Incorporation of azapeptoid residues into collagen

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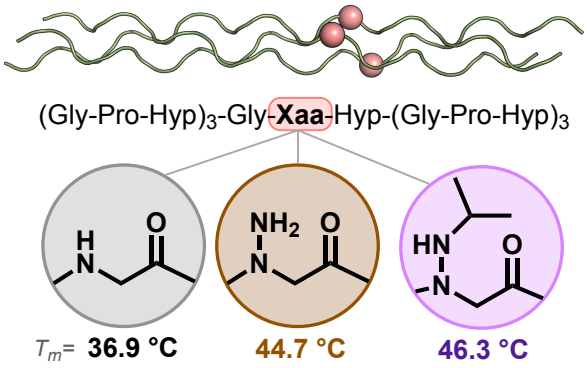


ABSTRACT

Collagen, the major structural protein in connective tissue, adopts a right-handed triple helix composed of peptide chains featuring repeating Gly-Xaa-Yaa tripeptide motifs. While the cyclic residues proline (Pro) and hydroxyproline (Hyp) are prevalent in the Xaa and Yaa positions due to their PPII-favoring conformational properties, diverse acyclic peptoid (N-alkylated Gly) residues can also stabilize the collagen fold. Here, we investigated the effects of N-aminoglycine (aGly) and its N′-alkylated derivatives – so-called 'azapeptoid' residues – on the thermal stability of collagen mimetic peptides (CMPs). Substitution of Pro at the central Xaa11 position with aGly resulted in destabilization of the triple helix, yet introduction of select N′-alkyl groups (isopropyl, butyl) partially restored thermal stability. Moreover, the N-amino group of azapeptoid residues enhanced thermal CMP stability relative to an unsubstitued Gly analogue. Kinetic studies revealed that the introduction of the hydrazide bonds in aGly and (iPr)aGly CMPs did not significantly impact triple helix refolding rates. Their modular late-stage derivatization and tunable properties highlight azapeptoid residues as potentially valuable tools for engineering CMPs and probing the structural determinants of collagen folding.

1. INTRODUCTION

Collagen, the principle proteinaceous component of connective tissue in mammals, is a right-handed triple-helical assembly of peptide chains that each adopt polyproline II (PPII) secondary structure.[1,2] Collagen is characterized by repeating Gly-Xaa-Yaa tripeptide subunits, where Xaa and Yaa are commonly proline (Pro) or *trans*-4-hydroxyproline (Hyp).[3] Although other residues are frequently encountered at these positions, the pyrrolidine ring in Pro and Hyp stabilizes the collagen fold by favoring PPII backbone ϕ and ψ dihedral angles (–60° and +150°).[4,5] However, the tertiary amide bonds formed by Pro and Hyp also exhibit an enhanced propensity to adopt a *cis* geometry relative to other canonical residues. Given that collagen and other PPII folds require all-*trans* amide geometry (ω = 180°), significant effort has been devoted to the design of unnatural proline analogues with increased *trans* rotamer bias.[3,6–11] While the *trans* propensity of monosubstituted proline derivatives generally correlates with collagen stability, pyrrolidine ring puckering also plays an important role in conformational pre-organization.

N-Alkylated Gly (peptoid) residues represent an interesting class of Pro surrogates that are devoid of cyclic ϕ constraint and, like Pro, form tertiary amide bonds when incorporated into peptides. The combination of enhanced *cis* amide population and potentially increased backbone flexibility would appear to be detrimental to collagen folding. However, Goodman and coworkers demonstrated that replacement of 9 Pro (Yaa) residues with N-isobutylglycine (Nleu) afforded a triple-helical collagen mimetic peptide (CMP) with increased thermal stability.[12] More recently, several other peptoid residues were shown to stabilize the collagen triple helix, thus greatly expanding the chemical diversity possible in the design of folded CMPs.[13,14] The stabilizing effect of peptoid residues was attributed to their strong preference for PPII ϕ and ψ torsions despite an increase in *cis* amide bond propensity.

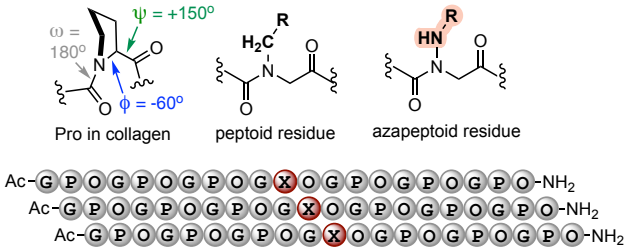


Figure 1. Residue substitutions in a collagen mimetic peptide (X = Xaa11 substitution site,G = Gly, P = Pro, O = Hyp).

Our interest in backbone N-heteroatom-substituted peptides prompted us to explore the impact of N-aminoglycine (aGly) and its N'-alkylated derivatives on collagen folding.[15] These 'azapeptoid' residues feature an NHR backbone amide substituent in place of the CH_2R group found in peptoids (Figure 1). We previously demonstrated that aGly and (N'-isobutyl)aGly promote PPII conformation in a single-strand folding model.[16] Here, we asked whether azapeptoid residues would be similarly well-tolerated in a triple-helical CMP. Our results show that, unlike several peptoid residues, incorporation of aGly into the Xaa position of collagen destabilizes the collagen fold relative to Pro. However, late-stage N'-alkylation with select substituents restores some degree of thermal stability. Moreover, backbone amide substitution of a Gly residue in the Xaa position with amino and alkylamino groups results in a significant increase in melting temperature.

2. MATERIALS AND METHODS

2.1. Fmoc-based solid-phase peptide synthesis (SPPS). Automated SPPS carried out on a CEM Liberty Blue peptide synthesizer or a PurePrep Chorus peptide synthesizer using ProTide Protide Rink amide MBHA resin (100–200 mesh, 0.63 mmol/g, 0.1 mmol scale). The following derivatives suitable for Fmoc SPPS were used: Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Hyp(*t*Bu)-OH, and H-a(Boc)Ala-OH.[17] Fmoc deprotection steps were carried out by treating the resin with a solution of 20% (w/v) piperidine/DMF at room temperature (5 min), then at 75 °C (2 min). After Fmoc deprotection, the resin was washed with DMF 4×. Coupling of Fmoc-protected building blocks was achieved using 5 equiv HCTU (0.25 M in DMF), 10 equiv NMM (1 M in DMF), and 5 equiv of Fmoc protected amino acid or H-(Boc)aAla-OH[17] (0.2 M in DMF) at 50 °C (10 min) then at 75 °C (5 min). Deprotection and coupling steps were repeated until peptide synthesis was complete and then a final Fmoc deprotection of the N-terminus. The resin was transferred to a suitable vessel, washed with DCM (5 mL × 4) and dried under vacuum. Peptides were N-terminally acetylated using 5% acetic anhydride and 10% pyridine in DCM (15 min) and the resin was washed with DCM (5 mL × 4) and dried under vacuum. Cleavage from the solid support and global deprotection was effected by incubating the dried resin in 10 mL of TFA:TIPS:H_2O (95:2.5:2.5) for 2.5 h. The resin was filtered and the filtrate was collected in a 50 mL centrifuge tube. The resin was washed with DCM (10 mL), filtered, and crude peptides were precipitated from the combined filtrate by the addition of cold Et_2O (45 mL). The mixture was centrifuged and the supernatant decanted. The pellet was washed with Et_2O (25 mL × 2) and dried thoroughly under vacuum. All peptides were purified by preparative HPLC with a reverse-phase column (C12 or C18, 250 mm × 21.2 mm, 4 μm, 90 Å) using linear gradients of MeCN in H_2O (mobile phases modified with 0.1% formic acid) over 30 min.

2.2. Submonomer synthesis of aGly. For incorporation of aGly residues, the resin was removed from the peptide synthesizer after the deprotection of the residue preceding aGly in the synthesis procedure. 2-Bromoacetic acid (70 mg, 0.50 mmol) was pre-activated with DIC (77 μL, 0.50 mmol) for 10 min in DMF (10 mL) before being added to the resin. The resin was allowed to stir for 30 min, then drained and washed with DMF (5 mL × 3), and the process was repeated. The resin was then stirred in a solution of 2 M *t*-butyl carbazate in DMF (10 mL) overnight at 50 °C. The resin was then drained and washed with DMF (5 mL × 3) before being treated with 5 equiv of Fmoc-Pro-Cl[18] and 2,4,6-collidine (130 μL, 1.0 mmol) in DMF (10 mL) for 30 min.

2.3. Incorporation and on-resin acylation of aAla. For incorporation of aAla into **aAla11-CMP**, H-(Boc)aAla-OH was added to the peptide on resin under standard conditions. The resin was then drained and washed with DMF (5 mL × 3) before being treated with 5 equiv of an Fmoc-Pro-Cl[19] and 10 equiv of 2,4,6-collidine (130 μL, 1.0 mmol) in DCM (10 mL) for 30 min.

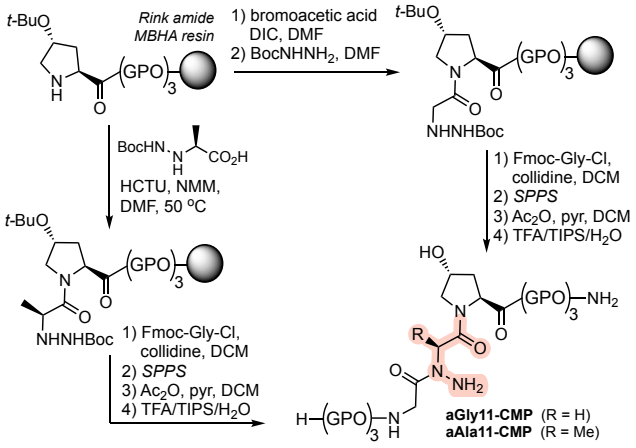
2.4. N'-Alkylation of crude aGly-containing peptides. Hydrazide N'-alkylation was achieved by carrying out cleavage of the aGly-containing peptides from the resin with TFA:TIPS:H_2O: (95:2.5:2.5) in the presence of 0.5 mmol of either benzaldehyde, *p*-anisaldehyde, acetone, cyclohexancarboxaldehyde, trimethylacetaldehyde, butyraldehyde, isobutyraldehyde, or 4-chlorobenzaldehyde. After 2.5 h, the resin was filtered and the filtrate was collected in a 50 mL centrifuge tube. The resin was washed with DCM (10 mL), filtered, and crude peptides were precipitated from the combined filtrate by the addition of cold Et_2O (45 mL). The mixture was centrifuged and the supernatant decanted. The pellet was then resuspended in 5 mL of H_2O containing 1.00 mmol $NaBH_3CN$ and stirred for 1 h before being frozen and lyophilized. In cases where incomplete conversion to the product was observed by analytical LCMS, peptides were treated again with 0.5 mmol of ketone/aldehyde in 5 mL of H_2O (adjusted to pH 2 with TFA) and stirred for 3 h, at which point 1.00 mmol $NaBH_3CN$ was added and the reaction followed by stirring for 1 h. The reaction was then frozen and lyophilized. Peptides were purified by preparative HPLC with a reverse-phase column (C12 or C18, 250 mm × 21.2 mm, 4 μm, 90 Å) using linear gradients of MeCN in H_2O (mobile phases modified with 0.1% formic acid) over 30 minutes.

2.5. Circular dichroism (CD). All peptides were prepared by dissolving lyophilized powder in 1x aq. PBS (pH 7.4) at a concentration of 150 μM. CD spectra were acquired using a JASCO J-1700 CD spectrometer in a 1 mm path length quartz cell with 2 s digital integration time, 1 nm bandwidth, 0.5 nm datapitch, and a scan speed of

100 nm/min at 20 °C. Mean residue ellipticity at a given wavelength (MRE; deg cm^2 $dmol^{-1}$ $residue^{-1}$) was calculated based on the equation MRE = q / (10 × b × M × n), where q is ellipticity (mdeg), b is the pathlength (cm), M is the peptide concentration (mol L^{-1}), and n is the total number of residues. All ellipticity values are reported as the mean across 3 scans. Temperature-dependent CD spectra were acquired using the same parameters from 7–90 °C in 1 °C increments at a ramp rate of 2 °C per minute and monitoring the maximum at 225 nm. Non-linear regression curves and melting temperatures were calculated according to the method described by Shortle and coworkers.[18] Melting temperatures are reported as the mean and SD across two independent denaturation experiments. Hysteresis studies were conducted by monitoring the maximum ellipticity at 225 nm using the same parameters from 7–90 °C and from 90–7 °C in 1 °C increments at a ramp rate of 2 °C per minute.

3. RESULTS AND DISCUSSION

To assess the impact of azapeptoid residues on CMP stability, we selected a parent sequence comprised of seven Gly-Pro-Hyp (GPO) repeats. Variants featuring aGly and other substitutions at the central position (Xaa11) were prepared by Fmoc SPPS using HCTU/NMM activation on Rink amide MBHA resin (Scheme 1). In the case of **aGly11-CMP**, bromoacetic acid was coupled to resin-bound Hyp12 using DIC, which was followed by an S_N2 displacement using *tert*-butyl carbazate. The subsequent Gly residue was coupled to the Boc hydrazide as its acid chloride[20] and elongation of the peptide was completed under standard conditions. For the CMP containing aAla at position 11 (**aAla11-CMP**), the N'-Boc-protected hydrazino acid[17] was coupled to Hyp12 using HCTU/NMM activation and the subsequent Gly residue was coupled as the acid chloride. Each peptide was then acetylated using acetic anhydride and pyridine followed by deprotection and cleavage from the resin using TFA. All crude peptides were purified using RP-HPLC and their identities were confirmed using HRMS (Table 1). In addition to **aGly11-CMP** and **aAla11-CMP**, CMPs harboring Gly and Ala residues (**Gly11-CMP** and **Ala11-CMP**) were synthesized in order to compare against their N-aminated counterparts.



Scheme 1. Synthesis of CMPs containing backbone N-aminated residues at the Xaa position.

Table 1. Synthesized CMPs featuring Xaa substitutions.

peptide	calculated *m/z*	observed *m/z*	yield %
Pro11-CMP	1951.8797 $[M+Na]^+$	1951.8793 $[M+Na]^+$	5
aGly11-CMP	1926.8593 $[M+Na]^+$	1926.8575 $[M+Na]^+$	21
aAla11-CMP	1940.8749 $[M+Na]^+$	1940.8733 $[M+Na]^+$	13
Gly11-CMP	1911.8484 $[M+Na]^+$	1911.8466 $[M+Na]^+$	14
Ala11-CMP	1925.8640 $[M+Na]^+$	1925.8646 $[M+Na]^+$	11

The peptides in Table 1 were first analyzed by far UV CD at pH 7.4 to compare their spectral signatures to that of the parent peptide (**Pro11-CMP**). All of the peptides exhibited PPII triple-helical structure characterized by maxima and minima near 225 and 204 nm, respectively (Figure 2). This indicates that substitution of Pro11 with aGly, aAla, and their amino acid analogues maintains the overall structure of the parent CMP.

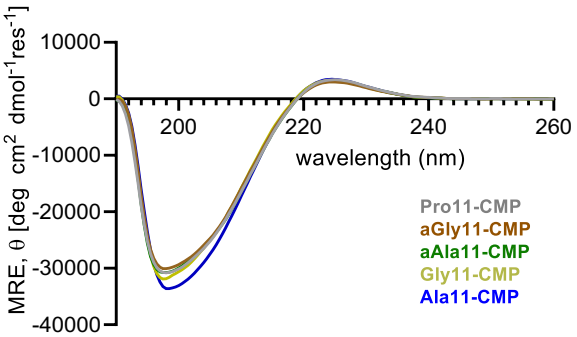


Figure 2. Far UV CD spectra of CMPs analyzed at 150 μM in aq PBS (pH 7.4).

Thermal denaturation was then carried out for each CMP by monitoring mean residue ellipticity (MRE) at 225 nm as a function of temperature and fitting of the data to a two-state unfolding model (Figure 3). **Pro11-CMP** exhibited a melting temperature (T_m) of 53.2 °C (Table 2). Substitution of Pro11 with Gly or Ala resulted in destabilization of the triple-helical fold (T_m of 36.9 °C and 46.6 °C, respectively). This is consistent with previous PPII propensity trends revealing that Ala is better accommodated into a PPII model peptide than Gly.[20] However, N-amination of these residues resulted in disparate effects on CMP stability. Thus, aGly11 substitution enhanced thermal stability relative to Gly, whereas aAla further destabilized the triple helix relative to Ala. This result is also in agreement with data from a single-strand PPII model showing that N-amination of Gly promotes PPII structure while N-aminated residues bearing Cα substituents prefer β-strand-like conformations.[16,21]

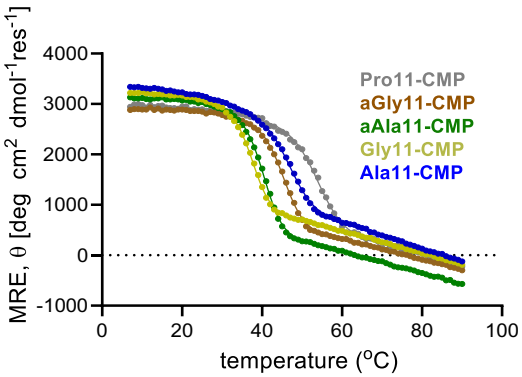
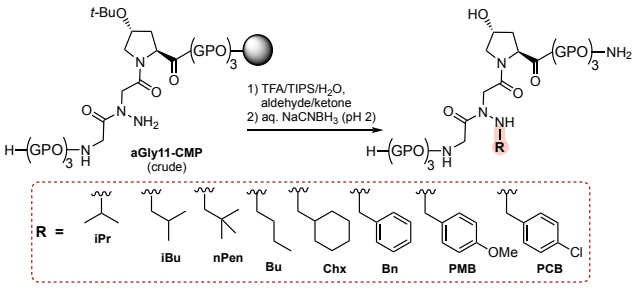


Figure 3. Thermal denaturation of Pro11-substituted CMPs at 150 μM in aq PBS (pH 7.4). Representative plots of MRE at 225 nm as a function of temperature determined by CD.

Table 2. Melting temperatures (T_m) for Pro11-substituted CMPs derived from non-linear regression. T_m values represent the mean and standard deviation from two separate denaturation experiments.

peptide	T_m (°C)
Pro-CMP	53.2 ± 0.1
aGly11-CMP	44.7 ± 0.2
aAla11-CMP	39.7 ± 0.5
Gly-CMP	36.9 ± 0.4
Ala-CMP	46.6 ± 0.5

A key feature of N-amino peptides is the presence of a reactive hydrazide handle that allows for late-stage diversification of the backbone.[8,22] As mentioned above, several hydrophobic peptoid residues are known to stabilize CMPs.[23,24] Wennemers and co-workers also showed that the thermal stability of a collagen peptide can be increased by introduction of alkyl groups onto the sidechain of 4-azaproline.[25] We hypothesized that alkylation of aGly would thus result in more thermally stable CMPs. Using seven different aldehydes and one ketone, **aGly11-CMP** was derivatized by reductive alkylation to obtain eight N'-substituted analogues (Scheme 2). After alkylation, the peptides were purified using RP-HPLC and their identities were confirmed using HRMS (Table 3).



Scheme 2. Late-stage derivatization of **aGly11-CMP** *via* reductive alkylation.

Table 3. Synthesized CMPs featuring N'-alkylated aGly residues at position 11.

peptide	calculated *m/z*	observed *m/z*	yield %
(iPr)aGly11-CMP	1968.9062 $[M+Na]^+$	1968.9072 $[M+Na]^+$	8
(iBu)aGly11-CMP	1982.9219 $[M+Na]^+$	1982.9241 $[M+Na]^+$	11
(nPen)aGly11-CMP	1996.9375 $[M+Na]^+$	1996.9350 $[M+Na]^+$	7
(Bu)aGly11-CMP	1982.9219 $[M+Na]^+$	1982.9172 $[M+Na]^+$	9
(Chx)aGly11-CMP	2022.9532 $[M+Na]^+$	2022.9560 $[M+Na]^+$	14
(Bn)aGly11-CMP	2016.9062 $[M+Na]^+$	2016.9096 $[M+Na]^+$	12
(PMB)aGly11-CMP	2046.9168 $[M+Na]^+$	2046.9178 $[M+Na]^+$	7
(PCB)aGly11-CMP	2050.8673 $[M+Na]^+$	2050.8675 $[M+Na]^+$	14

The alkylated derivatives of **aGly11-CMP** again showed native-like PPII triple-helical CD signatures. Thermal denaturation revealed that most of the hydrazide alkylations further destabilized the triple helix (Figure 4 and Table 4). However, **(iPr)aGly11-CMP** and **(Bu)aGly11-CMP** exhibited modest enhancements in stability relative to **aGly11-CMP** (approximately +1.5 °C). Previous studies using peptoid residues showed that hydrophobic amide substitutents generally enhance CMP stability relative to Pro while charged groups destabilize the triple helix. In constrast, high hydrophobicity in the azapeptoid series did not result in significantly enhanced melting temperatures.

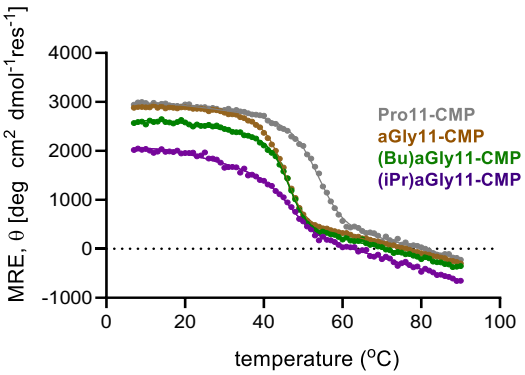


Figure 4. Thermal denaturation of alkylated **aGly11-CMP** derivatives at 150 μM in aq PBS (pH 7.4). Representative plots of MRE at 225 nm as a function of temperature determined by CD.

Table 4. Melting temperatures (T_m) for N'-alkylated CMPs derived from non-linear regression. T_m values represent the mean and standard deviation from two separate denaturation experiments.

peptide	T_m (°C)
aGly11-CMP	44.7 ± 0.2
(iPr)aGly11-CMP	46.3 ± 0.4
(iBu)aGly11-CMP	44.1 ± 0.3
(nPen)aGly11-CMP	38.8 ± 0.2
(Bu)aGly11-CMP	46.1 ± 0.2
(Chx)aGly11-CMP	41.6 ± 0.1
(Bn)aGly11-CMP	40.9 ± 0.1
(PMB)aGly11-CMP	41.1 ± 0.1
(PCB)aGly11-CMP	41.9 ± 0.1

Finally, we evaluated how incorporation of aGly at position 11 and alkylation of this residue impacts the rate of CMP refolding. The rate-determining step in collagen triple-helical assembly is *cis/trans* isomerization of the tertiary amide bonds.[23,24] We previously demonstrated that replacement of Pro11 with δ-oxaproline enhanced the rate of CMP refolding, likely due to the reduced isomerization barrier of the hydroxamate bond relative to the native Pro amide.[25] We hypothesized that the hydrazide bond in **aGly11-CMP** and **(iPr)aGly11-CMP** would exhibit a similar enhancement in isomerization rate. However, hysteresis experiments, wherein the peptides were denatured and re-cooled while monitoring by CD (at 225 nm), revealed that incorporation of aGly and (iPr)aGly did not significantly impact the rate of collagen triple-helix refolding (Figure 5).

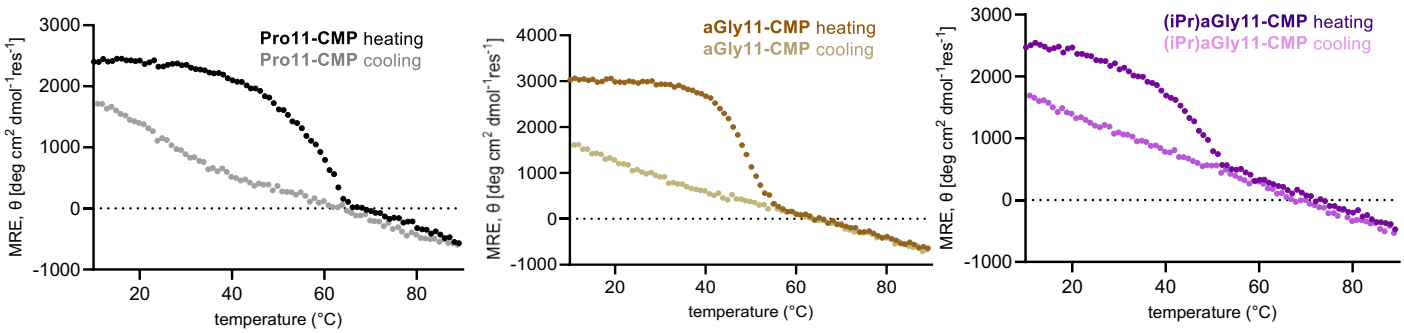


Figure 5. Representative hysteresis plots of **Pro11-CMP**, **aGly11-CMP,** and **(iPr)aGly11-CMP** obtained by monitoring at 225 nm by CD at 150 μM in aq PBS (pH 7.4).

4. CONCLUSIONS

In summary, we show that azapeptoid residues can be accommodated into the Xaa position of a collagen triple helix but incur an energetic folding penalty relative to Pro. The destabilizing effect of aGly incorporation was partially mitigated through late-stage reductive alkylation to generate N'-isopropyl and N'-butyl derivatives. Interestingly, the aGly and (iPr)aGly residues investigated here are directly analogous to the peptoid residues sarcosine and Nleu. Comparisons of their effects on CMP stability suggest that the azapeptoid N'H group may engage in detrimental cross-strand H-bonding interactions or destabilize PPII backbone torsions. Despite these effects, aGly and (iPr)aGly-containing CMPs were considerably more stable than an unsubstituted Gly11 analogue. Studies with dialkylated azapeptoid residues devoid of an H-bond donor will be useful in further probing the effects of amide-to-hydrazide substitution on collagen folding.

Acknowledgements
This work was supported by a grant from the National Science Foundation (CHE2109008).

Data Availability Statement
The data supporting this article have been included as part of the Supplementary Information.

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