Thr114 $O_{\gamma1}$ connects through two water molecules to Gly112 O in the same chain. Case 2: Thr114 $O_{\gamma1}$ connects through two water molecules to Thr114 N within the same residue. Case 3: Thr314 $O_{\gamma1}$ connects through two water molecules to Thr114 O of adjacent chain and Thr214 $O_{\gamma1}$ connects through two water molecules to Pro317 O of adjacent chain. In the residue name, the first digit corresponds to a chain number and the next two digits correspond to a residue number. (b) Hydration patterns involving OH group of 4(*R*)Hyp and carbonyl groups in (Pro-4(*R*)Hyp-Gly)$_{11}$ structure.[16] Three chains in a molecule are shown by different shedding; light-, middle- and dark-gray. Spheres are water molecules. Intra-chain and inter-chain water-mediated networks are shown in broken and solid lines, respectively.

carbonyl group of Gly112 in the same chain (broken lines). In the second case, two water molecules interact between the OH and the amide groups within the same Thr114 residue (broken lines). And the third case occurs at two positions; one is two water molecules are linked between the OH group of Thr314 and the carbonyl group of Thr114 of neighboring chain and another is the similar pattern between the OH group of Thr214 and the carbonyl group of Pro317 (solid lines). The average hydrogen bond distance in these three water-mediated patterns is 2.95 Å. The hydration pattern in the second case could not be found at 4(*R*)Hyp due to the lack of hydrogen at the amide group. However, hydration patterns in the first and the third cases, which are inter- and intra-chain hydrogen bond networks, are generally observed at 4(*R*)Hyp in the Yaa position in the peptides including Pro-4(*R*)Hyp-Gly tripeptide unit.[16,17] These inter- and intra-chain hydration networks occur repeatedly along the triple-helical molecule, for example, in (Pro-4(*R*)Hyp-Gly)$_{11}$ structure[16] as shown in Figure 2b. They are the dominant feature in the repetitive patterns of 4(*R*)Hyp in peptides having Pro-4(*R*)Hyp-Gly repeating sequence.[16,17] Thus, this result indicates that the OH group of Thr in the Yaa position participates in water-mediated inter- and intra-chain hydrogen bonds in the similar way to the OH group of 4(*R*)Hyp. Moreover, the position of the OH group of Thr is located close to that of 4(*R*)Hyp when Thr in the OTG structure is superimposed over the 4(*R*)Hyp in the (Pro-4(*R*)Hyp-Gly)$_{11}$ structure (Figure 3). The distance between both hydroxyl oxygen atoms is about 1 Å. The close location of the OH groups of Thr to 4(*R*)Hyp could contribute to the similar formation of water-mediated hydrogen bonds. Therefore, the OTG structure demonstrates that Thr could act like 4(*R*)Hyp at OH group side-chain to make similar water-mediated networks. The thermal stability of Ac-(Gly-4(*R*)Hyp-Yaa)$_{10}$-NH_2 peptides containing the Thr is higher than those containing the Ser, *allo*Thr, Ala and Val,[9] which suggests the importance of the side-chain conformation in the triple-helical structure.

So far only the T3-785[15] peptide has a reported structure of Thr in a triple-helical structure. The X-ray determination of the OTG peptide provides insight into detailed structure of frequently observed residues in *Riftia pachytila* cuticle collagen. Although the stabilization mechanism of the OTG peptide is not clearly understood, the fine structure of the OTG peptide provides valuable information of Thr conformation including diversity of water-mediated hydrogen bonds around Thr in the triple-helical structure. Interestingly, the observed hydration patterns of Thr are similar to those of 4(*R*)Hyp and moreover, OH group side-chain characteristic of Thr and 4(*R*)Hyp is similar as well.

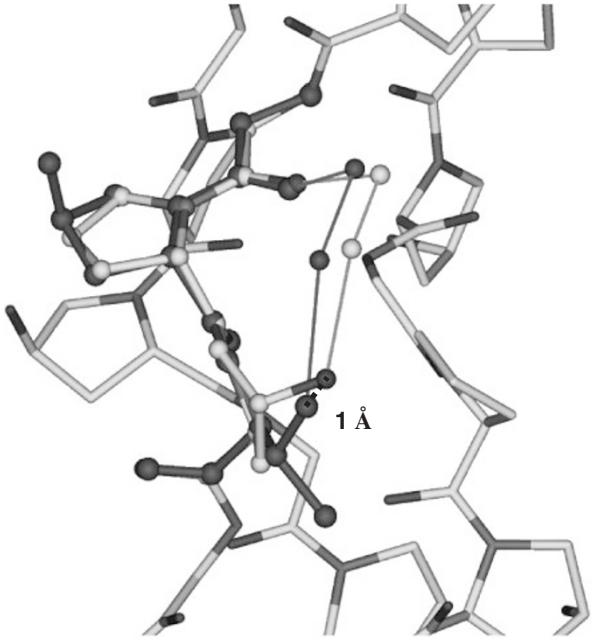


Figure 3. Superimposition of Thr in the OTG peptide (dark-gray) on the (Pro-4(*R*)Hyp-Gly)$_{11}$ triple-helix (light-gray).[16] The distance between OH groups of Thr and 4(*R*)Hyp in the Y position of two peptides is about 1 Å and both peptides show the similar hydration pattern. Gly-4(*R*)Hyp-Thr in the OTG and Gly-Pro-4(*R*)Hyp in the (Pro-4(*R*)Hyp-Gly)$_{11}$ are shown in ball and stick diagram.

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4(*R*)Hyp: 4(*R*)-hydroxyproline
O: 4(*R*)-hydroxyproline
Gal: galactose
T3-785 peptide: (Pro-Hyp-Gly)$_3$-Ile-Thr-Gly-Ala-Arg-Gly-Leu-Ala-Gly-Pro-Hyp-Gly-(Pro-Hyp-Gly)$_3$