Glycosylation/Hydroxylation-induced stabilization of the collagen triple helix: 4-trans-hydroxyproline in the Xaa position can stabilize the triple helix

James G. Bann‡ and Hans Peter Bächinger‡§¶

From the ‡Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, and §Shriners Hospital for Children, Portland, Oregon 97201

Running Title: 4-Hyp in the Xaa position can stabilize the collagen triple helix

¶To whom correspondence should be addressed:
Shriners Hospital for Children
Research Department
3101 SW Sam Jackson Park Road
Portland, OR 97201
Tel.: 503-221-3433
Fax.: 503-221-3451
E-mail: hpb@shcc.org
Abstract

We have shown recently that glycosylation of threonine in the peptide Ac-(Gly-Pro-Thr)_{10}-NH_{2} with β-D-galactose induces the formation of a collagen triple helix, while the non-glycosylated peptide does not. In this report, we present evidence that a collagen triple helix can also be formed in the Ac-(Gly-Pro-Thr)_{10}-NH_{2} peptide, if the proline (Pro) in the Xaa position is replaced with 4-trans-hydroxyproline (Hyp). Furthermore, replacement of Pro with Hyp in the sequence Ac-(Gly-Pro-Thr(β-D-Gal))_{10}-NH_{2} increases the T_{m} of the triple helix by 15.7 °C. It is generally believed that Hyp in the Xaa position destabilizes the triple helix, since (Pro-Pro-Gly)_{10} and (Pro-Hyp-Gly)_{10} form stable triple helices but the peptide (Hyp-Pro-Gly)_{10} does not. Our data suggest that the destabilizing effect of Hyp relative to Pro in the Xaa position is only true in the case of (Hyp-Pro-Gly)_{10}. Increasing concentrations of galactose in the solvent stabilize the triple helix of Ac-(Gly-Hyp-Thr)_{10}-NH_{2}, but to a much lesser extent than achieved by covalently linked galactose. The data explain some of the forces governing the stability of the annelid/vestimentiferan cuticle collagens.
Introduction

The collagens of both vertebrates and invertebrates share the same characteristic repeating Gly-Xaa-Yaa tripeptide units that are required for the formation of the collagen triple helix. In vertebrates and most invertebrates, Xaa is often proline and Yaa is often 4-trans-hydroxyproline, and the thermal stability of a triple helix from a particular species increases as the total content of these imino acids increases (1). An exception to this correlation is the cuticle collagen of the deep-sea hydrothermal vent worm Riftia pachyptila. This collagen has a relatively high thermal stability ($T_m = 37 \degree C$), despite a low content of Pro/Hyp residues ($\sim 5\%$) (2). Amino acid analysis and partial sequencing of the cuticle collagen revealed that there is a high content of threonine residues, and that these are glycosylated, mainly with di- and tri-saccharides of galactose (3).

The observation of di- and tri-saccharides of galactose is consistent with the carbohydrate compositions of the cuticle collagens of Lumbricus terrestris (earthworm) and Nereis virens (clamworm). Also consistent is the positional specificity of Hyp, which for these collagens seems to primarily occur in the Xaa position rather than the Yaa position (4, 5). For the L. terrestris cuticle collagen, the sequence -Gly-Hyp-Ser- accounts for 4-5% of the total hydroxyproline content (5).

For the vertebrate collagens, 4-Hyp is found exclusively in the Yaa position, and studies of collagen-like peptides show that although the peptides (Pro-Pro-Gly)$_{10}$ and (Pro-Hyp-Gly)$_{10}$ formed stable triple-helices, the peptide (Hyp-Pro-Gly)$_{10}$ did not (6). From this study it was concluded that Hyp in the Xaa position does not contribute to the stability and is destabilizing to the triple helix. Indeed, even though the Hyp content of the L. terrestris cuticle collagen is high (16%-18%), the thermal stability is low ($T_m = 22 \degree C$) (7). Also, for the R. pachyptila cuticle
collagen, which has a low content of Pro/Hyp residues (~5%), it was suggested that glycosylation of Thr with galactose must compensate for the presence of Hyp in the Xaa position in order to achieve the high observed thermal stability (3).

We have recently shown that substitution of Thr by Thr-\(O-(\beta\)-D-galactose) in the peptide Ac-(Gly-Pro-Thr)\(_{10}\)-NH\(_2\) induces the formation of a collagen triple-helix, with the latter peptide having essentially a random coil structure in aqueous solution (8). In this report we extend these studies to investigate the replacement of Pro by Hyp in the Xaa position of the peptides Ac-(Gly-Pro-Thr)\(_{10}\)-NH\(_2\) and Ac-(Gly-Pro-Thr(\(\beta\)Gal))\(_{10}\)-NH\(_2\).

**Experimental Procedures**

*Peptide synthesis and purification.*

Peptides were synthesized using a Milligen 9050 peptide synthesizer. Couplings were carried out on a PAL-PEG-PS resin (Perseptive Biosystems, 0.16 mmol/g) using \(N\alpha\)-9-fluorenlymethoxycarbonyl (FMOC) amino acids (Fmoc-Gly-OH, Fmoc-Hyp(tBu)-OH, Perseptive Biosystems, 4.0 eq.) and (O-(7-azabenzotriazol-1-yl)-1.1.3.3.tetramethyluronium hexafluorophosphate (HATU) (Perseptive Biosystems, 4.0 eq.) mediated peptide couplings. The glycopeptide was synthesized using FMO\(C\)-\(O-(2,3,4,6\)-tetra-O-acetyl-\(\beta\)-D-galactopyranosyl)-Thr-OH, which was prepared according to Elofson, et al. (9). The peptides were cleaved from the resin and purified by semi-preparative high performance liquid chromatography (HPLC). Treatment of the glycopeptide with 6 mM sodium methoxide in methanol overnight removed the acetyl protecting groups on the sugar (10). The peptide was again purified by HPLC, and all peptides were characterized by amino acid analysis and MALDI-TOF mass spectrometry.
Circular Dichroism spectroscopy.

Circular dichroism spectra were recorded on an Aviv 202 spectropolarimeter using a Peltier thermostatted cell holder and a 1 mm pathlength rectangular cell. All measurements were performed in water, and peptide concentrations in all cases were 100 µM. Concentrations were determined by amino acid analysis. The wavelength spectra represent an average of 8 scans. Thermal transitions were recorded from 5°C to 70°C at 223 nm, and the temperature was raised at a rate of 12°C/h. The thermal transitions were fit to the all-or-none equation as described (11) in order to calculate the Tm, ΔH° (van’t Hoff) and ΔS°. D-Galactose was purchased from Sigma. A stock solution of galactose (0.41 g/ml) was prepared by dissolving galactose in water and measuring the optical rotation in a Perkin Elmer Model 241 MC polarimeter, using a 10 cm cell at room temperature. The concentration was determined based upon an [α]D value of +80.2 as listed in the Merck Index.

Analytical ultracentrifugation.

Equilibrium sedimentation experiments were carried out on a Beckman Model E analytical ultracentrifuge using double-sector cells. Peptides were dissolved in water to 0.5 and 0.3 mg/ml for the peptides Ac-(Gly-Hyp-Thr)10-NH2 and Ac-Gly-Hyp-Thr(βGal)10-NH2, respectively. The temperature was regulated to 4.3°C, and the wavelength for monitoring was at 230 nm. Weight-average molecular weights were determined by fitting the data using Scientist® (Micromath, Salt Lake City, Utah). Partial specific volumes of 0.71 cm³/g for the GZT peptide and 0.67 cm³/g for the GZT(βGal) peptide were calculated from the amino acid composition and galactose content.
(12). However no value has been published for hydroxyproline, so the value for proline was used.

**Differential scanning calorimetry.**

The temperature dependence of the partial heat capacity was measured in an MC-2 differential scanning calorimeter (MicroCal Inc., Northampton, MA). The heating rate was 13 °C/h and the data was collected and analyzed using the software provided by the manufacturer.

**Results and Discussion**

Previous evidence using circular dichroism (CD) spectroscopy showed that the peptide Ac-(Gly-Pro-Thr(β-D-Gal)₁₀-NH₂ formed a collagen triple helix while the peptide Ac-(Gly-Pro-Thr)₁₀-NH₂ could not (8). Figure 1A compares the CD spectra of Ac-(Gly-Pro-Thr)₁₀-NH₂ (8) to that of Ac-(Gly-Hyp-Thr)₁₀-NH₂ and in water at 5 °C. Only the latter peptide exhibits the CD spectral characteristics of a collagen triple helix, with a positive ellipticity [θ] at 223 nm and a large negative minimum at 198 nm (13). This ellipticity value at 198 nm for Ac-(Gly-Pro-Thr)₁₀-NH₂ (-30,000 deg cm² dmol⁻¹) is similar to what is observed for the peptide (Pro-Ser-Gly)ₙ ([θ]₁₉₈nm = -27,400 deg cm² dmol⁻¹) which, even at fairly high molecular weights (18,000 Da) exhibits a mostly random-coil structure (13). The temperature-dependence of the CD spectrum of Ac-(Gly-Hyp-Thr)₁₀-NH₂ is shown in figure 1B, and exhibits a highly cooperative transition with a Tₘ of 19.2 °C. Equilibrium sedimentation of the peptide in water gave a weight-average molecular weight of 9780 +/- 100 Da, which is slightly higher than the expected
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mass of the trimer (8315 Da), and is presumably due to an error in the calculated partial specific volume and/or the presence of a small amount of aggregates.

The far-UV CD spectra of Ac-(Gly-Hyp-Thr(βGal))_{10}-NH₂ is shown in figure 2A, along with the CD spectrum of Ac-(Gly-Pro-Thr(βGal))_{10}-NH₂, which has been reported recently (8). The overall shape of the spectra is the same, with the Hyp peptide having a slightly lower ellipticity at 198 nm and a slightly larger ellipticity at 220 nm. The inset shows the CD spectra at 70°C for both peptides, which is above their respective Tₘ (see figure 2B). The ellipticity values at 200 nm are similar, suggesting that the differences in the CD at 5 °C are not due to a concentration artifact. Figure 2B shows the dependence of the ellipticity at 223 nm as a function of temperature for both Ac-(Gly-Hyp-Thr(βGal))_{10}-NH₂ and Ac-(Gly-Pro-Thr(βGal))_{10}-NH₂. The Tₘ of the Gly-Hyp-Thr(βGal) peptide is 54.8 °C, while the Tₘ of the Gly-Pro-Thr(βGal) peptide is 39.2 °C. Equilibrium sedimentation of the former peptide gave a weight-average molecular weight of 12,650 +/- 100 Da, slightly less than the calculated mass of the trimer (13,150 Da), probably due to an error in the calculated partial specific volume and/or the presence of a small amount of monomeric chains.

In order to determine if galactose, by itself, could have a similar influence as having the galactose covalently linked to threonine, we measured the thermal stability of both Ac-(Gly-Pro-Thr)_{10}-NH₂ and Ac-(Gly-Hyp-Thr)_{10}-NH₂ in increasing concentrations of galactose in water (Figure 3A and B). Figure 3A shows the influence of galactose at 0, 0.5, 1.0 and 2.0 M on the CD spectra of Ac-(Gly-Pro-Thr)_{10}-NH₂ and Ac-(Gly-Hyp-Thr)_{10}-NH₂. Little difference in the CD of either peptide is observed at around 220 nm. For the peptide Ac-(Gly-Hyp-Thr)_{10}-NH₂, the Tₘ was measured by again monitoring the change in ellipticity at 223 nm as a function of temperature. A linear increase in Tₘ is observed as the concentration of galactose increases.
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(Figure 3B). We estimated of the effective concentration of galactose required to match that of having the carbohydrate covalently linked to threonine. Based upon the predicted dimensions from molecular modeling of the peptide Ac-(Gly-Pro-Thr(βGal))5-NHCH₃, the effective concentration is about 1.6 M. However, the Tₘ at 2 M galactose is only about 33 °C, compared to the near 55 °C Tₘ for Ac-(Gly-Hyp-Thr(βGal))₁₀-NH₂.

The above data show the favorable influence of glycosylation on the thermal stability of the triple-helix, as has been shown (8). Previous measurements on the effects of various sugars and polyols on the stability of the triple helix of calf-skin collagen suggested that the observed linear increase in Tₘ with increasing concentrations of sugars is due to an effect on the structure of water (14). The influence of various solvents on the stability of the peptide (Pro-Ser-Gly)ₙ was studied (15). Although (Pro-Ser-Gly)ₙ could not form a triple helix in water, this polypeptide had the propensity to form a triple helix, since in the solvent 1,3-propanediol the molecules exhibited the CD spectral features of a triple helix. A similar observation was made with the peptide H-(Gly-Pro-Thr)₁₀-Gly-Pro-Cys-Cys-OH, which in the solvent 1,2-propanediol also forms a triple-helix (3).

The ability of other solvents to stabilize the triple-helix was also investigated by Brown and coworkers, and it was found that both neat trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP) destabilize the triple-helix, whereas the polyhydric alcohols glycerol, ethylene glycol, diethylene glycol, 1,4-butanediol and 1,3-propanediol are stabilizing (15). It was suggested from these studies that the weaker hydrogen bonding solvents provide a more favorable environment than water in which to form the inter-chain hydrogen bonds. Presumably the effect of sugars and polyols would be manifested in a decrease in the activity of water and the ability of water to interact with the polypeptide backbone. Thus, peptide backbone-water hydrogen bonds would be
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disfavored relative to peptide-peptide hydrogen bonds. A possible mechanism of stabilization by O-glycosylation then is to change the local environment of the peptide backbone such that water-backbone interactions are disfavored, and the inter-chain hydrogen bonds become favored.

While the values for $\Delta H^0$ and $\Delta S^0$ for the galactosylated peptides are very similar to the values reported for (Pro-Pro-Gly)$_{10}$ and (Pro-Hyp-Gly)$_{10}$ (see Table 1), the transition curve for (Gly-Hyp-Thr)$_{10}$ is much steeper. This is reflected by a much larger enthalpy change for this peptide. This large change in enthalpy was confirmed by calorimetry. One possible explanation is that when threonine and hydroxyproline are contiguous, there is a strongly enthalpic interaction (probably a hydrogen bond) between the side-chain OH groups within a chain, between chains, or with water molecules. This interaction is compensated by a very large loss in entropy, possibly the side-chain movement of threonine or in the movement of water molecules (bound to the side-chains).

The stabilizing influence of Hyp in the Xaa position may be explained by either an increase in the number of stable hydrogen bonds with water (16) or through a stereoelectronic inductive effect (17). We favor the latter hypothesis, since based upon the water binding mechanism the peptide Gly-Pro-Thr (and Gly-Pro-Ser) should form a stable triple helix, as was suggested recently (18). The OH group of Hyp is effective at withdrawing electrons away from the imide bond, and results in a change in the equilibrium constant for cis $\leftrightarrow$ trans isomerization, favoring the trans isomer. Since all peptide bonds in the triple helix are trans, there is a cumulative increase in stability as the number of stable trans isomers increases. Thus, having Hyp in the Xaa position would increase the stability of the triple-helix by also increasing the number of trans isomers.
Why then does (Hyp-Pro-Gly)\textsubscript{10} not form a triple-helix? One possibility is that the stereoconfiguration of the proline ring in the Yaa position is altered if the Pro in the Xaa position is Hyp. The recent crystal structures (19, 20) show that the Pro in the Xaa position is in general puckered down, while the Hyp or Pro in the Yaa position is \textit{always} puckered up. This configuration in puckering between two contiguous prolines or with Pro-Hyp may not be favored if the Hyp is in the Xaa position, and may result in an upward puckering in the Xaa and a downward puckering for Pro in the Yaa. This could then in turn change the Xaa psi angle between the two proline residues, favoring a value other than around 160° as reported for collagen and collagen-like peptides (19, 20).

The presence of Hyp in the Xaa position for the cuticle collagens of \textit{L. terrestris}, \textit{N. virens} and \textit{R. pachyptila} suggest that a novel hydroxylating enzyme is present in these organisms that is distinct in specificity from the normal vertebrate prolyl-4-hydroxylase (21). Such an enzyme has been described from the subcuticular epithelium of \textit{L. terrestris} (22). From these studies, this enzyme would also be required for the normal stability of the triple helix of these collagens.
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Footnotes

This work was supported by a grant from Shriners Hospital for Children. We thank J. Gambee for help with peptide synthesis and amino acid analysis.

Figure Legends

**Figure 1.** A. The CD spectra of Ac-(Gly-Hyp-Thr)$_{10}$-NH$_2$ (solid line) and Ac-(Gly-Pro-Thr)$_{10}$-NH$_2$ (dotted line) in water at 5°C. B Melting transition curves for Ac-(Gly-Hyp-Thr)$_{10}$-NH$_2$ (solid line) and Ac-(Gly-Pro-Thr)$_{10}$-NH$_2$ (dotted line) recorded at 223 nm. Concentrations in both A and B were 100 µM.

**Figure 2.** A. The CD spectra of Ac-(Gly-Hyp-Thr-(O-β-D-galactose))$_{10}$-NH$_2$ (solid line) and Ac-(Gly-Pro-Thr-(O-β-D-galactose))$_{10}$-NH$_2$ (dotted line) in water at 5°C and at 70°C (inset). B Melting transition curves for Ac-(Gly-Hyp-Thr-(O-β-D-galactose))$_{10}$-NH$_2$ (solid line) and Ac-(Gly-Pro-Thr-(O-β-D-galactose))$_{10}$-NH$_2$ (dotted line) recorded at 223 nm. Concentrations in both A and B were 100 µM.

**Figure 3.** A. Dependence of galactose concentration on the far-UV CD spectra of Ac-(Gly-Hyp-Thr)$_{10}$-NH$_2$ (solid line) and Ac-(Gly-Pro-Thr)$_{10}$-NH$_2$ (dotted line) at 5°C, 100 µM peptide. The darker lines are the CD traces up to 2M galactose, and no change in the region near 220 nm is observed. B. Dependence of the melting curve transition on galactose concentration of Ac-(Gly-Hyp-Thr)$_{10}$-NH$_2$ (100 µM). A = 0 M, B = 0.5 M, C= 1.0 M, D = 2.0 M galactose.
Table 1

Comparison of thermodynamic parameters for the triple helix ⇄ coil transition of various peptides.

| Peptide                     | $T_m$ (K)$^a$ | $\Delta H^0_{\text{VH}}$ (kJ/mol) | $\Delta S^0$ (J/mol/K) | $\Delta H^0_{\text{cal}}$ (kJ/mol) | c (mM) | Ref  |
|-----------------------------|---------------|-----------------------------------|------------------------|------------------------------------|--------|------|
| (Pro-Pro-Gly)$_{10}$        | 297.8         | -7.91                             | -22.4                  | -7.68                              | 2.6    | 11   |
| (Pro-Hyp-Gly)$_{10}$        | 330.5         | -13.4                             | -36.4                  | -13.4                              | 2.4    | 11, 23 |
| (Gly-Pro-Thr($\beta$Gal))$_{10}$ | 312.0       | -14.0                             | -39.6                  | 0.1                                | this study |      |
| (Gly-Hyp-Thr($\beta$Gal))$_{10}$ | 323.2       | -11.1                             | -29.0                  | 0.1                                | this study |      |
| (Gly-Hyp-Thr)$_{10}$        | 291.2         | -27.1                             | -87.1                  | -27.5                              | 0.1    | this study |

$^a$ Melting temperatures $T_m$ are concentration dependent and are indicated for the concentration c. $\Delta H^0_{\text{VH}}$ and $\Delta S^0$ values were obtained by fitting the experimental data to equation (2) in reference 11. The thermodynamic parameters are expressed per mol tripeptide units in a triple helix.
Figure 1
Figure 2
Figure 3
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J. Biol. Chem. published online May 25, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M003336200

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