

A Single Stereodynamic Center Modulates the Rate of Self-Assembly in a Biomolecular System

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Abstract: Chirality is a property of asymmetry important to both physical and abstract systems. Understanding how molecular systems respond to perturbations in their chiral building blocks can provide insight into diverse areas such as biomolecular self-assembly, protein folding, drug design, materials, and catalysis. Despite the fundamental importance of stereochemical preorganization in nature and designed materials, the ramifications of replacing chiral centers with stereodynamic atomic mimics in the context of biomolecular systems is unknown. Herein, we demonstrate that replacement of a single amino acid stereocenter with a stereodynamic nitrogen atom has profound consequences on the self-assembly of a biomolecular system. Our results provide insight into how the fundamental biopolymers of life would behave if their chiral centers were not configurationally stable, highlighting the vital importance of stereochemistry as a pre-organizing element in biomolecular folding and assembly events.

One of the most ubiquitous chiral elements in nature is the sp^3 -hybridized tertiary carbon stereocenter, exemplified by its presence in the α -amino acid building blocks of biomolecules.^[1] Chirality^[2] in molecular systems can be thought of as a form of stereochemical preorganization that limits the conformational space. Understanding how molecular systems respond to perturbations in their chiral building blocks can provide insight into diverse areas, such as biomolecule self-assembly, protein folding, drug design, materials, and catalysis.^[3] Biomolecular structure is governed by a delicate balance of non-covalent intra- and intermolecular interactions that drive macromolecular assembly and recognition events that are critical to all life processes. These events are intimately coupled to stereochemistry, which is a property that is hard-wired into the building blocks of life.^[1a,b,4,5] For proteins and peptides, the building blocks are amino acids with sp^3 -hybridized tertiary carbon stereocenters in the L-configuration. Natural amino acid building blocks are typically enantiopure and lack the ability to readily interconvert between D and L forms in the absence of enzymatic or harsh chemical conditions.^[5] The C–H bond of an sp^3 -hybridized tertiary carbon requires more than 80 kcal mol^{−1} for thermal homolysis (Figure 1a).^[6] The unique asymmetry imposed by the configurationally stable stereocenters of amino acids also restricts conformational mobility, as shown in Ramachandran

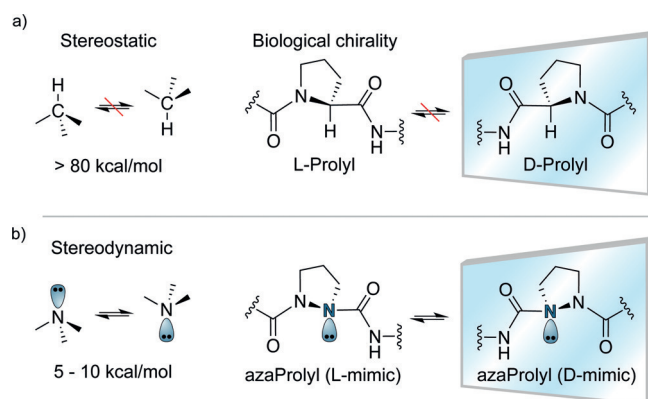


Figure 1. Example of configurational stability and stereodynamics with respect to the prolyl amino acid. a) Configurationally stable sp^3 -hybridized tertiary carbon stereocenter and the prolyl amino acid example. b) A stereodynamic sp^3 -hybridized tertiary nitrogen center and an example of how a stereodynamic pyramidalized nitrogen (as in aza-proline, AzPro) could potentially mimic both prolyl stereochemical configurations.

maps.^[7] Nitrogen atoms in their sp^3 -hybridized form can adopt a pyramidalized geometry that is very similar to a tertiary carbon stereocenter, with the exception of a lone pair of electrons occupying the position once held by a hydrogen atom on the tertiary carbon.^[8] Despite a similar electron pair geometry between sp^3 -hybridized nitrogen and sp^3 -hybridized carbon, there is a striking difference in the energy of activation required for interconversion between the two mirror image forms in each case (Figure 1). A typical activation barrier for pyramidal inversion of sp^3 -hybridized nitrogen ranges from 5–10 kcal mol^{−1}, corresponding to interconversion between the two mirror image forms.^[8]

In addition to the conformational constraints imposed by amino acid stereochemistry, living systems have proline at their disposal. Proline is a unique amino acid representing a limiting case in the continuum of preorganization.^[4,7,9] Proline has a privileged structure, where side chain cyclization to form the pyrrolidine ring results in the most conformationally restricted amino acid building block present in nature (Figure 1). The pyrrolidine ring constraint has a dramatic impact on biomolecular conformation, folding, assembly, and recognition events. Collagen represents one of the most striking examples of a highly preorganized, proline-rich, self-assembling protein structure in nature.^[10] Collagen adopts a densely packed triple helical structure requiring a precise backbone conformation for proper self-assembly (Figure 2).^[10,11] A major gap in our understanding of biomolecular folding and self assembly involves grasping the

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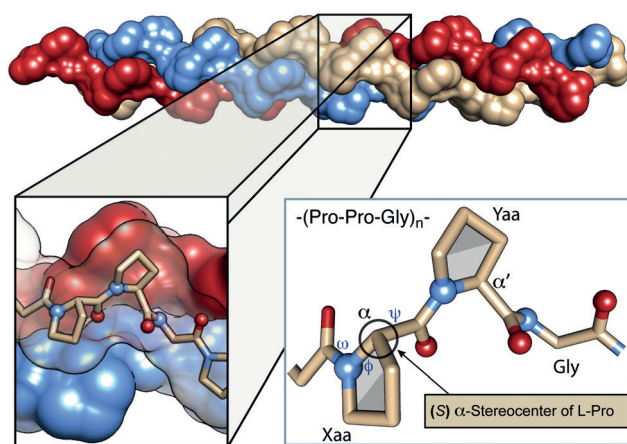


Figure 2. The collagen triple helix and an expanded view of the -Pro-Pro-Gly- tripeptide repeat with adjacent view of the chemical structure. Individual polypeptide chains are differentially colored red, blue, and beige. Detailed view of the tripeptide repeat (Xaa-Yaa-Gly) showing designated torsional descriptors and proline stereocenters. Heteroatoms are differentially colored red = oxygen, blue = nitrogen, and beige = carbon.

importance of stereochemistry and its impact on these processes. The achiral nature of aza-proline (AzPro or AzP) and the ability of nitrogen to readily adopt either of two pyramidalized forms provides a unique opportunity to glean insight into the vital role that stereochemistry plays in biomolecular assembly (Figure 1b). Prior studies have not examined the effect of AzPro modifications on folding or self-assembly in the context of longer peptide chains, prompting our investigation of the impact of AzPro in a collagen model peptide system^[11e] to assess the influence of stereodynamics on the triple helix self-assembly process.

Herein, we demonstrate that replacing a single amino acid stereocenter with a nitrogen atom results in a fluxional system with the capacity to mimic amino acid stereochemistry. First, we evaluated a proline rich dipeptide by replacing a single stereocenter with a nitrogen atom to create a stereodynamic perturbation at a central location. Next, this perturbation was assessed in the context of a triple helix forming collagen model peptide system. We demonstrate that a single stereodynamic atom dramatically alters the rate of triple helix self-assembly with little to no effect on the thermal unfolding, highlighting the vital importance of stereochemistry as a pre-organizing element in biomolecular folding and assembly events.

The tertiary structural motif adopted by collagen is a right-handed triple helix composed of three staggered polypeptide chains in a left-handed polyproline type-II (PPII) helical conformation. Over the past several decades, collagen has been the focus of many research efforts aimed at developing a molecular level understanding of its self-assembling properties and for the development of designed materials.^[11,12] The most common repeating amino acid motif is XaaYaaGly, where Xaa is proline 28 % of the time, Yaa is either hydroxyproline (Hyp) or proline 38 % of the time, with the glycine remaining intolerant to substitution.^[10c] To date, nearly all amino acid substitutions beyond the ProHypGly

motif have led to significant destabilization of the collagen triple helix except for a few rare examples. The few instances of stabilizing amino acid substitutions that have been discovered to date have led to significant insight into protein structure, such as the stereoelectronic effects and $n \rightarrow \pi^*$ interactions pioneered by the Raines group.^[13] These studies have revealed that side-chain modification using unnatural amino acids can modulate the self-assembly properties of the triple-helical structure, pointing to a delicate balance of noncovalent interactions.^[14,15] Seminal studies on peptoid substitutions have also shown that *N*-alkylated glycine can be substituted for proline when a bulky hydrophobic side chain is used. This is the one example of an achiral proline replacement that has led to collagen triple helix stabilization.^[16] In contrast to side chain modifications, collagen backbone modifications have resulted in significantly destabilized structures, often resulting in a complete inability to form a triple helical structure.^[17] Backbone modifications in the form of stereochemical inversion (L to D amino acids) and heteroatom replacement have all resulted in either severe destabilization or a complete lack of triple helix formation.^[17,18] Amide-to-ester substitutions also have a detrimental impact on collagen triple helix stability and many other protein secondary structures.^[18a] In addition, *trans*-alkene amide bond isosteres greatly destabilize the triple helical structure of collagen irrespective of positioning and involvement in hydrogen bonding.^[18b,c] A recent example of thioamide substitution in collagen was demonstrated to have a minimally perturbing and modest stabilizing effect on the triple helix structure, depending on position, highlighting the importance of minimally perturbing backbone substitutions in proteins.^[19] To date, these seminal studies have revealed a wealth of fundamental insight into protein structure and point to a general intolerance of the collagen peptide backbone to molecular editing.^[10c,17,18]

Intrigued by the possibility of nitrogen serving as a stereodynamic stereochemical mimic, we analyzed a model of the collagen triple helix based on existing crystal structures.^[10d,11h] The model revealed that aza-proline substitution in the Xaa position would potentially result in a solvent exposed nitrogen devoid of any deleterious electronic effects with main chain functional groups (Figure 3a). In contrast, substitution at the Yaa position revealed a potential detrimental inter-strand electrostatic interaction. After determining a strategic position for nitrogen backbone incorporation, we conducted a thorough survey of pyramidalized nitrogen containing small molecule crystal structures in the Cambridge Structural Database (see Supporting Information (SI), Figures S1–S4). This survey revealed that small molecule crystal structures containing AzPro and other trisubstituted amines display a broad range of pyramidal character (Figure 3c, entry 1–26; Figure S1). To quantify the extent of pyramidalization (*d*), a triangular pyramid was defined with nitrogen at the apex and the substituent atoms positioned at the remaining vertices of the base. The distance from the apex normal to the base plane was measured as the *d* value, in angstroms, for each structure. The *d* value of aza-proline and other nitrogen containing small molecules was found to range from 0 to 0.6 Å (Figure 3c). Applying this same measure to the proline

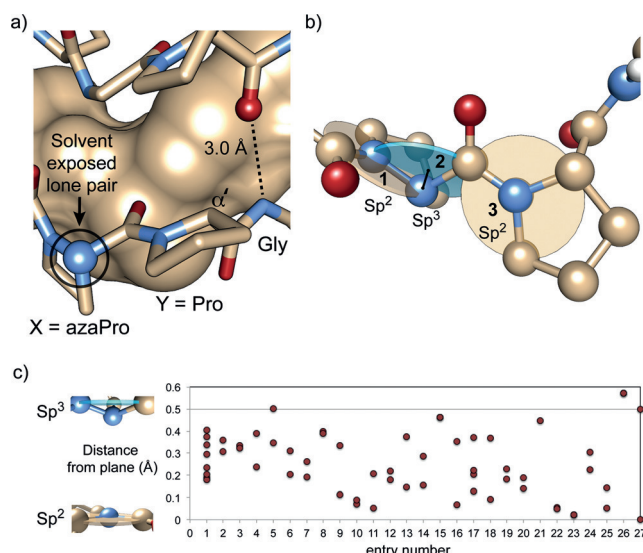


Figure 3. a) Model showing the position of AzPro containing collagen peptide peptide 5. b) ball-stick model of the dipropyl urea moiety in CMP 5; black arrow and cyan plate show the distance d between N and the plane defined by its three connecting atoms, indicating the degree of pyramidalization c) Survey of the degree of pyramidalization of nitrogen atoms in AzPro-containing small molecule crystal structures found in the CCDC and other trisubstituted nitrogen containing small molecules. Details for entry numbers can be found in Figure S1 of the Supporting Information.

α carbon in high-resolution crystal structures of triple helical collagen reveals an average pyramidalization distance of 0.53 ± 0.02 Å ($n=36$) for effectively mimicking the stereogenic center with a nitrogen atom (Figure 3c, top marker in entry "27").

To gauge the impact of AzPro incorporation we synthesized two small molecule model systems: Ac-Pro-Pro-OMe (**1**) containing natural L-proline residues and Ac-AzPro-Pro-OMe (**2**) containing a single AzPro residue in which the α -CH stereocenter has been replaced by nitrogen, rendering the residue achiral (Figure 4, Scheme S1). AzPro has been previously incorporated into small peptide systems and pioneering work on aza-amino acids has led to the development of many important bioactive aza-peptides often showing increased biological activity and/or improved pharmacokinetic properties.^[20,21] The proton NMR spectra of our dipeptide model systems provide a striking picture of the difference in the conformational dynamics between the parent dipeptide (**1**) and the AzPro containing dipeptide (**2**) (Figure 4). The parent dipeptide system (**1**) exhibits well-resolved 1H resonances in the NMR with visible spin-spin splitting patterns. In contrast, the NMR spectrum of the dipeptide containing AzPro (**2**) is severely line broadened and featureless, illustrating the fluxional nature of the nitrogen atom replacement (Figure 4).

We performed variable temperature 1H NMR experiments to gain insight into the dynamics of the AzPro containing dipeptide **1**. Upon cooling dipeptide **1**, we observed that the broad $\alpha 1$ proton signal split into four broad but defined conformer populations. Coalescence of the $\alpha 1$ proton peaks begins at $-15^\circ C$ and continues until all of the

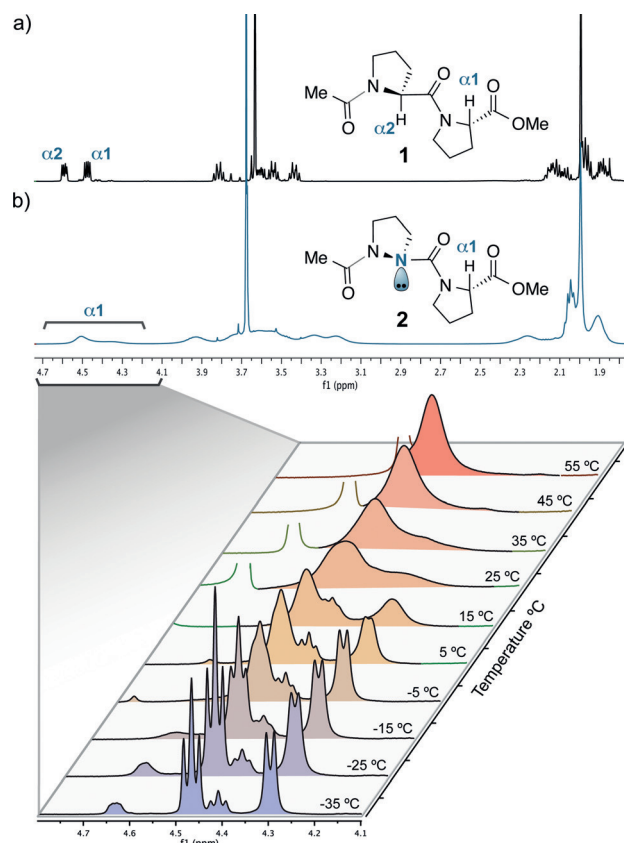


Figure 4. Impact of replacing an α -stereocenter with a nitrogen atom. Prolyl dipeptide model system and the nitrogen substituted analogue shown with 1H NMR data (taken in $CDCl_3$). a) Chemical structures of the Ac-Pro-Pro-OMe (**1**) dipeptide model system and 1H NMR spectrum. b) Chemical structure of the Ac-AzPro-Pro-OMe (**2**) dipeptide model system and 1H NMR spectrum. NMR spectra show severe broadening after replacement of the carbon stereocenter with a nitrogen atom. The nitrogen atom replacement is shown in blue with the lone pair. The $\alpha 1$ proton region is expanded and shown at variable temperatures. Upon cooling, distinct conformer populations are observed.

peaks coalesce near $40^\circ C$. At temperatures of $-15^\circ C$, discrete sets of signals were observed as exemplified by the expanded $\alpha 1$ proton region shown in Figure 4b. At $-35^\circ C$, the apparent triplet and doublet peaks could be observed together with peaks that are still relatively broad in the aliphatic region, suggesting sub-populations of fluxional conformers with a certain degree of dynamism even at low temperature. Therefore, only qualitative conclusions could be drawn from the low temperature NMR experiments regarding the four different conformer populations in solution. The rate constants for the interconversion between conformer populations were estimated to be between $k_{ic} = 60\text{--}200$ s $^{-1}$ for the $\alpha 1$ conformer sub-populations with activation energies ranging from $\Delta G^\ddagger = 13\text{--}16$ kcal mol $^{-1}$ and relative ground state energies of ≤ 1 kcal mol $^{-1}$. The dynamic nature of dipeptide **2** is extraordinary given the perturbation of only a single atom, providing a qualitative representation of the AzPro stereodynamics.

To gain insight into the accessible conformational space and the pyramidalization preference of AzPro dipeptide **2**,

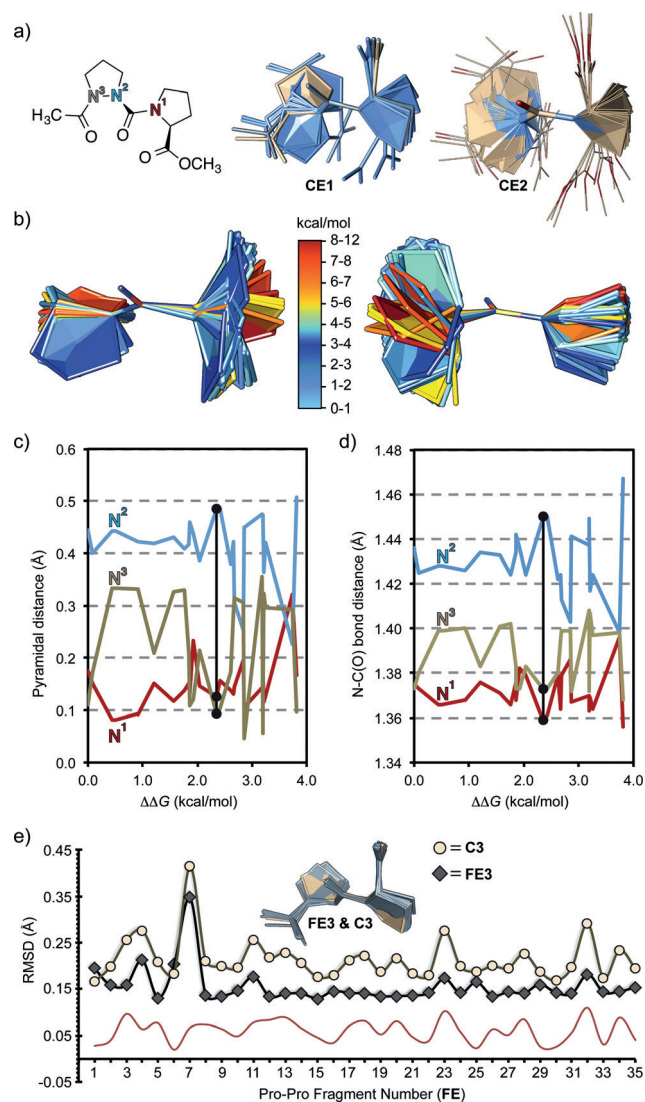


Figure 5. Computational analysis of the AzPro-Pro dipeptide. a) (left) Chemical structure with labeled nitrogen atoms. (middle) Ensemble (**CE1**) of lowest energy conformers with relative energies less than 4 kcal mol⁻¹. (right) Ensemble (**CE2**) of all conformers found for the AzPro-Pro dipeptide **2** by DFT. b) Front and back view of the conformational ensemble calculated for the AzPro-Pro dipeptide color-coded by relative ground state energy shows the stereodynamic nature of AzPro (oriented toward middle) versus the stereostatic nature of proline (oriented toward left or right side). Low energy conformers exhibit both D and L proline mimicking conformations. Conformations where N2 is planarized correlate with higher relative ground state energies. c) Pyramidalization distance for conformer ensemble **CE1** plotted as a function of relative ground state energy for each nitrogen atom. Pyramidalization is defined as the distance *d* between N and the plane defined by its three connecting atoms, indicating the degree of pyramidalization. d) Plot of the nitrogen-carbonyl bond length for each of the three nitrogen atoms. e) Comparison of Pro-Pro dipeptide units from collagen crystal structures (**FE3**) to the conformer (**C3**) showing the small structural RMSD. Deviation between **C3** and each member of **FE3** is shown as the bottom line.

employed DFT calculations utilizing the 6-31g(d) basis sets to assess the possible low energy conformations available to the AzPro-Pro dipeptide (Figure 5 and Figures S5–S10). Nine low

energy conformers (**CE1**) were determined with relative ground state energies less than 1 kcal mol⁻¹ with approximately half of the population occupying a conformation mimicking D-proline and the other half of the population occupying a conformation mimicking L-proline. The full calculated ground state conformational ensemble (**CE2**) contained a total of 48 structures with relative energies less than 12 kcal mol⁻¹ (Figure 5a,b). The results in Figure 5b provide an overlay of all 48 ground state structures colored according to their relative energies and a glimpse into the stereodynamic nature of aza-proline in the context of the sterically congested proline containing peptide **2** (Figure 5b and supporting information), which is in contrast to the stereostatic nature of the adjacent proline residue with locked alpha carbon stereochemistry. A subset of 35 conformers with relative energies less than 4 kcal mol⁻¹ were further analyzed for pyramidal character and nitrogen-carbonyl bond lengths at each of the three nitrogen atoms shown in Figure 5a. In general, N² exhibited a clear preference for adopting a highly pyramidalized form and a longer N–CO bond, which is consistent with the capacity to mimic both D and L forms of proline. Nitrogen atoms N¹ and N³ exhibited less pyramidal character with N¹ showing a propensity toward a more sp² amide-like geometry. The pyramidalization preferences paralleled the expectations for N–CO bond length trends, where structures with less pyramidal character exhibited shorter N–CO bond lengths (Figure 5d). A comparison of the RMSD between the calculated structures and their dipeptide analogues found in the crystal structure of collagen yielded several low energy conformations capable of mimicking orientations compatible with the collagen triple helix, implying the feasibility of proline with AzPro. The calculated structure (**C3**) with the lowest average pairwise RMSD compared to the ensemble of fragments from the crystal structures is shown in Figure 5e, and conformer **C3** is indicated with black markers on the plots in Figure 5c and d. The presence of additional low energy calculated structures suggest the conformationally dynamic nature of the dipeptide with the capacity to mimic both the D and L stereochemistry of proline.

Previous studies of small molecule and short peptide systems containing AzPro reveal that substitution of the alpha-CH by a nitrogen atom in the prolyl ring can result in perturbations to ring puckering, backbone conformation, and rotational barriers of the *cis-trans* amide bond preceding the prolyl residue compared to that of proline. Crystallography and computational studies have demonstrated the ability of both nitrogen atoms in AzPro to adopt a pyramidalized geometry; the bond lengths of CO–N and N^α–CO are longer by up to 0.1 Å and shorter by 0.01–0.03 Å compared to a Pro residue, respectively, although this result most certainly varies with dihedral angle and molecular environment.^[22–24] NMR, IR, X-ray and computational studies support a *cis* amide bond preference for AzPro in certain situations, such as short peptides prone to adopt a type VI β-turn structure in organic solvents.^[22,23] Preference for the *cis* geometry in organic solvents has been attributed to the potential for a lp–lp interaction between N^α and the preceding carbonyl in the *trans* conformation. This repulsive interaction is reported to

be reduced in water resulting in a significant decrease in the *cis* amide preference.^[22b,c] Simulations on tetrapeptides containing AzPro indicate that the *cis-trans* preference is highly variable and context dependent.^[22a] Rotational barriers for *cis-trans* isomerization of the aza-prolyl amide bond have been calculated to be 16.7 kcal mol⁻¹ in water, which is 2.1–4.3 kcal mol⁻¹ lower than estimates of the cognate proline dipeptide.^[22d] Building on previous studies and our current observations of AzPro incorporation into peptide **2**, AzPro appears well suited as a stereodynamic mimic for proline at the X position within collagen model peptide systems.

Next, we focused on the impact of stereodynamics in the context of a full-length collagen model peptide system. Guided by insights gained from analysis of dipeptide fragments, we targeted the alpha stereocenter of proline in the Xaa position of the collagen peptide for replacement with a stereodynamic probe. Inspection of the Xaa position revealed a minimally perturbing solvent exposed environment, providing a logical choice for AzPro replacement (Figure 3a). We synthesized peptides **3**, **4** and **5**, where peptide **5** constitutes replacement of a single stereogenic carbon atom with a single nitrogen atom at the central Xaa location in a 260 atom (21 amino acid) peptide system (Figure 6a). Circular dichroism (CD) spectroscopy was used to evaluate self-assembly of the triple helix for each of our collagen model peptides. CD measurements of solutions containing collagen model peptides **3** and **5** exhibit characteristic maxima at approximately 224 nm, which is typical for the collagen triple helix structure (see SI).

Control peptide **4** containing D-Proline exhibits a CD spectrum at 4 °C similar to that of **3** and **5**, but with a reduced ellipticity maximum and a shallower minimum, indicating a lower propensity toward triple helix formation (SI).^[11] Thermal CD denaturation experiments revealed that peptide **5** underwent a cooperative unfolding transition upon heating (Figure 6 and SI). In contrast, peptide **4** lacked the ability to self-assemble into a triple-helix structure, as evidenced by a linear decrease in molar ellipticity as a function of temperature. These results indicate that replacement of the stereocenter between two proline residues with a nitrogen atom (peptide **5**) results in a stable triple helix that cooperatively unfolds in a manner similar to the parent all L-amino acid configured collagen model peptide, **3**, and at a similar unfolding temperature. The data from CD thermal denaturation experiments were fitted to a two-state model, as previously described.^[25a,15b,c] For peptide **3** and **5**, the melting temperatures, at which 50% of the triple helix unfolds, are nearly identical (< 1 °C) (Figure 6d). This observation reveals that an sp³-hybridized nitrogen atom can effectively mimic the geometry of a tertiary carbon stereogenic center in the context of a collagen model peptide system. The implication is that the L-proline conformation is effectively mimicked by the stereodynamic nitrogen in peptide **5**, further supported by data from compound **4**, showing that D-amino acids are detrimental to the formation of a stable triple helix in addition to previous reports.^[17]

To probe the impact of stereodynamics on the kinetics of triple helix self assembly, we monitored triple helix formation for peptides **3** and **5** using isothermal CD refolding experi-

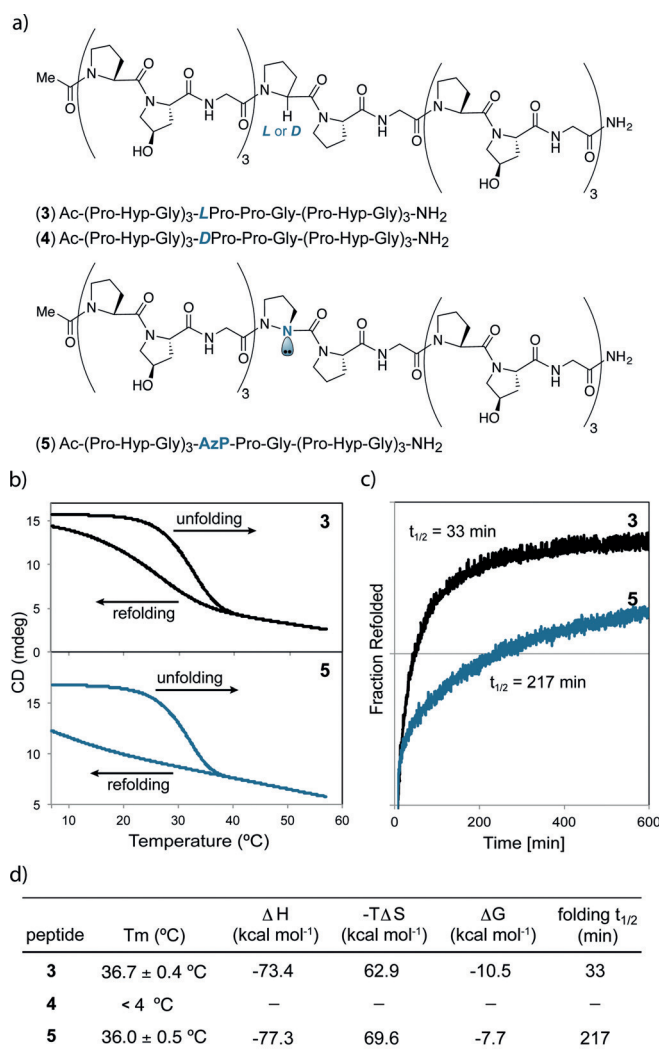


Figure 6. Unfolding and folding data for collagen model peptides. a) Chemical structure of collagen model peptide **3**, **4** and model peptide **5** containing a stereodynamic nitrogen atom. b) Unfolding and folding data for collagen model peptides showing the hysteresis difference. c) Kinetic refolding showing a 7-fold decrease in folding half time. d) Table of thermodynamic data and refolding half-times.

ments over a time course of 600 min. Peptides **3** and **5**, at a concentration of 0.2 mM in PBS buffer, were denatured at 80 °C for 15 min and their CD profiles were monitored at 4 °C until both peptides recovered > 50% ellipticity at 224 nm. A major difference was observed as peptide **5** approached 50% recovery at a rate approximately 7 times slower than that of control peptide **3** (Figure 6c). Hysteresis studies were performed to gain further insight into the stability of peptide **5**.^[15b,c,25b] The free energy difference between peptide **3** and **5** is relatively large compared to the difference in *T*_m (Figure 6b,d). Based on the hysteresis data and refolding studies, the origin of the free energy difference is consistent with an increase in the entropy term for peptide **5**. There appears to be a compensatory effect in the enthalpy term even though the overall Δ*G* is less favorable. The Δ*G* was found to be -10.5 kcal mol⁻¹ for peptide **3** and -7.7 kcal mol⁻¹ for peptide **5** with a ΔΔ*G* of 2.8 kcal mol⁻¹. The difference

appears to be primarily rooted in the entropy term, considering the observed enthalpic benefit after replacing the stereocenter with nitrogen, although further calorimetric data is needed to reach a firm conclusion. The large difference in half time values for triple helix self-assembly are a striking demonstration of the important role that stereochemistry plays in biopolymer preorganization; however, there are several other factors pertaining to AzPro that must be taken into consideration.

Taken collectively, the results reported herein support the notion that the nitrogen atom of the AzPro system can adopt an sp^3 -hybridized form, with a pyramidalized geometry effectively mimicking the stereocenter of proline in the context of a collagen triple helix. Substitution at the Xaa position places the AzPro residue in the most sterically congested environment possible among the naturally occurring amino acids between two proline residues. A pyramidalized geometry implies decreased resonance stabilization when there is an electron withdrawing carbonyl substituent on nitrogen. However, in the case where the AzPro residue is adjacent to another proline residue, a ground state destabilized situation is created with enough steric congestion to preclude a planarized resonance stabilized pseudo-urea as supported by our calculations (Figure 5 d,e). This destabilized ground state may decrease the N–C(O) rotational barrier, allowing easy access to fluxional conformations spanning the continuum between fully delocalized and fully pyramidalized. In addition to a subtle but intricate balance of electronic, steric, and stereoelectronic effects the fluxional nature of a stereodynamic perturbation also exerts an entropic cost during the process of triple-helix self-assembly, as reflected in the refolding kinetics of peptide **5** (Figure 6).

In conclusion, our study suggests that configurationally stable sp^3 -hybridized tertiary carbon stereocenters serve as preorganizing elements to lower the entropic barrier of biomolecular folding and self-assembly events. Pyramidalized nitrogen is able to effectively mimic stereogenic carbon atoms in biomolecular systems. The low nitrogen pyramidalization barrier creates a fluxional system capable of sampling conformational space. Conformational sampling can allow for enthalpic tuning of inter- and intramolecular interactions albeit with an entropic cost. In addition to insight into the fundamental importance of stereochemistry as a preorganizing element in natural systems, these studies may provide insight into diverse areas ranging from self-assembling materials and drug design to catalysis and synthetic receptors. Despite increased entropy being manifest in dynamic systems, herein lies the first experimental realization of the impact of stereodynamics in the context of a self-assembling biopolymer system utilizing a stereodynamic single atom probe as an atomic mimic of an sp^3 -hybridized tertiary carbon stereocenter.

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