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Received 21 September 2000;  
accepted 6 December 2000

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## Contribution of Tertiary Amides to the Conformational Stability of Collagen Triple Helices

**Abstract:** The collagen triple helix is composed of three polypeptide strands, each with a sequence of repeating (Xaa–Yaa–Gly) triplets. In these triplets, Xaa and Yaa are often tertiary amides: L-proline (Pro) and 4(R)-hydroxy-L-proline (Hyp). To determine the contribution of tertiary amides to triple-helical stability, Pro and Hyp were replaced in synthetic collagen mimics with a non-natural acyclic tertiary amide: N-methyl-L-alanine (meAla). Replacing a Pro or Hyp residue with meAla decreases triple-helical stability. Ramachandran analysis indicates that meAla residues prefer to adopt  $\phi$  and  $\psi$  angles that are dissimilar from those of the Pro and Hyp residues in the collagen triple helix. Replacement with meAla decreases triple-helical stability more than does replacement with Ala. All of the peptide bonds in triple-helical collagen are in the trans conformation. Although an Ala residue greatly prefers the trans conformation, a meAla residue exists as a nearly equimolar mixture of trans and cis conformers. These findings indicate that the favorable contribution of Pro and Hyp to the conformational stability of collagen triple helices arises from factors other than their being tertiary amides. © 2001 John Wiley & Sons, Inc. *Biopolymers* 59: 24–28, 2001

**Keywords:** collagen triple helices; polypeptide strands; tertiary amides

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### INTRODUCTION

Collagen is the most abundant protein in animals. The tertiary structure of collagen is a right-handed helix composed of three chains, each resembling a polyproline type II helix. The primary sequence of each polypeptide chain is comprised of repeating Xaa–Yaa–Gly triplets, where Gly is glycine and Xaa and Yaa are often L-proline (Pro) and 4(R)-hydroxy-L-

proline (Hyp), respectively. Together, Pro and Hyp comprise nearly one fourth of the residues in type I collagen, which is the most common type in humans.<sup>1</sup> The abundance of these cyclic tertiary amides correlates with the conformational stability of the collagen triple helix.<sup>2,3</sup>

Pro–Hyp–Gly is the most common triplet in type I collagen.<sup>1</sup> Yet, Pro is not an especially common residue in other proteins, comprising 5.1% of all residues

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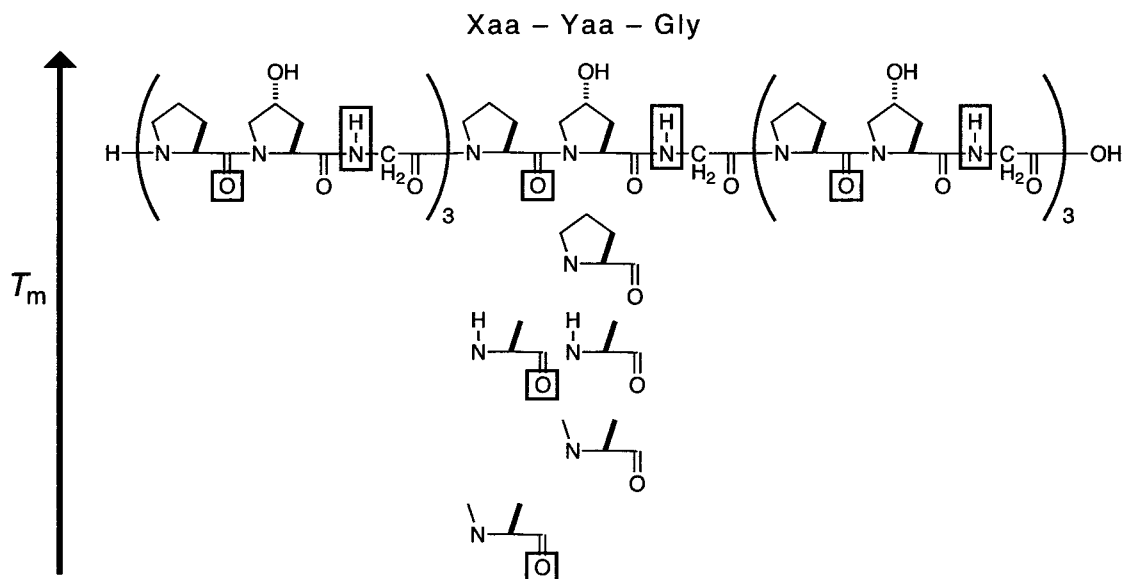
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Contract grant sponsor: NIH

Contract grant number: AR44276

*Biopolymers*, Vol. 59, 24–28 (2001)

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**FIGURE 1** Relative conformational stability of (Pro-Hyp-Gly)<sub>3</sub>(Xaa-Yaa-Gly)(Pro-Hyp-Gly)<sub>3</sub> triple helices in which the central triplets are (Pro-Hyp-Gly), (Pro-Pro-Gly), (Ala-Hyp-Gly), (Pro-Ala-Gly), (Pro-meAla-Gly), or (meAla-Hyp-Gly). Boxed atoms participate in interstrand hydrogen bonds in a collagen triple helix.

in known proteins.<sup>4</sup> In some proline-rich proteins, Nature seems to have chosen Pro simply because it is the only naturally occurring residue that forms a tertiary amide. For example, Src homology 3 (SH3) and WW domains retain high-specificity recognition for proline-rich ligands when proline in these ligands is replaced with the non-natural amino acid N-methylglycine (sarcosine; Sar).<sup>5</sup> In other words, proline is recognized by SH3 and WW domains because it lacks a main-chain NH. Inspired by this finding, we decided to probe the role of the prevalent tertiary amides in the conformational stability of collagen.

The effect of tertiary amides on collagen stability is unclear from previous studies. Peptides in which Sar has been substituted at the Yaa position, poly-(Gly-Pro-Sar), do not form stable triple helices.<sup>6,7</sup> But like Gly, Sar has much conformational flexibility.<sup>8</sup> In contrast, N-isobutylglycine (Nleu) has been used in place of Pro and Hyp without sacrificing stability. Both (Gly-Nleu-Pro)<sub>9</sub> and (Gly-Pro-Nleu)<sub>9</sub> form more stable triple helices than does (Gly-Pro-Pro)<sub>10</sub>, and Nleu is more stabilizing in the Xaa position than in the Yaa position.<sup>7,9</sup> Hydrophobic interactions between the Nleu side chain and the Pro ring contribute to this stability.<sup>10,11</sup>

Here, we use synthetic collagen mimics to reveal the contribution of tertiary amides to the conformational stability of collagen triple helices. Three residues, Pro, N-methyl-L-alanine (meAla), and L-alanine

(Ala) were placed in the Xaa and Yaa positions of the central triplet of a synthetic (Pro-Hyp-Gly)<sub>3</sub>(Xaa-Yaa-Gly)(Pro-Hyp-Gly)<sub>3</sub> peptide. Each peptide forms a triple helix. Thermal denaturation experiments reveal that meAla destabilizes the triple helix more than does Ala, and that meAla is more destabilizing in the Xaa position than in the Yaa position (Figure 1).

## MATERIALS AND METHODS

### Peptide Synthesis

Triple-helical peptides were prepared with the sequence (Pro-Hyp-Gly)<sub>3</sub>(Xaa-Yaa-Gly)(Pro-Hyp-Gly)<sub>3</sub> in which the (Xaa-Yaa-Gly) triplet was (meAla-Hyp-Gly), (Pro-meAla-Gly), (Ala-Hyp-Gly), (Pro-Ala-Gly), and (Pro-Pro-Gly). Peptides were prepared by solid-phase synthesis on a Perkin-Elmer/Applied Biosystems Model 432A synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Fmoc-protected amino acids (Nova-Biochem, San Diego, CA) were coupled to Fmoc-glycine Wang resin (Advanced ChemTech, Louisville, KY). The peptides were deprotected and cleaved from the resin with trifluoroacetic acid (TFA) containing triisopropylsilane (TIS; 2.5% v/v) and water (2.5% v/v), and then precipitated with diethyl ether.

Peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a Waters

system (486 detector, 510 pumps) and a Pharmacia C-18 semi-preparatory column. Peptides were eluted with a gradient of aqueous acetonitrile (5–40% v/v) containing TFA (0.1% v/v) at a flow rate of 3 mL/min. Purified peptides were obtained by collecting the major peak with absorbance at 215 nm, which occurred between 17 and 19% v/v acetonitrile. HPLC-purified peptides were analyzed by electrospray mass spectrometry using a PE Sciex API 365 triple quadrupole with an ionspray source, or by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using a Bruker REFLEX II (Billerica, MA) equipped with a 337 nm laser, reflectron, and delayed extraction.

### Triple Helix Formation

Triple helices were formed by incubating peptides in 50 mM acetic acid (0.2 mM final concentration) for 24 h at 4°C. Concentrations of peptides were determined by measuring absorbance at 214 nm ( $\epsilon = 4.48 \times 10^4 M^{-1} \text{ cm}^{-1}$ ). Collagen has a characteristic CD spectrum that contains a peak at 225 nm. Triple helix formation was assessed at 5°C by CD spectrometry on an Aviv 62A DS or an Aviv 202 SF instrument, both of which are equipped with an automated temperature controller.

### Thermal Denaturation

Values for  $T_m$  for each triple helix were determined in triplicate by thermal denaturation experiments monitored by CD spectroscopy on an Aviv 62A DS or an Aviv 202 SF instrument, both equipped with an automated temperature controller. Ellipticity at 225 nm was monitored as the temperature was increased from 5 to 50°C in 3°C increments with a 3- or 5-min equilibration time at each temperature. As the temperature was increased, the ellipticity decreased. Data were fitted to a two-state model for unfolding to determine the value of  $T_m$ , which is the temperature at the midpoint of the thermal transition.

## RESULTS AND DISCUSSION

The  $\gamma$  carbon of proline is particularly fateful for the conformational stability of triple-helical collagen. Electron-withdrawing substituents on the  $\gamma$  carbon can increase stability. For example, a (Pro-Hyp-Gly)<sub>10</sub> triple helix has a  $T_m$  of 58°C, whereas a (Pro-Pro-Gly)<sub>10</sub> triple helix has a  $T_m$  of only 24°C.<sup>12</sup> Replacing the hydroxyl group of Hyp with fluorine, the most electronegative atom, increases further the conformational stability of triple-helical collagen.<sup>13,14</sup> An alternative approach to revealing the role of  $C_\gamma$  in collagen stability is to remove it. L-Azetidine-2-carboxylic acid (Aze) is a Pro analogue that lacks a carbon, as it has a four-membered ring. Both poly-

**Table I** Values of  $T_m$  for Synthetic (Pro-Hyp-Gly)<sub>3</sub>(Xaa-Yaa-Gly)(Pro-Hyp-Gly)<sub>3</sub> Triple Helices

Xaa-Yaa-Gly	$T_m$ (°C) <sup>a</sup>
Pro-Hyp-Gly <sup>b</sup>	36 ± 2
Pro-Pro-Gly	30.5 ± 2.2
Ala-Hyp-Gly	26.1 ± 0.7
Pro-Ala-Gly	25.0 ± 1.0
Pro-meAla-Gly	21.7 ± 0.8
meAla-Hyp-Gly	17.5 ± 1.6

<sup>a</sup> Values of  $T_m$  were determined by CD spectroscopy for peptides (0.2 mM) in 50 mM acetic acid, and are the average ( $\pm$ SE) of at least 3 determinations.

<sup>b</sup> From Ref. 21.

(Gly-Pro-Aze) and poly(Gly-Aze-Pro) form less stable triple helices than does poly(Gly-Pro-Pro).<sup>15</sup> Here, we take a more subtle approach to removing  $C_\gamma$  while retaining a tertiary amide—replacing Pro with meAla.

Five triple-helical collagen-like peptides were synthesized by solid-phase methods, and the integrity of each was confirmed by mass spectrometry (data not shown). There was no evidence of diketopiperazine formation, based on HPLC analysis and mass spectrometry. (Xaa-Yaa-Gly) triplets of the sequence (meAla-Hyp-Gly), (Pro-meAla-Gly), (Ala-Hyp-Gly), (Pro-Ala-Gly), and (Pro-Pro-Gly) were introduced into the middle of a (Pro-Hyp-Gly)<sub>3</sub>(Xaa-Yaa-Gly)(Pro-Hyp-Gly)<sub>3</sub> peptide. CD spectroscopy indicated that each peptide formed a triple helix at low temperatures (data not shown). At a concentration of 0.2 mM in 50 mM acetic acid, the CD spectrum for each peptide contained the positive peak at 225 nm that is characteristic of the collagen triple helix.

With increasing temperature, the ellipticity of a collagen triple helix at 225 nm decreases as the concentration of the triple helix decreases. The resulting  $T_m$  value provides an indication of the conformational stability of a triple helix. The values of  $T_m$  for the five (Pro-Hyp-Gly)<sub>3</sub>(Xaa-Yaa-Gly)(Pro-Hyp-Gly)<sub>3</sub> triple helices are affected significantly by the central triplet, increasing in the order: (meAla-Hyp-Gly) < (Pro-meAla-Gly) < (Ala-Hyp-Gly), (Pro-Ala-Gly) < (Pro-Pro-Gly) (Table I; Figure 1). All five of these triple helices have a  $T_m$  significantly lower than that of (Pro-Hyp-Gly)<sub>7</sub>. Substitution of meAla is highly destabilizing in both the Xaa and Yaa positions, and is more destabilizing in the Xaa position. Ala substitution is also destabilizing, in agreement

with previous studies,<sup>3</sup> but less so than meAla substitution. There is no significant difference in  $T_m$  between Ala substitution in the Xaa vs Yaa position. Finally, a (Pro–Pro–Gly) triplet destabilizes the (Pro–Hyp–Gly)<sub>7</sub> triple helix, but less so than a triplet containing either Ala or meAla.

Why is meAla so destabilizing to a collagen triple helix? Instability could result from the unfavorable conformation of meAla. Structural data have shown that the average values for the dihedral angles of Pro in the (Pro–Hyp–Gly)<sub>10</sub> triple helix are  $\phi = -72^\circ$  and  $\psi = 161^\circ$ , and of Hyp in a (Pro–Hyp–Gly)<sub>10</sub> triple helix are  $\phi = -58^\circ$  and  $\psi = 152^\circ$ .<sup>16</sup> Empirically derived conformational energy maps for N-methylated analogues of three tripeptide hormones show that N-methyl substitution greatly restricts the conformational freedom of the  $\phi$  and  $\psi$  dihedral angles.<sup>17</sup> Ramachandran plots indicate that the most favorable dihedral angles for N-methylated residues in the tripeptides are approximately  $\phi = -130^\circ$  and  $\psi = 70^\circ$ . These preferred angles are far from those of Pro and Hyp in a collagen triple helix. In addition, the average  $\phi$  and  $\psi$  values for Pro and Hyp in collagen lie completely outside the broad low-energy region (bounded by the 10 kcal/mol contour) for N-methylated residues. In comparison to meAla, Ala has a broad low-energy region in tripeptides.<sup>17</sup> Although the most favorable dihedral angles for Ala are approximately  $\phi = -80^\circ$  and  $\psi = 80^\circ$ , the average  $\phi$  and  $\psi$  angles for Pro and Hyp in (Pro–Hyp–Gly)<sub>10</sub> lie well within the energy contour bounded by 10 kcal/mol.

Substitution of meAla decreases the stability of the triple helix more than does substitution of Ala. In part, the difference may be due to the tendency of meAla to adopt a *cis* peptide bond conformation. All of the peptide bonds in the collagen triple helix are in the *trans* conformation, and *cis* to *trans* isomerization is the rate-limiting step in the folding of a triple helix.<sup>18</sup> Although Ala exists almost exclusively in the *trans* conformation, meAla exists predominantly in the *cis* conformation, at least in organic solvents.<sup>19</sup> These studies provide an explanation for our finding that meAla is more destabilizing to the collagen triple helix than is Ala. Interestingly, a Pro–Sar dipeptide has a lower percentage of *cis* conformers (15–35%) than does a Pro–meAla dipeptide.<sup>19</sup> N-Substituted glycine residues (such as Nleu) might therefore be less destabilizing than meAla due to a lower tendency to adopt the *cis* conformation.

The hydrogen-bonding pattern in triple-helical collagen is strictly conserved between the amide N–H of glycine and the amide oxygen of the Xaa residue.<sup>16,20</sup> It is reasonable that meAla substitution in the

Xaa position causes conformational changes that weaken the interchain hydrogen bonds more than does substitution in the Yaa position. This reasoning can explain meAla substitution being significantly more destabilizing in the Xaa position ( $T_m = 17.5^\circ\text{C}$ ) than in the Yaa position ( $T_m = 21.7^\circ\text{C}$ ). Weaker hydrogen bonds caused by meAla substitution in the Xaa position would translate into a less stable triple helix.

## CONCLUSION

In collagen, unlike in the ligands of SH3 and WW recognition domains, a tertiary amide is not enough. The conformational stability of collagen relies on more than the mere presence of tertiary amides. Instead, the conformational restrictions imposed by the pyrrolidine ring of Pro and Hyp are critical for the structural integrity of the collagen triple helix.

We are grateful to C. L. Jenkins and K. M. Taylor for advice. EAK was supported by a WARF predoctoral fellowship and Biotechnology Training Grant GM08349 (NIH). CD data were obtained at the University of Wisconsin—Madison Biophysical Instrumentation Facility, which is supported by the University of Wisconsin—Madison and grant BIR-9512577 (NSF). This work was supported by grant AR44276 (NIH).

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