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We have analyzed thermal unwinding behavior of a biologically relevant collagen mimetic peptide (PDB ID: 1bkv) through molecular dynamics simulations in explicit water. Conformational changes of the triple helix were monitored by introducing a set of local triad vectors and measuring variations in their torsional angles. Although the molecule fluctuates thermally at 273 K, unwinding becomes pronounced at 300 K and 330 K. We found that the region containing Gly-Ile, which is a common cleavage site in collagen, to be an initiation site for unwinding. Our results suggest that local unwinding of collagen is spontaneous at physiological temperatures, and it could be a property utilized for binding by other proteins, such as cleavage enzymes or fibril associated collagens.

1. Introduction

Collagen is the most abundant protein in the human body and forms the major component of the extracellular matrix in many tissues and organs [Alberts et al. 2000; Brodsky and Persikov 2005]. It is involved in diverse processes related to growth, maintenance, and remodeling of tissues. Currently more than twenty-seven types of collagens have been identified [Boot-Handford et al. 2003; van der Slot 2005], which can self-assemble into higher-level polymeric structures such as fibrils, fibers, and networks. Mutations in collagen genes can cause several diseases, some of which are fatal [Myllyharju and Kivirikko 2001]. Due to such biological significance, its structure, function, and relationship with other extracellular components have been a major focus of attention during the past six decades.

High-resolution X-ray crystallography of collagen mimetic peptides have provided useful structural insights [Bella et al. 1994; Kramer et al. 2000; 2001]. Collagen molecules have a distinct triple helical structure with three α (polyproline-II-type) chains; three left-handed triple helices are wound together to form a rope-like right-handed superhelix (Figure 1) [Ramachandran and Kartha 1955; Rich and Crick 1961; Brodsky and Persikov 2005]. Individual α chains have a regular amino acid repeating sequence $–(\text{G-X-Y})_n–$, where G is glycine and X,Y are other amino acids. The three chains are staggered by one amino acid along the length of the molecule (Figure 2c), making glycine, the smallest amino acid, always fall in the central part of the triple helix. This, along with the constraint on the backbone imposed by the imino rings in proline (Pro), gives a tight triple helical tertiary structure to the molecule. During posttranslational modifications, prolines in the Y position are specifically hydroxylated into (4R)hydroxyprolines (Hyp). Raines and coworkers have shown that the electron withdrawing effect of the hydroxyl group in Hyp favors an *exo*-ring puckering, giving a stable *trans*-conformation to the Y residue [Holmgren et al. 1998; 1999; Jenkins and Raines 2002; Hodges and Raines 2005] . Gly-Pro-Hyp (GPO) triplets thus form the most stable structural domains [Shah et al. 1996] and are commonly chosen as major constituents of

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short collagen mimetic peptides. These imino-rich regions have a tighter 7-fold (7/2) helical symmetry (7 amino acids per two helical turns) compared to imino-poor regions which have a 10-fold (10/3) symmetry (10 amino acids per three helical turns) [Kramer et al. 1999]. GPO domains are found at the C-terminal region of collagen and nucleate the triple helix formation [McLaughlin and Bulleid 1998]. Thus, the local sequence of a collagen domain determines its stability, helicity, and functionality.

The complex process of collagen turnover is intricately related to its mechanics [Wright and Humphrey 2002; Tomasek et al. 2002]; for example, mechanical strain affects collagen cleavage [Ruberti and Hallab 2005] and local lability in its structure can help in fibril formation [Kadler et al. 1988]. Functionally, non-GPO domains, being labile and flexible, are cleavage sites for matrix metalloproteinases (MMPs) or binding sites for integrins and heparin [Emsley et al. 2000; Persikov and Brodsky 2002]. Such interactions make collagens crucial members in the feedback loop through which cells can alter their environment to promote homeostasis in tissues. Static and cyclic mechanical loads on collagen substrates can induce differential responses from cells, including cell proliferation, collagen deposition, collagenase activity, and release of growth factors that modulate growth and remodeling of tissues [Carver et al. 1991; Hsueh et al. 1998; MacKenna et al. 2000]. Structurally, collagen cleavage is linked to possible unwinding by collagenases [Chung et al. 2004]. Isolated collagen molecules are known to be unstable at body temperature [Leikina et al. 2002], while collagens cross-linked as a bundle are much more stable. Hence collagen turnover in tissues most likely involves local conformational fluctuations of individual collagen molecules that are sensitive to their thermomechanical environment.

To characterize local conformational variations in collagen, we developed a systematic way of assigning a set of local Cartesian basis triads along the length of the molecule and monitored relative changes in torsional (helical) angles among them. Using molecular dynamics (MD) simulations in explicit water environments, we characterized temperature-dependent changes in the triple helical twist of a collagen mimetic peptide (PDB ID: 1bkv) [Kramer et al. 1999]. This peptide is of special interest, compared to other collagen mimetic peptides, since it contains a biologically relevant 12 amino acid sequence immediately downstream of the unique collagenase cleavage site in human collagen type III. This sequence is known to be important in collagenase specificity [Fields 1991]. We found that the molecule can locally unwind within 1 ns at temperatures of 300 K and above. Importantly, unwinding is initiated around the Gly-Ile bond in the imino-poor region. Such region-specific unwinding could be an initiating event of collagen denaturation or it may facilitate collagenase binding [Fields 1991; Chung et al. 2004]. Our results will thus be useful for developing structure-based physical models for collagen stability and turnover.

2. Simulation methods

For MD simulations we used CHARMM [Brooks et al. 1983] version 31b1 with the param22 all-atom force field [MacKerell Jr. et al. 1998]. The crystal structure 1bkv was taken from Protein Data Bank (www.pdb.org) and hydrogens were added using the HBUILD facility in CHARMM [Brünger and Karplus 1988]. The charmm 22 force field does not contain parameters for Hyp which were added from an earlier study [Anderson 2005].

The peptide was solvated by putting it in a water box containing preequilibrated TIP3 water molecules. The box had dimensions $112\times37\times37$ Å^3 with an approximate density of 1080 kg/m^3. The box size was

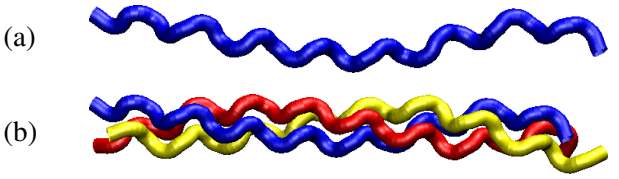

Figure 1. Collagen Structure. Each α chain backbone is shown as a tube to reveal its helical twist: (a) a left-handed helical α-chain, and (b) a collagen triple helix formed by three α-chains in a right-handed manner. Drawings rendered using VMD [Humphrey et al. 1996]; same color schemes used for α chains in Figure 2.

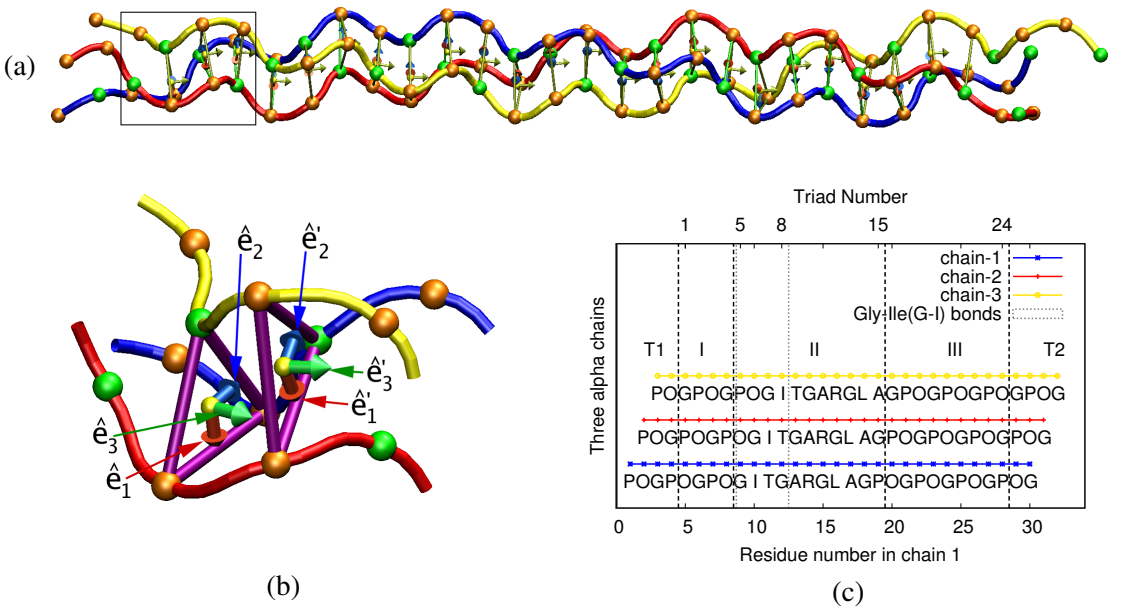

Figure 2. Triads along the helix. The three α-chains are shown in blue (chain-1), red (chain-2), and yellow (chain-3) (see Figure 1). Spheres are C_α atoms of Gly (green) and X/Y (orange). (a) Overview of 1bkv with the 24 triads. (b) Magnified view of the box in (a). To show face of triangle, molecule is rotated about the vertical axis of the paper plane. Two neighboring triads are denoted $\{\hat{e}_1, \hat{e}_2, \hat{e}_3\}$ and $\{\hat{e}'_1, \hat{e}'_2, \hat{e}'_3\}$. (c) Triad numbers versus amino acid sequence. The horizontal shift of the chains reflects staggered structure in the triple helix; grouped into domains I, II, and III. T1 and T2 are unconsidered regions to eliminate end effects. Triads 5–8, between grey dotted vertical lines, contain Gly-Ile bonds. First proline (P) residue in chain-1 in (c) is shown only for clarity and is invisible in the X-ray crystal structure of 1bkv.

chosen such that its boundaries were at least 11 Å away from the peptide. Periodic boundary conditions were imposed around the solvation box. Water molecules whose oxygen atoms were within a radius of

2.2 Å from heavy atoms in the peptide were deleted during solvation. After solvation, there were 5075 water molecules in the simulation box.

To eliminate close contacts and geometric strain in the system, we used the following energy minimization scheme. The peptide molecule was initially fixed and water molecules were minimized for 100 steps by steepest descent (SD) and for 400 steps by adapted basis Newton–Raphson (ABNR) method. As the next step, only the backbone was fixed and side chains along with water molecules were minimized chain by chain for 200 steps and then together for 500 steps by ABNR. The side chains were minimized chain by chain to solvate them better and to eliminate close sidechain-sidechain contacts. Finally, all constraints were removed and the system was minimized for 200 steps using ABNR.

The system was heated from 0 K and then equilibrated at target temperatures for 35 ps, with the peptide harmonically constrained to its original position. The heating rate was 2 K every 0.2 ps to target values ($T = 273$ K, 300 K, and 330 K). After heating, equilibration followed during which temperatures were rescaled if they deviated more than ± 2 K from target temperatures. The final production run was performed for 1 ns using the leap-frog Verlet integration algorithm with a time step of 1 fs at each target temperature with all harmonic constraints removed. The nonbonded pair and image atom lists were updated at each simulation step. A 12 Å cutoff was used for nonbonded interaction energies. Trajectories were stable during production runs, where relative root-mean-square fluctuations of temperature and energy were less than 0.7 % and 0.3 %, respectively.

3. Characterization of unwinding

To characterize the conformation of the triple helix, local coordinate triads $\{\hat{e}_1, \hat{e}_2, \hat{e}_3\}$ were generated as follows. We constructed triangles joining the C_α atoms of Gly, X, and Y of adjacent chains along the length of the molecule; see Figure 2a. The centroid of each triangle was chosen as the local coordinate origin and $\hat{e}_1$ was defined as the unit vector along the line joining the centroid to the midpoint of the line segment joining C_α atoms of chain-1 and chain-2. Setting $\hat{e}_3$ perpendicular to the plane fixes $\hat{e}_2 = \hat{e}_3 \times \hat{e}_1$; see Figure 2b. The first two triads and the last two triads were omitted in the analysis to reduce end effects; see Figure 2c. The remaining 24 triads within the middle were grouped into three (I,II,III) sets: domains I (triads 1–4) and III (triads 15–24) are the regular GPO repeats at the N and C termini, while domain II (triads 5–15) is the central imino-poor region.

We characterized orientations of triads using Euler angles [Goldstein 1980]. Let $\mathbf{X} = \{\hat{e}_1, \hat{e}_2, \hat{e}_3\}$ and $\mathbf{X}' = \{\hat{e}'_1, \hat{e}'_2, \hat{e}'_3\}$ be two adjacent triads; see Figure 2b. Denoting a Cartesian coordinate basis fixed in space as $\mathbf{E} = \{\hat{x}, \hat{y}, \hat{z}\}$, we have $\mathbf{X} = A\,\mathbf{E}$, $\mathbf{X}' = B\,\mathbf{E}$, where A and B are 3×3 matrices containing the Cartesian components of $\mathbf{X}$ and $\mathbf{X}'$, respectively. We then have

$$\mathbf{X}' = T\,\mathbf{X} \qquad \text{with} \quad T \equiv BA^{-1}. \tag{1}$$

On the other hand, $\mathbf{X}$ and $\mathbf{X}'$ are related by Euler transformation matrices by

$$\mathbf{X}' = R_x R_y R_z \mathbf{X} \qquad \Longrightarrow \qquad T = R_x R_y R_z, \tag{2}$$

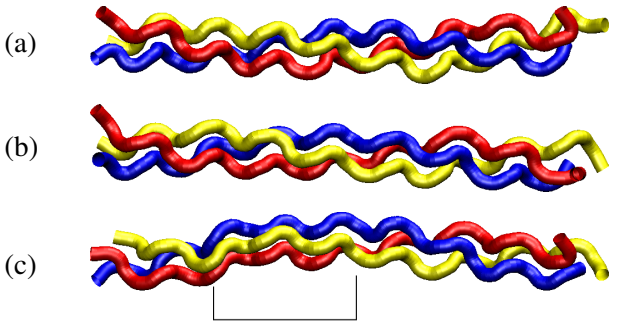

Figure 3. Unwinding of collagen. Triple helix at 330 K at: (a) 0 ns, (b) 0.5 ns, and (c) 1 ns. Marked region in (c) shows unwound domain II.

where

$$R_x = \begin{pmatrix} 1 & 0 & 0 \\ 0 & \cos\theta_x & \sin\theta_x \\ 0 & -\sin\theta_x & \cos\theta_x \end{pmatrix}, \qquad R_y = \begin{pmatrix} \cos\theta_y & 0 & \sin\theta_y \\ 0 & 1 & 0 \\ -\sin\theta_y & 0 & \cos\theta_y \end{pmatrix}, \qquad R_z = \begin{pmatrix} \cos\theta_z & \sin\theta_z & 0 \\ -\sin\theta_z & \cos\theta_z & 0 \\ 0 & 0 & 1 \end{pmatrix}.$$

As A and B are easily measurable in a given conformation of the molecule, T can be calculated from Equation (1), which in turn gives Euler angles θ_x, θ_y, and θ_z by solving Equation (2). If we set $\hat{z}$ along the major axis of the triple helix, changes in θ_z represent the degree of the helical twist (torsion). This can be seen by plotting the torsional map as the difference $\Delta\theta_z$ between a triad under consideration and a reference triad. For example, if we set the first triad as the reference, $\Delta\theta_z$ will monotonically increase with the triad number for a right-handed triple helix, and the triad that reaches $\Delta\theta_z = 360°$ will define the period of the triple helix. Because θ_z is the rotation angle of triads with respect to the z-axis, our method is not affected by the fact that the triangles in Figure 2a are not exactly perpendicular to the helical axis. For our analysis, we chose the reference point either as the first or the last triads, which gave consistent results. If there was unwinding of the triple helix in a particular region, it would show up as a change in slope of the torsional map; see Figure 4. We also calculated differences in θ_z between successive triads as another measure by which unwinding can be detected as a dip in the curve; see Figure 5.

4. Results

The peptide maintained its stable triple helical structure at 273 K, but unwinding was observed within 1 ns of the production run at 300 and 330 K; see Figure 3. We considered the statistics (average and standard deviation) of the torsional angles during time intervals 0.0–0.2, 0.2–0.4, 0.4–0.6, 0.6–0.8, and 0.8–1.0 ns at different temperatures; see Figure 4. Maximum $\Delta\theta_z$ was approximately 360°, as this peptide has only one helical turn. The torsional map at 273 K gives straight lines because the peptide does not unwind during the 1 ns simulation, see Figure 4a. However, even at such low temperature the G-I region shows some flexibility. Its lower slope (see Figure 4a, right) indicates that this region is less tightly wound compared to other regions. Within the 1 ns simulation time, variation in the profile at 273 K is more like a fluctuation rather than unwinding. Interestingly, the profile for the 0.8–1.0 ns interval shows the slowest growth of $\Delta\theta_z$ until triad 8 (see Figure 4a, right), but in triads 9–15 the slope is steeper than

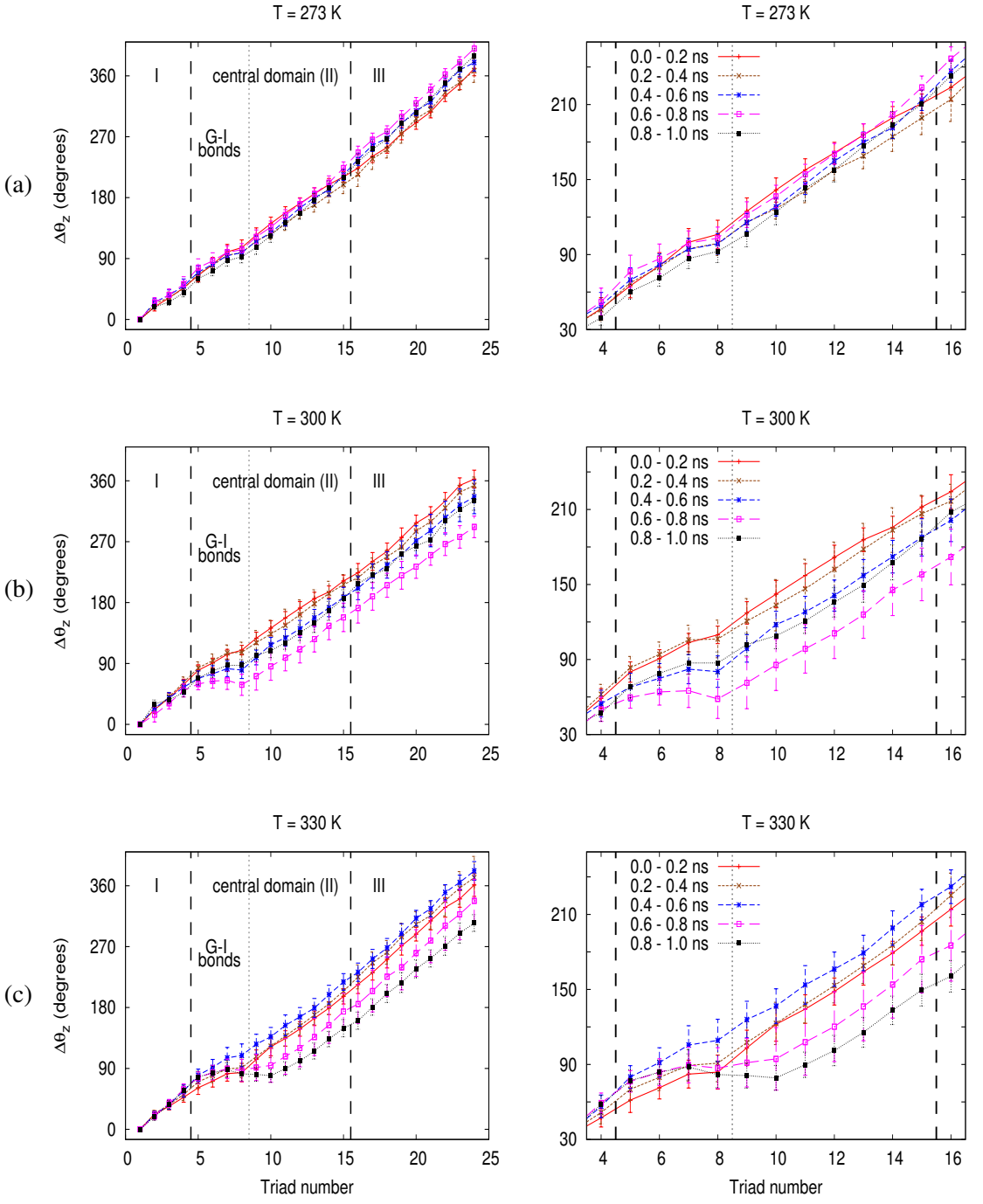

Figure 4. Torsional map with triad 1 as reference at (a) 273 K, (b) 300 K, and (c) 330 K. Data points give average value during time interval ± standard deviation: overview on left, close-up of domain II on right. Vertical lines divide different domains.

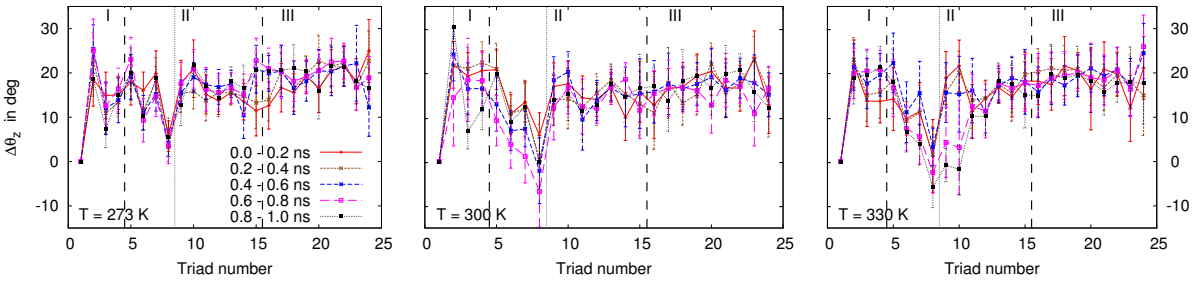

Figure 5. Differences in θ_z between successive triads.

those of other time intervals, so that triad 24 has $\Delta\theta_z$ above that from the 0.0–0.2 ns interval (see Figure 4a, left). This indicates that the unwinding near the G-I region is compensated by an overtwisting of triads 9–15 in domain II, indicative of the fluctuating nature of the imino-poor domain II.

The above trend becomes stronger at 300 K; see Figure 4b. A magnified view of the G-I region (see Figure 4b, right) shows greater fluctuations; unwinding increases until the 0.6–0.8 ns interval, where triad 8 is overunwound (left-handed) with a decreasing $\Delta\theta_z$. During this interval, the change in $\Delta\theta_z$ at triad 24 was 70.6° compared to the interval 0.0–0.2 ns. (This profile was obtained *with respect to* triad 1. In the simulations, triads at both ends rotate in the opposite direction to generate unwinding.) The molecule rewinds again during 0.8–1.0 ns, with a profile similar to the 0.4–0.6 ns interval. Due to the finite simulation time, it is not clear whether the conformation will eventually return to the original or if it will stay partially unwound. Note that overtwisting of triads 9–15 is not present at this temperature, suggesting that triads 1–4 (domain I) and 9–24 (domain II and III, except the G-I region) rotate rigidly relative to each other with the G-I region as a rotational hinge. This is presumably due to shorter relaxation times of the structure at higher temperatures.

At 330 K, initial overtwisting is observed (0.0–0.6 ns), but unwinding manifests at later times. Moreover, it propagates out of the G-I region into triads 9–10 in domain II; see Figure 4c, right. Experimentally, the melting temperature of 1bkv is $\approx$ 293 K [Kramer et al. 2001]. We thus expect that at 330 K the unwinding will proceed eventually into denaturation of the whole molecule. If the molecule becomes heavily disrupted, our approach will become inadequate for characterizing the triple helical conformation. For longer simulations in which unwinding proceeds further, it may be useful to introduce the area of each triangle (Figure 2) as an additional measure of the integrity of the three-chain bundle.

While Figure 4 identifies local unwinding within the global helicity of the molecule, a sensitive way of monitoring conformational behavior triad-by-triad is by comparing differences in θ_z along successive triads; see Figure 5. Decreased helicity is evident at 273 K between triads 7 and 8, and overtwisting can be seen to be localized mostly in triad 14–15, at the boundary between domains II and III. Yet, profiles at different time points overlap within standard deviations, thus no clear unwinding behavior is present other than fluctuations. At 300 K a negative value of $\Delta\theta_z$ between triads 7 and 8 again shows an opposite helicity (left-handed), which propagates to the right within domain II at 330 K.

5. Concluding discussion

In the present study we have introduced a local triad basis method to monitor delicate conformational changes in collagen triple helix conformation. This approach enabled characterization of local unwinding as an early event preceding denaturation and was sensitive enough to elucidate the lability of the G-I region even at 273 K when unwinding was not noticeable; see Figure 5.

As a comparison, Stultz previously used umbrella sampling to study localized unfolding of 1bkv and an imino-rich peptide [Stultz 2002]. His approach mainly consisted of increasing the radius of gyration of *one* chain near its center of mass, while the atoms outside the *reaction region* were harmonically constrained to their crystallographic coordinates. Although radius of gyration may be a good parameter for the foldedness of globular proteins, it is not suitable for describing conformations of filamentous proteins. It is thus difficult to appreciate the transition state or unfolded conformations obtained in his study as physically meaningful. In contrast, our approach naturally follows the conformational fluctuation of the peptide; it thus offers a higher degree of accuracy and is applicable to characterizing torsional behaviors of other filamentous systems. Other MD simulations on collagen mimetic peptides tested imino-rich structures with simple repeating sequences [Lorenzo and Caffarena 2005; Handgraaf and Zerbetto 2006; Radmer and Klein 2006] and do not address the conformational behavior of imino-poor domains. As a control, we have tested a model collagen which consists only of the GPO repeats. Consistent with experimental results [Bella et al. 1994], we did not observe any unwinding at 300 K (data not shown). We suspect that surface-bound water bridges are involved in the stabilization of the imino-poor region, which are disrupted at higher temperatures. Further analysis of the role of water will be published elsewhere. To our knowledge, the present work is the first to characterize spontaneous unwinding of a collagen triple helix through MD simulations.

Our results indicate that the G-I region within the imino-poor region is most labile and is an initiating site for unwinding; see Figure 4c. The lability of the G-I region might originate from the strongest β-sheet propensity of the isoleucine residue [Kim and Berg 1993], which prefers an extended backbone conformation. In type III collagen there is an MMP cleavage site [Fields 1991; Lauer-Fields and Fields 2002] (denoted by Gly∼Ile) that is upstream of the domain II of 1bkv: -Gly∼Ile-Ala-Gly-Ile-Thr-Gly-Aln-Arg-Gly-Leu-Ala-Gly-, where Gly-Ile is the G-I region in 1bkv. This region, which contains two G-I bonds, is thus likely to be highly flexible in collagen III and could unwind at physiological temperatures.

MMPs [Overall 2002] cleave fibrillar collagen (type I, II, or III) only at a specific site (-Gly∼Ile or -Gly∼Leu-) approximately three-fourths from the amino terminus of the molecule. A distinctive feature of this site is that it is followed by a loose helical region [Fields 1991]. In gelatin — that is, denatured collagen — apart from the unique collagen cleavage site, other Gly-Ile and Gly-Leu bonds are cleaved by collagenases [Welgus et al. 1982], suggesting that MMP specificity in targeting those bonds could be due to their tendency to unwind.

Structurally, the groove of the collagen cleaving domain in MMPs is too narrow to accommodate the triple helix, but can accommodate only one α-chain [Chung et al. 2004]. Collagen unwinding is thus a requirement for cleavage, for which the hemopexin-like domain in MMP is believed to unwind collagen [Nagase and Woessner 1999; Overall 2002; Lauer-Fields and Fields 2002; Chung et al. 2004]. While further studies are needed for MMP-mediated collagen unwinding, our results suggest that the cleavage sites can spontaneously unwind via thermal fluctuations even at body temperature. Hence, the function

of a hemopexin-like domain would be to help stabilize the unwound conformation rather than forcibly unwind a domain.

Our view is supported by the report that proteolysis of collagen does not require much energy and can occur in the absence of ATP molecules [Lauer-Fields and Fields 2002]. It is possible that the hinge domain in MMP, which is a polyproline chain, may invade the loose nonhelical strands, further disturbing the helical structure and facilitating cleavage [De Souza et al. 1996]. Spontaneous unwinding also explains why trypsin, though it does not have a hemopexin-like domain to unwind collagen, can still cleave collagen despite with less efficiency [Ryhänen et al. 1983; Lauer-Fields and Fields 2002].

In preliminary simulations, we also observed unwinding of the 'labile domain' in collagen-I (63 residues per α chain) [Miles and Bailey 2001] that is low in imino acid content (data not shown), in accordance with previous experimental results that local relaxation exists in collagen even below melting temperatures [Ryhänen et al. 1983; Kadler et al. 1988]. Strand invasion by Fibril Associated Collagen with Interrupted Triple helices (FACITs) is another example by which spontaneous collagen unwinding likely plays a role – in an intact collagen fiber, nano-particle coated FACIT mimetic peptides associate with the labile domain in the gap region [Mo et al. 2006], presumably due to the spontaneous unwinding of individual collagens. These peptides bind along the fiber after the sample was heat treated to 313 K, indicating extensive damage to the triple helical structure. As local unwinding facilitates binding to other extracellular components (FACITs, fibronectin, etc.) [Engvall et al. 1978] and render flexibility to collagen molecules, it would play a key role in the self-assembly of collagen into fibrils and networks.

Our simulations were performed without any external load on the molecule which provides a bound on its behavior [Chen et al. 1998]. In the extracellular matrix, however, collagen is likely stressed due to cell traction and external loads. Experimentally mechanical load on collagen matrix enhances resistance to degradation and thermal denaturation [Chen et al. 1998; Ruberti and Hallab 2005]. Based on our results we suspect that the resistance is based on a decreased tendency for unwinding under tension. Further extension of our simulation with mechanical loads is under way.

Our results suggest that a collagen molecule can be conceptually divided into *structural* and *functional* domains. The structural domains are composed of imino-rich residues where electro-negativity plays an important role in stability [Holmgren et al. 1998; 1999]. Being highly stable and tightly packed, these domains play a structural role giving shape and strength to the molecule. The imino-poor functional domains are thermally fluctuating metastable regions. By having such domains, degradation of a collagenous matrix can be easy and systematic, which is important in making collagen a highly adaptable material.

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