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General Solution for Stabilizing Triple Helical Collagen

Yitao Zhang, Madison Herling, and David M. Chenoweth*

Department of Chemistry, University of Pennsylvania, 231 South 34th Street, Philadelphia, Pennsylvania 19104-6323, United States

Supporting Information

ABSTRACT: One of the most ubiquitous stabilizing forces in nature is the hydrogen bond, exemplified by the folded secondary, tertiary, and higher-order structure of biomolecules. Despite the fundamental importance of hydrogen bonding, dependence on this stabilizing force places limitations on nature's proteinogenic building blocks. Herein, we demonstrate that replacement of the strictly conserved glycine in collagen with aza-glycine has profound consequences on the stability and self-assembly of collagen peptides by providing an extra hydrogen bond donor. The additional hydrogen bond provided by aza-glycine allows for complete replacement of glycine residues in collagen peptides and truncation to the smallest self-assembling collagen peptide systems observed to date. Our results highlight the vital importance of hydrogen bonding at desolvated interfaces, providing a new strategy for optimization of designed peptide materials and a general solution for stabilizing the collagen triple helix.

Collagen is the most prevalent protein in the animal kingdom, serving as a fibrous material in nature with diverse structural applications in a broad array of biomaterials ranging from the hair, bone, and skin of humans to the gelatinous bodies of jellyfish and sea anemones.[1] Collagen has remained the subject of intense research for decades due to its importance as a biomaterial and its involvement in human disease.[2] The most basic structural element of collagen is the right-handed triple helix composed of three parallel, left-handed polypeptide chains that mimic the polyproline II helix.[3] The component chains of collagen may be described as repeating units of XaaYaaGly, where Xaa and Yaa are variable, with Gly remaining strictly conserved. Glycine mutations severely disrupt the structural integrity of the collagen triple helix and underscore the molecular basis for several debilitating diseases such as osteogenesis imperfecta and various forms of achondrogenesis.[4] Variation in the Xaa and Yaa positions of the repeat collagen sequence provide a diverse array of recognition motifs for many collagen binding proteins of vital importance.[5] The most frequently encountered amino acids in the Xaa and Yaa positions are (2*S*)-proline and (2*S*,4*R*)-hydroxyproline (Hyp), respectively.[3]

Many elegant studies have focused on elucidating the fundamental forces responsible for stabilizing the collagen triple helix as well as introducing synthetic modifications to enhance its structural properties.[3,6,7] Side chain modifications using unnatural amino acids have been used to modulate or enhance the stability of the collagen triple helix structure.[8] In contrast, backbone modifications typically result in a destabilized triple helix,[9] although this notion has been challenged by recent findings. In 2015, our group reported that the stereodynamic aza-proline residue could replace proline in the Xaa position.[10] The Raines group recently discovered that incorporation of a thioamide in a Yaa proline residue stabilizes the collagen triple helix, possibly due to enhanced interchain hydrogen bonding.[11] Recently, we reported that replacing a single α-carbon of glycine with a nitrogen atom (substituting glycine for aza-glycine) in a collagen model peptide (CMP) leads to significantly enhanced triple helix stability (Figure 1, top).[12] Our molecular dynamics calculations pointed toward the possibility of a new hydrogen bond from aza-glycine to one of two carbonyls on adjacent peptide strands or a bifurcated hydrogen bond to both, in

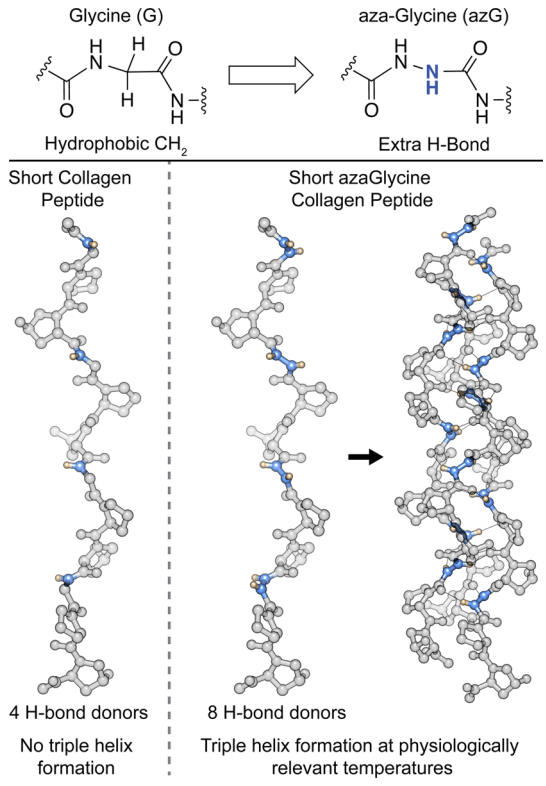

Figure 1. (Top) Illustration of the aza-glycine (azG) substitution. The new -NH hydrogen bond donor is shown in blue. (Bottom) Three-dimensional representation of a $(GPO)_4$ CMP (left) compared to the azG-substituted analogue, $(azGPO)_4$ (right). The all azG-containing peptide spontaneously assembles into a triple helix at physiologically relevant temperatures, while the short unaltered peptide remains single stranded (this work).

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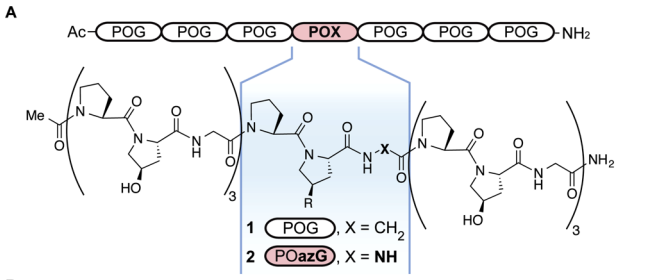
addition to the canonical cross-strand hydrogen bonds already present in collagen.[10,12] In our previous report, a single aza-glycine (azGly or azG) substitution was placed at the central location of a 21-mer CMP, providing the first favorable glycine replacement in collagen. Despite this result, the generality of aza-glycine as a stabilizing residue has not been demonstrated, and many important questions regarding aza-glycine incorporation linger. For instance, elucidation of the positional preference for azGly substitution and the impact of multiple azGly substitutions remain unresolved.

Herein, we define the impact of azGly incorporation on collagen triple helix self-assembly by answering the following three important, yet unresolved questions: First, what are the effects of azGly positioning along the collagen peptide chain? Second, what is the effect of incorporating multiple azGly residues and the impact of spacing between adjacent azGly residues? Third, what is the effect of replacing all glycine residues with azGly and what is the minimum peptide length necessary for self-assembly?

To investigate whether there is a positional effect of azGly incorporation, CMPs **3**–**7** were obtained via solid-phase peptide synthesis (SPPS) (Figure 2A,B top). Purified peptides were incubated in phosphate buffered saline (PBS) for at least 24 h.



B

CMP	CMP sequence	T_m	ΔT_m
1	Ac-POG POG POG POG POG POG POG-NH_2	40	–
3	Ac-POazG POG POG POG POG POG POG-NH_2	46	+6
4	Ac-POG POazG POG POG POG POG POG-NH_2	48	+8
5	Ac-POG POG POazG POG POG POG POG-NH_2	50	+10
2	Ac-POG POG POG POazG POG POG POG-NH_2	51	+11
6	Ac-POG POG POG POG POazG POG POG-NH_2	49	+9
7	Ac-POG POG POG POG POG POazG POG-NH_2	47	+7

CMP	CMP sequence	T_m	ΔT_m
8	Ac-POG POazG POG POG POG POazG POG-NH_2	57	+17
9	Ac-POG POG POazG POG POazG POG POG-NH_2	59	+19
10	Ac-POG POG POG POazG POazG POG POG-NH_2	61	+21
11	Ac-POG POazG POG POazG POG POazG POG-NH_2	69	+29
12	Ac-POG POG POazG POazG POazG POG POG-NH_2	74	+34

CMP	# aa's	CMP sequence	T_m	ΔT_m
13	9	Ac-azG POazG POazG PO-NH_2	–	–
14	12	Ac-azG POazG POazG POazG PO-NH_2	36	-4
15	15	Ac-azG POazG POazG POazG POazG PO-NH_2	78	+38

Figure 2. (A) General structure of CMPs composed of multiple units of Pro-Hyp-Gly/azGly. (B) Schematic structures of CMPs **1**–**15**, along with experimentally determined melting temperatures (T_m). Values of T_m were determined in triplicate at scan rates of 12 °C/h. All values of ΔT_m displayed to the right of T_m values are calculated relative to the all POG-containing control peptide **1**. Schematic presentation of the CMP sequences where ellipsoids represent triplet repeats. P = (2*S*)-proline, O = (2*S*,4*R*)-hydroxyproline, and azG = *aza*-glycine. The trimeric state of CMP **14** and CMP **15** was confirmed by SEC-MALS and AUC (see Supporting Information).

Circular dichroism (CD) spectra of aqueous solutions of mono-azGly peptides **3**–**7** all exhibit characteristic local maxima at approximately 224 nm, commonly associated with triple-helical structure. Thermal denaturation experiments showed cooperative unfolding transitions for all peptides, as observed for control peptide **1** and peptide **2** (see SI for CD spectra). The CD data were fitted to a two-state model as previously described.[13,14] The melting temperatures (T_m), at which 50% of the triple helix unfolds, together with ΔT_m are listed next to the peptides in Figure 2. A general stabilization was observed in peptides **2**–**7** (Figure 2B, top), as azGly afforded enhanced stability at all positions investigated in these peptides. Furthermore, it is evident that azGly stabilizes the triple helix more when it is closer to the central position, with peptide **2** being the most stabilized.

Next, we synthesized peptides **8**–**12** to determine the effect of multiple azGly substitutions on the thermal stability of triple helical CMPs (Figure 2B, middle). Confirmation of triple helix self-assembly, thermal denaturation experiments, and curve fitting were carried out as described for **2**–**7** (see SI). Increases in T_m of 17–21 °C relative to **1** were observed for disubstituted peptides **8**, **9**, and **10**; while increases of 29 and 34 °C were observed for trisubstituted peptides **11** and **12**. For ease of comparison, the ΔT_m value relative to control peptide **1** is shown next to the T_m of each peptide in Figure 2. Surprisingly, an extra 1–2 °C of stabilization beyond simple additivity is observed for di-azGly peptides **8**–**10**, while an extra 3 or 4 °C of stabilization beyond additivity is seen in tri-azGly peptides **11** and **12**. Despite this nonadditivity, it should be noted that ΔT_m values are not always linearly related to folding free energy values, especially when comparing peptides with large ΔT_m differences. The amount of extra thermal stability seems to depend on the number of azGly residues introduced into the peptides and their relative proximity, with enhanced stabilization observed for central positioning. The effect is clearly seen in peptide **12** where the combined effect of incorporating three azGly residues leads to an increase in T_m of 34 °C over that of control peptide **1** and 5 °C over that of skip-spaced tri-azGly peptide **11** (Figure 2).

We attribute the extra stability conferred by additional azGly insertion to a cumulative and synergistic H-bond network positioned at the desolvated interface of the three collagen peptide strands. Each azGly residue adds one extra hydrogen bond donor capable of interaction with proximal carbonyls on adjacent peptide strands. In addition to adding an extra hydrogen bond, aza-glycine could strengthen the existing hydrogen bond and provide extra stabilization by virtue of increased preorganization due to restricted N–C(O) bond rotation. The increased preorganization may be reflected in the faster refolding times observed for azGly-containing CMPs. A bathochromic shift in λ_{max} of 1–2 nm and a decrease in CD ellipticity at the positive maximum are observed as the number of azGly residues increases (Table S1). In addition, there is a significant shift in the negative CD band to higher wavelengths upon incorporation of multiple azGly residues. To further probe the impact of aza-glycine we replaced all glycine residues in a series of collagen model peptides as discussed below and shown in Figure 2.

Our observation that peptides with multiple azGly insertions exhibit a cooperative stabilizing effect provided the unique opportunity for replacing all glycine residues in a CMP with azGly, in an effort to define the minimal length peptide capable of triple helix self-assembly. Peptides **13**–**15** were synthesized via sequential coupling of the protected peptide trimer Fmoc-azGly-Pro-Hyp(*t*Bu)-OH on a solid phase (see SI). Peptide **15** contains 15 amino acid residues, five of which are azGly. CD scanning

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experiments reveal a clear shift to higher wavelength for the overall CD signals of all azGly-containing peptides and an increase in the strength of the negative band characteristic of a collagen triple helix (see Figure S4). Variable-temperature CD scanning experiments were performed on peptide **15** (Figure S5). Thermal transition experiments were monitored at 210, 215, 220, and 225 nm. Cooperative melting transitions were observed at all wavelengths monitored (Figure 3A, 3B, and Figure S6). The

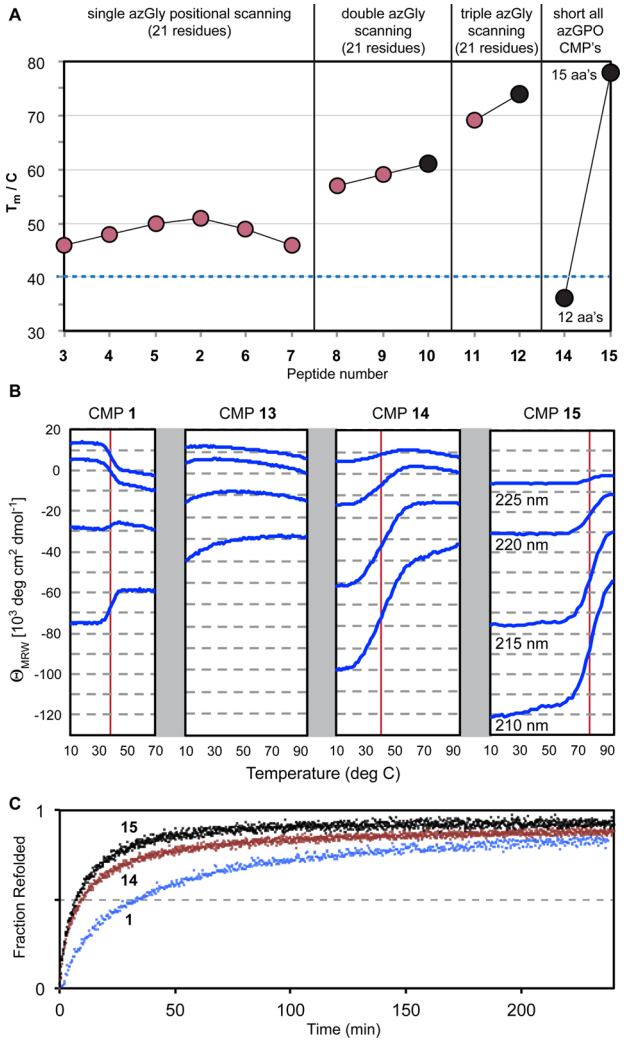

Figure 3. (A) Summary chart of the T_m values for all peptides. The dotted blue line indicates the T_m of collagen model peptide **1**. Peptides are grouped by number of azGly substitutions. (B) Representative unfolding data for CMPs **1**, **13**, **14**, and **15**, as monitored at 210, 215, 220, and 225 nm (in order from bottom to top) by CD spectroscopy. Approximate melting temperatures are indicated by red lines. (C) Kinetic refolding of peptides **1**, **14**, and **15** observed by monitoring the recovery of ellipticity after thermal denaturation using CD spectroscopy. The trimeric state of CMPs **14** and **15** was confirmed by SEC-MALS and AUC (see SI).

midpoint of the melting curve of **15** was determined to be 78 °C. Similar CD thermal transition experiments were performed on CMPs **13** and **14**, all-azGly-containing peptides with nine and twelve residues, respectively. While CMP **13** maintained a single-strand conformation, compound **14** showed a sigmoidal unfolding curve with a T_m of 36 °C (Figure 3B). Compared to CMP **15**, the large thermal shift in the cooperative unfolding curve clearly indicates the presence of a stable, higher-order structure that is highly dependent on peptide length. Peptide **13** serves as an all azGly-containing CMP single strand control that lacks a cooperative unfolding curve. To further define the higher-order structure of CMP **14** and **15** we conducted size exclusion chromatography-multi-angle light scattering (SEC-MALS) and analytical ultracentrifugation (AUC) analysis (see SI). The results from both methods clearly indicate that CMPs **14** and **15** form trimeric structures consistent with triple helical CMPs.

The melting temperatures of all peptides used in this study are plotted on the same temperature scale in Figure 3A with their peptide lengths indicated. Incorporating three azGly residues (CMPs **11** and **12**) provides triple helical 21-mer CMPs with T_m values up to 74 °C. Incorporating five azGly residues and truncating the CMP to 15 residues (CMP **15**) raises the T_m even higher to 78 °C. This represents the shortest CMP to date with the highest triple helical thermal stability. Decreasing the peptide length to 12 residues (CMP **14**) represents the shortest peptide sequence to date that is capable of triple helix self-assembly at a physiologically relevant temperature. Interestingly, the positive bands at 224 nm in the CD spectra of the triple helical assemblies decrease as a function of increasing azGly residues. This effect can be seen with mono-azGly peptides **3**–**7**, where there is a slight decrease in the positive CD band at 224 nm. The di-azGly-containing peptides **8**–**10** show a more substantial decrease in the 224 nm peak and this is even more pronounced in the tri-azGly-containing peptides **11** and **12** (see SI). The most dramatic example of the perturbation in the positive CD band can be seen in the spectra of the all-azGly-containing peptides **14** and **15**, where the positive CD band at 224 nm is completely absent and there is instead a more pronounced and red-shifted negative CD band. Peptides **14** and **15** are both trimeric as determined by SEC-MALS and AUC analysis (see SI) and both undergo cooperative unfolding transitions (Figure 3b and SI).

To assess the kinetics of triple helix self-assembly for fully azGly-substituted peptides, a refolding CD experiment was performed on CMPs **14** and **15**. The two peptides, at concentrations of 0.2 mM in PBS, were denatured at 95 °C for 15 min, and their CD signals were monitored at 4 °C until both peptides recovered >50% ellipticity at 215 nm. Raw CD data were normalized into fraction refolded plots (Figure 3C) from which half-refolding times ($t_{1/2}$), the times at which 50% of each peptide recovered triple helicity, were obtained. The $t_{1/2}$ values determined for **14** and **15** are 8 ± 2 and 6 ± 1 min, respectively. Compared to the $t_{1/2}$ of the natural control peptide **1**, 24 ± 6 min, compounds **14** and **15** exhibit much faster folding kinetics. Taken together, our results demonstrate that aza-glycine is a general solution for stabilization of triple helical collagen model peptides.

In summary, we have defined the impact of azGly incorporation on collagen triple helix self-assembly by addressing three key questions. First, we demonstrated that azGly has a positional preference with respect to added thermal stability when introduced into CMPs, with a slight preference for central substitution over N- or C-terminal substitution. Second, the inclusion of multiple azGly residues in CMPs leads to synergistic stabilization of the triple helical form. Third, peptides with all glycine residues replaced by azGly result in the most stable self-assembled collagen peptides reported to date. Notably, a 12-mer peptide can now be self-assembled into a defined protein tertiary structure. In addition to elucidating the fundamental importance of hydrogen bonding in natural systems, these studies may provide insight into diverse areas ranging from self-assembling materials and drug design to catalysis and synthetic receptors as

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we move toward more non-natural, biomimetic "foldamer" architectures.[15] The unique CD signature for the trimeric form of the azGly-containing collagen peptides raises interesting questions regarding their structure and conformation. Do azGly collagen peptides form collagen-like triple helix assemblies or some new trimeric structure? Are these assemblies hydrogen bonded at their interfaces like collagen triple helix assemblies, and do they form extra hydrogen bonds as a result of the azGly residues? Do the azGly collagen peptides tolerate other common residues found in collagen triple helix assemblies? Do azGly collagen peptides recognize common collagen-binding proteins and how do they interact in biological settings? Alternative collagen peptide sequences and structural investigations will be needed to answer these exciting questions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b03823.

General methods, synthesis of small molecules, synthesis of collagen model peptides, CD spectra, NMR spectra, and circular dichroism experimental protocols (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*dcheno@sas.upenn.edu

Notes

The authors declare no competing financial interest.

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